CURRENT TOPICS IN OPIOID RESEARCH

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CURRENT TOPICS IN OPIOID RESEARCH

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EDITORIAL
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Editorial: Current Topics in Opioid Research

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Although natural opiates have been used for centuries, and semi-synthetic, and synthetic opiates have been used and abused for decades, these last few years have witnessed an incredible increase in opioid abuse and deaths due to overdose. With this opioid crisis has come an increase in research into the mechanisms of analgesia, abuse, and addiction, interaction with other drugs, anatomical and imaging studies, as well as ethical discussions of opioid use and abuse. This Research Topic, Current Topics in Opioid Research, presents 7 minireviews, 2 hypotheses/perspectives, and 16 original research articles, from 13 different countries, and has articles that span the field of opioid research, gives insight into ongoing topics, and provides a basis for further study and potential reduction in severity of the opioid crisis.

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HYPOTHESIS AND THEORY/PERSPECTIVE ARTICLES

Drug Addiction: From Neuroscience to Ethics by Farisco et al. presents a novel hypothesis concerning drug addiction. The authors suggest that in addition to well-described neuronal/neurochemical factors contributing to addictive dynamics, the socioeconomic status also plays a causal role in drug addiction through epigenetic processes that require additional reward in the brain. This provides a strong base for a sociopolitical form of responsibility for preventing and managing the addiction crisis. For this reason, the authors consider addiction to be a social disorder in addition to a medical and mental disorder.

The Clinical Concept of Opioid Addiction Since 1877: Still Wanting After All These Years by Schütz et al. proposes a comprehensive theory of addiction that uses life and social sciences, dynamic and complex systems theory, and philosophical–phenomenological approaches to understand the full complexity of addiction while integrating neurobiological, psychological, and sociocultural aspects. According to this theory, addiction can be viewed as a habit, induced by a network of mental, behavioral, and social processes, which not only shape the addict's perceptions and actions, but also cause one to self-maintain.

REWARD/ADDICTION

In Management of Opioid Addiction With Opioid Substitution Treatments: Beyond Methadone and Buprenorphine, Noble and Marie discuss drug therapies for addiction treatment. They discuss the benefits and detriments of the two mu opioid agonists, with a brief discussion of drug characteristics leading to these properties. They also discuss the use of the opioid antagonist naltrexone and potential leads toward next-generation medications, including the use of biased

agonists, nociception/orphanin (NOP) receptor agonists, or potentially enkephalin degradation inhibitors.

In Enkephalin as a Pivotal Player in Neuroadaptations Related to Psychostimulant Addiction Mongi-Bragato et al. address changes in enkephalin levels in the mesocorticolimbic reward circuitry due to administration of psychostimulants. The authors discuss how these changes affect signaling of mu and delta opioid receptors and the importance of receptor activation with respect to cocaine- and amphetamine-induced behavioral sensitization, conditioned place preference (CPP), and self-administration.

Previously, Li et al. had demonstrated that activation of Trx-1, an important redox regulating protein, could protect mice from the rewarding effects of morphine. In Overexpression of Thioredoxin-1 Blocks Morphine-Induced Conditioned Place Preference Through Regulating the Interaction of γ-Aminobutyric Acid and Dopamine Systems, these authors demonstrate that morphine-induced CPP was blocked in Trx-1 overexpressing transgenic mice. Furthermore, Trx-1 expression was induced by morphine in the ventral tegmental area (VTA) and nucleus accumbens (NAc) in wild-type (WT) mice. The level of dopamine, expression of tyrosine hydroxylase (TH), and D1 dopamine receptor as well as levels of GABA and $GABA_B$-receptors were altered by chronic morphine. Therefore, Trx-1 may play a role in blocking CPP induced by morphine through regulating the expressions of D1, TH, and $GABA_B$ receptors in the VTA and NAc.

Two papers described effects on drug abuse models subsequent to activation of NOP receptors, the fourth member of the opioid receptor family. In NOP receptor agonist Ro 64-6198 decreases escalation of cocaine self-administration in rats genetically selected for alcohol preference, Li et al. examine the effect of NOP receptor agonist Ro 64-6198 on alcohol self-administration in Marchigian Sardinian alcohol-preferring (msP) rats that have an upregulated NOP receptor system and in Wistar control rats. Ro 64-6198 was better able to attenuate cocaine self-administration in msP than in Wistar rats.

In The Nociceptin Receptor (NOP) Agonist AT-312 Blocks Acquisition of Morphine- and Cocaine-Induced Conditioned Place Preference in Mice, Zaveri et al. discuss the actions of AT-312, a selective NOP agonist, on morphine and cocaine CPP. AT-312 blocked acquisition of both morphine and cocaine as well as locomotor stimulation in WT but not NOP receptor knockout (KO) mice. These results demonstrate that NOP agonists may have a potential as pharmacotherapy for opioid and psychostimulant addiction or for treating polydrug addiction.

Three papers focused on the relationship between opiates and alcohol abuse. In Binge-Like Exposure to Ethanol Enhances Morphine's Anti-nociception in B6 Mice, Chang et al. hypothesize that binge drinking potentiates onset and progression of opioid use disorder (OUD). To examine this, the authors examined and found an increase in inflammatory cytokines and mu receptor mRNA in the striatum after binge ethanol drinking. This corresponded with an increase in potency of morphine at 3 mg/kg in the hotplate test. Such effect might initiate the onset and progression of OUDs.

Granholm et al. described the effects of ethanol exposure on the level of opioid peptides in Episodic Ethanol Exposure in Adolescent Rats Causes Residual Alterations in Endogenous Opioid Peptides. To mimic binge drinking in adolescents, the authors administered ethanol to rats 3 days per week from weeks 4 to 9. Beta-endorphin, dynorphin B, and Met-enkephalin-Arg^6Phe^7 (MEAP) were then analyzed 2 h and 3 weeks after the final ethanol administration. Changes were observed for each peptide in selected brain regions. These alterations in opioid networks after adolescent ethanol exposure could explain, in part, the increased risk for high ethanol consumption later in life.

In Critical Role for Gi/o-Protein Activity in the Dorsal Striatum in the Reduction of Voluntary Alcohol Intake in C57Bl/6 Mice, Robins et al. explore the hypothesis that dorsal striatal Gi/o-protein activation is sufficient to reduce voluntary alcohol intake. The authors examined this hypothesis in two ways. In one set of experiments, they expressed the inhibitory, Gi/o-coupled, M4 DREADD in the dorsal striatum. In these animals, receptor activation with CNO reduced consumption of 10% ethanol in a two-bottle choice paradigm. In other experiments, delta opioid receptor activation with the Gi/o-biased agonist TAN-67 reduced alcohol consumption in WT and β-arrestin-2 KO animals, while activation with the β-arrestin-2-biased agonist SNC80 increased alcohol intake in WT but decreased intake in β-arrestin-2 KO animals. These results suggest that activation of Gi/o-coupled receptors in the striatum, with biased agonists, could be a mechanism for treating alcohol use disorder.

In CB1 Agonism Alters Addiction-Related Behaviors in Mice Lacking Mu or Delta Opioid Receptors, Roeckel et al. investigate the interaction between opioid and cannabinoid CB1 receptors with respect to pain, withdrawal, anxiety, and depression using the selective CB1 agonist ACEA, as well as mu and delta opioid receptor KO mice. The authors demonstrated that ACEA had no antinociceptive activity of its own in the warmwater tail withdrawal test. Naloxone was able to precipitate withdrawal from chronic ACEA in mice of all genotypes. Anxiety-like behavior was independent of genotype and ACEA treatment, but a pro-depressive effect of ACEA was absent in mu KO mice. These studies indicate an interaction between opioid and CB1 receptors in withdrawal and depression.

Butelman et al. examine opioid-dependent patients to determine if other drug use was a predictor of ultimate opioid dependence. In Non-medical Cannabis Self-Exposure as a Dimensional Predictor of Opioid Dependence Diagnosis: A Propensity Score Matched Analysis, the authors used an outpatient observational study to examine age of onset of heaviest use of cannabis, cocaine, and alcohol and how that correlated with the onset of opioid dependence. They concluded that the maximal self-exposure to cannabis and cocaine, but not to alcohol, was greater in volunteers with opioid dependence and that increasing self-exposure to cannabis and cocaine, but not alcohol, was a positive predictor of opioid dependence.

PAIN

Pain Therapy Guided by Purpose and Perspective in Light of the Opioid Epidemic, by Severino et al., discusses the history behind the current opioid epidemic then goes on to review potential methods for treatment of pain without off-target effects. To

this end, the authors discuss the potential for ligand bias and bifunctional opioid agonists as potential methods of reducing side effects and also outline how the pharmacokinetic profile of opioids contribute to their potential for addiction and abuse.

In The Contribution of the Descending Pain Modulatory Pathway in Opioid Tolerance, Lueptow et al. review mechanisms that underlie opioid tolerance development, concentrating on the descending periaqueductal gray matter (PAG)–rostral ventromedial medulla (RVM)–spinal cord pain pathway. The authors describe how tolerance in the PAG is mediated by mu receptor uncoupling from downstream G-protein mediated signaling. Other experiments describe the relationship between tolerance development and glial activation; particularly, the role of TLR4 in that relationship is also discussed.

In Cutting-Edge Search for Safer Opioid Pain Relief: Retrospective Review of Salvinorin A and Its Analogs, Zjawiony et al. review the development of salvinorin A analogs from the perspective of a medicinal chemist. Salvinorin A, a natural product purified from *Salvia divinorum*, is a selective and high-affinity kappa agonist. *Salvia* is currently used as a recreational drug, since it alters consciousness but, like kappa drugs, is often dysphoric. Nevertheless, it has been used as a template for drug discovery leading to the production of many kappa and mu selective agonists. In particular, many analogs appear to have ligand bias, which might be useful in developing drugs with lower abuse potential.

In Advances in Achieving Opioid Analgesia Without Side Effects, Machelska and Celik review emerging opioid-based strategies to develop effective analgesics with reduced side effect profile. The novel concepts discussed include biased agonism, peripherally active compounds, heteromeric compounds, receptor splice variants, and use of endogenous opioids by inhibiting degradation or enhancing production. Compounds in clinical trials and undergoing preclinical studies are identified.

Zhang et al. examined the involvement of NOP receptors on a post-traumatic stress disorder (PTSD) model in male and female rats in Sex Differences in Nociceptin/Orphanin FQ Peptide Receptor-Mediated Pain and Anxiety Symptoms in a Preclinical Model of Post-traumatic Stress. Interestingly, male NOP receptor KO rats did not develop single prolonged stress-induced allodynia and thermal hypersensitivity, while female NOP receptor KO rats exhibited tactile allodynia and thermal hypersensitivity to the same extent as WT rats. These experiments demonstrate the distinct role that the NOP receptor system plays in males and females after exposure to sustained stress.

Stötzner et al., in Mu-Opioid Receptor Agonist Induces Kir3 Currents in Mouse Peripheral Sensory Neurons—Effects of Nerve Injury explore the activity of mu receptors in the dorsal root ganglia (DRG) to examine the potential of peripherally active mu agonists as analgesics with greatly reduced side effects. Receptor activity was determined by measuring activity of the G protein-coupled inwardly rectifying potassium channels, Kir3 in the DRG of naïve and chronic pain (chronic constriction injury CCI) mice. The authors determined that the mu agonist DAMGO could activate Kir3 currents and the percentage of mu-containing DRG neurons was equivalent in naïve and CCI mice.

PAIN/NOVEL LIGANDS

In *In Vitro* and *in Vivo* Pharmacological Activities of 14-O-Phenylpropyloxymorphone, a Potent Mixed Mu/Delta/Kappa-Opioid Receptor Agonist With Reduced Constipation in Mice, Lattanzi et al. discuss *in vitro* and *in vivo* properties of this novel compound (POMO). POMO is a 14-*O*-phenylpropyl-substituted analog of the mu opioid agonist 14-*O*-methyloxymorphone (14-OMO). POMO has subnanomolar affinity at mu, delta, and kappa receptors and is a full agonist at mu and delta. Importantly, it is a very potent analgesic, with an ED_{50} of 0.7 nmol/kg, 9,000 times more potent than morphine. Despite the extremely potent antinociceptive activity, it has reduced inhibition of gut transport, suggesting a greater therapeutic window.

Kumar et al. performed a structure–activity relationship study of oxymorphinone analogs in Synthesis, Biological Evaluation, and SAR Studies of 14β-phenylacetyl Substituted 17-cyclopropylmethyl-7,8-dihydronoroxymorphinones Derivatives: Ligands With Mixed NOP and Opioid Receptor Profile. Affinity and *in vitro* efficacy were determined at mu, delta, kappa, and NOP receptors. All compounds were partial agonists at each receptor, and structure activity relationship results were consistent with molecular modeling predictions that a binding site within the NOP receptor could be accessed by an appropriate 14β side chain. This resulted in compounds with much higher affinity at NOP receptors than the parent compound naltrexone.

MU RECEPTOR

Heteromerization Modulates mu Opioid Receptor Functional Properties *in vivo* by Ugur et al. reviews the evidence for heteromers of the mu receptor and discusses how heteromers affect mu opioid receptor signaling, trafficking, and subsequent behavioral responses. The authors describe how selective targeting of heteromers to modulate mu opioid receptor activity has attracted significant interest as a method for developing novel innovative therapeutics.

In Microglia Express Mu Opioid Receptor: Insights From Transcriptomics and Fluorescent Reporter Mice, Maduna et al. examine microglia for the presence of the mu receptor. The reasoning behind these experiments was the observation that microglia appear to mediate certain adverse effects of opiates including analgesic tolerance and opioid-induced hyperalgesia. Using transcriptomic databases from both mice and humans, as well as imaging of newly created Cx3cr1l-eGFP-MOR-mCherry mice, the mu receptor was found in the vast majority of mouse and human microglia datasets. Furthermore, mu receptors could be found in roughly 40% of microglia in both brain and spinal cord. These results are consistent with functional studies showing the actions of mu receptor agonists on microglia.

In Oxycodone Self-Administration Induces Alterations in Expression of Integrin, Semaphorin and Ephrin Genes in the Mouse Striatum, Yuferov et al. examined the effect of chronic oxycodone treatment on molecules that affect axon guidance including integrin, semaphorin, and ephrin gene families. It was the author's hypothesis that opioid-induced changes in axon-target

connections and synaptogenesis may be implicated in the behaviors associated with opiate addiction. Chronic oxycodone caused either an increase or decrease in the majority of the 38 known genes in these gene families. The relationship between expression of these genes and specific behaviors is under investigation.

MU RECEPTOR IMAGING

In Deformation-based Morphometry MRI Reveals Brain Structural Modifications in Living Mu Opioid Receptor Knockout Mice, Nasseef et al. used a structural magnetic resonance imaging (MRI) approach to determine whether volumetric alterations also occur in mu opioid receptor KO mice. The authors measured deformation-based morphometry (DBM) for each voxel in subjects from mu KO and control groups. They found volumetric changes, both contractions and expansions in various brain regions, mainly in mu receptor-enriched regions and across reward/aversion centers. Some volumetric changes were in regions that showed functional connectivity changes identified in a previous resting-state functional MRI study, suggesting a possible function–structure relationship in mu KO-related brain alternations. These functional and structural MRI studies disclose whole-brain level mechanisms that likely drive mu-controlled behaviors.

Sasaki et al. demonstrated changes in tissue volume in the PAG using RMI voxel-based morphometry. In Larger Numbers of Glial and Neuronal Cells in the Periaqueductal Gray Matter of μ-Opioid Receptor Knockout Mice, these authors used immunohistochemistry to measure numbers of microglia, astrocytes, and neurons in four subregions of the PAG. They found larger numbers of each of these cell types in mu KO compared with WT mice, suggesting that these alterations might account for the hyperalgesic state in mu KO mice.

AUTHOR CONTRIBUTIONS

LT, KS, and DM all contributed to writing this Editorial of the Research Topic on Opioids that they edited in 2018.

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ORIGINAL RESEARCH
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NOP Receptor Agonist Ro 64-6198 Decreases Escalation of Cocaine Self-Administration in Rats Genetically Selected for Alcohol Preference

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Cocaine dependence is a psychiatric condition for which effective medications are still lacking. Published data indicate that an increase in nociceptin/orphanin FQ (N/OFQ) transmission by NOP receptor activation attenuates cocaine-induced place conditioning and the locomotor sensitization effects of cocaine. This suggests that the activation of the N/OFQ receptor (NOP) may attenuate the motivation for psychostimulants. To further explore this possibility, we investigated the effect of the potent and selective NOP receptor agonist Ro 64-6198 on cocaine intake under 1 h short access (ShA) and 6 h long access (LgA) operant self-administration conditions in rats. We used Marchigian Sardinian alcohol-preferring (msP) rats and Wistar control rats. msP rats were used because we recently found that this rat line, originally selected for excessive alcohol drinking and preference, exhibits a greater propensity to escalate cocaine self-administration following LgA training. msP rats are also characterized by innate overexpression of the N/OFQ-NOP system compared with Wistar rats. Wistar and msP rats both exhibited an increase in cocaine self-administration under LgA conditions, with a higher trend toward escalation in msP rats. In Wistar rats, the intraperitoneal administration of Ro 64-6198 (0. 1 and 3 mg/kg) significantly decreased ShA cocaine self-administration. In Wistar rats that underwent LgA cocaine self-administration training, Ro 64-6198 induced no significant effect either during the first hour of self-administration or after the entire 6 h session. In msP rats, Ro 64-6198 significantly reduced cocaine self-administration both under ShA conditions and in the first hour of the LgA session. At the end of the 6 h session, the effect of Ro 64-6198 was no longer observed in msP rats. The highest dose of Ro 64-6198 (3 mg/kg) did not affect saccharin self-administration in msP rats but reduced saccharin self-administration in Wistar rats. Altogether, these data suggest that NOP receptor activation attenuates cocaine self-administration, and this effect tends to be more pronounced in a rat line with innately higher NOP receptor expression and that more robustly escalates cocaine intake.

Keywords: abuse, addiction, psychostimulants, drug-seeking, opioids

INTRODUCTION

Cocaine is the most commonly abusedillicit psychostimulant, and its use is linked to serious physical, psychiatric, socioeconomic, and legal problems (1). Effective medications for the treatment of cocaine addiction are lacking. The development of medications that can control cocaine intake and seeking would represent a significant medical breakthrough.

Cocaine is often co-abused with alcohol. Cocaine dependence and alcohol dependence share several genetic traits, indicating that common predisposing factors may exist (2–4). We recently found that Marchigian Sardinian alcohol-preferring (msP) rats, which are genetically selected for excessive alcohol drinking and preference, also exhibit neurophysiological and pharmacological traits that confer a predisposition to psychostimulant abuse (5). We recently found that msP rats exhibited alterations of functional magnetic resonance imaging activity and an increase in nucleus accumbens dopamine release in response to an amphetamine challenge compared with Wistar rats. msP rats also exhibited a higher propensity to escalate cocaine intake under extended access (6 h/day) self-administration conditions (5). Compared with heterogeneous stock Wistars rats (i.e., the rat strain from which msP rats originate), the msP line appears to present an addiction-prone phenotype. msP rats are also characterized by inherited neurophysiological adaptations of several neurotransmitter systems that may contribute to their vulnerable phenotype (6–8). One such system consists of nociceptin/orphanin-FQ (N/OFQ) peptide and its NOP receptor, known for being structurally similar to dynorphin A and kappa opioid, respectively (9, 10). The activation of NOP receptors has been shown to attenuate the motivation for various drugs of abuse (11–18).

Intracranial N/OFQ administration inhibited psychostimulant-induced conditioned place preference and locomotor sensitization in rats (19, 20). NOP receptor knockout mice exhibited higher cocaine-induced conditioned place preference compared with their wildtype counterparts (21). Additionally, NOP knockout mice showed increased psychomotor sensitization to cocaine (22) and N/OFQ abolished cocaine-induced psychomotor sensitization in wildtype but not NOP knockout mice (23). Importantly, intracerebroventricular N/OFQ administration did not induce conditioned place preference or aversion, suggesting that NOP receptor agonists do not have motivational properties *per se* (24, 25). Although data on the effect of NOP receptor activation on cocaine-related behaviors have been published, direct evidence of an effect of NOP receptor agonists on cocaine self-administration are limited to buprenorphine and cebranopadol, two molecules that simultaneously activate NOP and μ opioid receptors (16–18). The present study investigated the effect of the potent and selective NOP receptor agonist Ro 646198 on cocaine intake in rats that were exposed to a daily 1 h short access (ShA) or 6 h long access (LgA) operant self-administration sessions. The study was performed in heterogeneous Wistar and msP rats. msP rats exhibit overexpression of the N/OFQ system and a greater propensity to escalate cocaine self-administration. msP rats also exhibit high sensitivity to NOP receptor agonists, and a history of dependence enhances NOP expression. Therefore, we predicted that Ro 64-6198 would be more effective in rats that were exposed to LgA cocaine self-administration and that msP rats would be more sensitive to Ro 646198 than Wistar rats (8, 26).

MATERIALS AND METHODS

Animals

The experiments were conducted with male Wistar rats (Charles River, Calco, Italy) and msP rats (bred at the School of Pharmacy, University of Camerino, Italy). The rats weighed 200–250 g at the beginning of the study. They were housed in pairs in a room under a reverse 12 h/12 h light/dark cycle (lights off at 9:00 a.m.) with constant temperature (20–22°C) and humidity (45–55%). Food and water were provided *ad libitum*. The animals were treated in accordance with the guidelines of the European Community Council Directive for Care and Use of Laboratory Animals. The experimental procedures were approved by the Italian Ministry of Health (authorization no. 414/2016-PR).

Drugs

Cocaine hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in sterile saline. Saccharin (Sigma, Italy) was dissolved in tap water. The NOP receptor agonist Ro 64-6198 was dissolved in 10% dimethylsulfoxide, 10% Tween-80, and 80% water. Doses timing and route of administration of Ro 64-6198 were chosen based on earlier NOP binding studies indicating that an acute IP injection of 3.2 mg/kg of Ro 64-6198 induced maximal NOP receptor occupancy after 30 min. Good receptor occupancy was maintained for approximately 3 h (27).

Catheter Implantation

The rats were anesthetized by an intramuscular injection of 100–150 μl of a solution that contained tiletamine chlorhydrate (58.17 mg/ml) and zolazepam chlorhydrate (57.5 mg/ml). For intravenous surgery, incisions were made to expose the right jugular vein. A catheter that was constructed from micro-renathane tubing (inner diameter = 0.020 inches, outer diameter = 0.037 inches) was subcutaneously positioned between the vein and back. After insertion into the vein, the proximal end of the catheter was anchored to the muscles that underlie the vein with surgical silk. The distal end of the catheter was attached to a stainless-steel cannula that was bent at a 90° angle. The cannula was inserted into a support that consisted of dental cement on the back of the animals and was covered with a plastic cap. Immediately after surgery, the rats were treated intramuscularly with 200 μl of enrofloxacin (50 mg/ml, Baytril, Germany).

The rats were allowed to recover for 1 week before self-administration training. Catheters patency was confirmed by an intravenous injection of 150 μl of sodium pentothal (25 mg/ml, Intervet, Italy). Before each self-administration session, the catheters were flushed with 100 μl of heparinized saline (20 UI/ml) that contained 0.5 mg/ml enrofloxacin.

Self-Administration Apparatus

The self-administration stations consisted of operant conditioning chambers (Med Associates, USA) that were enclosed in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with two retractable levers that were located in the front panel of the chamber. Cocaine was delivered intravenously through a plastic tube that was connected to an infusion pump. Saccharin was delivered in a receptacle that was connected to the infusion pump and located between the two levers. Responses on the right (active) lever activated the infusion pump, and responses on the left (inactive) lever were recorded but did not have any programmed consequences. In both the cocaine and saccharin sessions, activation of the pump resulted in the delivery of 0.1 ml of fluid. A computer controlled the delivery of cocaine solution and recorded the behavioral data.

General Cocaine Self-Administration Procedure

Wistar and msP rats were initially trained to self-administer cocaine (0.25 mg/infusion) under a fixed-ratio 1 (FR1) schedule of reinforcement for 1 h/day for 5 days. Afterward, the reinforcement contingency was switched to an FR5 schedule, and the rats of each line were split into two subgroups: self-administration in a 1 h short-access (ShA) session and self-administration in a 6 h long access (LgA) session. The LgA and ShA sessions under an FR5 schedule continued for 23 days, after which the effect of Ro 64-6198 on cocaine self-administration was tested.

Effect of Ro 64-6198 on Cocaine Self-Administration in Wistar Rats

Starting from the 24th FR5 session, we tested the effect of Ro 64-6198 and its vehicle on cocaine self-administration in Wistar rats that were trained under ShA ($n = 10$) and LgA ($n = 10$) conditions. Using a within-subjects counterbalanced design, 30 min before the session, the rats received Ro 64-6198 (1.0 and 3.0 mg/kg, i.p.) or its vehicle. The tests were repeated every third day. On the first intervening day, the rats remained in their home cage. On the second intervening day, they underwent a baseline cocaine self-administration session. The number of infusions was recorded after the first hour of self-administration and also at 6 h in the LgA group.

Effect of Ro 64-6198 on Cocaine Self-Administration in msP Rats

Similar to Wistar rats, starting from the 24th FR5 session, we tested the effect of Ro 64-6198 (1.0 and 3.0 mg/kg, i.p.) and its vehicle on cocaine self-administration in msP rats that were trained under ShA ($n = 9$) and LgA ($n = 9$) conditions. The tests were performed using a within-subjects counterbalanced design at intervals of 3 days. The number of infusions was recorded after the first hour of self-administration and also at 6 h in the LgA group.

Effect of Ro 64-6198 on Saccharin Self-Administration in Wistar and msP Rats

Two additional groups of Wistar ($n = 9$) and msP ($n = 9$) rats were trained in daily 1 h saccharin self-administration sessions under an FR1 schedule of reinforcement. When the rats reached a stable baseline of saccharin intake, we tested the effect of Ro 64-6198 (1.0 and 3.0 mg/kg, i.p.) and its vehicle on saccharin self-administration. Thirty minutes before the sessions, the rats received Ro 64-6198 (1.0 and 3.0 mg/kg, i.p.) and its vehicle in a counterbalanced Latin-square design. The tests were repeated every third day.

Statistical Analyses

The number of infusions that were received by ShA rats was compared with the number of infusions that were received by LgA rats during the first hour of the daily sessions using two-way repeated-measures analysis of variance (ANOVA), with session length (ShA *vs.* LgA) as the between-subjects factor and time (days) as the repeated measure. The escalation of cocaine self-administration was analyzed using one-way ANOVA, with time as the repeated measure. The effect of Ro 64-6198 on cocaine and saccharin self-administration was analyzed using one-way ANOVA, with dose as the within-subjects factor. Wistar and msP rats were analyzed separately. Significant main effects in the ANOVA were followed by the Newman-Keuls *post hoc* test for escalation and Dunnett's *post hoc* test for Ro 64-6198. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Escalation of Cocaine Self-Administration in Wistar and msP Rats

One LgA Wistar rat and one ShA Wistar rat became sick during training; therefore, only $n = 9$ LgA Wistar rats and $n = 9$ ShA Wistar rats were considered for the analyses. The escalation of cocaine intake reflects an increase in the number of infusions that are obtained daily over time. Escalation occurs during LgA sessions and usually is measured by analyzing the number of rewards that are earned during the first hour of intake in the LgA session compared with the ShA session (28).

In Wistar rats, the ANOVA indicated no effect of session length [$F_{(1,16)} = 1.5$, $p > 0.05$] but a significant effect of time [$F_{(22,352)} = 6.5$, $p < 0.0001$] and a significant session length $\times$ time interaction [$F_{(22,352)} = 1.7$, $p < 0.05$]. The two groups differed in intake on the first day under the FR5 schedule but earned a similar number of infusions during the remainder of training (**Figure 1A**). LgA Wistar rats exhibited an increase in lever pressing over time [$F_{(8,176)} = 8.1$, $p < 0.01$], with a progressive increase in the total number of daily (6 h) cocaine infusions that were earned (**Figure 1B**).

In msP rats, ANOVA was used to compare the number of infusions that were received in the ShA group and LgA group during the first hour of the session. The ANOVA indicated significant main effects of session length [$F_{(1,16)} = 40.9$, $p < 0.0001$] and time [$F_{(22,352)} = 24.7$, $p < 0.0001$] and a significant session length $\times$ time interaction [$F_{(22,352)} = 2.4$,

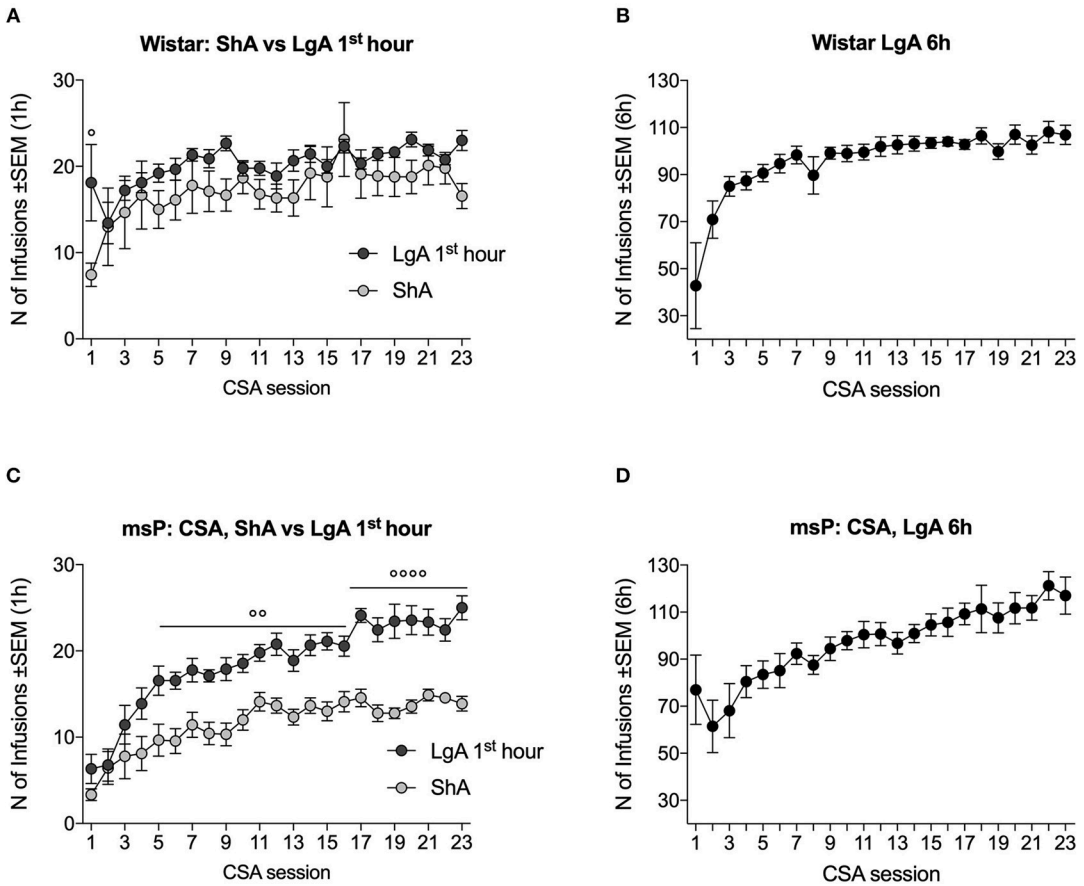

FIGURE 1 | Escalation of cocaine self-administration (CSA) in Wistar and msP rats. **(A)** The number of cocaine infusions that were received by Wistar rats in the first hour of ShA and LgA sessions was similar throughout training, with the exception of the first day. **(B)** The number of infusions that were received by Wistar rats during the entire 6 h LgA session increased over time. **(C)** The number of infusions that were received by msP rats during the first hour of the daily LgA sessions increased over training and was higher than ShA-trained msP rats beginning in the fifth session. **(D)** The number of infusions that were received by LgA-trained msP rats increased over training during the entire 6 h session. The data are expressed as mean ± SEM. $^{\circ}$ $p < 0.05$, $^{\circ\circ}$ $p < 0.01$, $^{\circ\circ\circ\circ}$ $p < 0.001$, compared with ShA.

$p < 0.001$]. The *post hoc* analysis indicated that LgA rats exhibited an increase in the number of infusions over time and differed significantly from ShA rats starting from the fifth session (**Figure 1C**). LgA msP rats exhibited an increase in lever pressing over time [$F_{(8,176)} = 8.9$, $p < 0.001$], with a progressive increase in the total number of daily (6 h) cocaine infusions that were earned (**Figure 1D**).

When the escalation ratio, calculated as a difference between the average infusions of the last 3 minus the first 3 LgA sessions, of msP and Wistar rats was compared, results indicated a significant difference between the two rat lines [$t_{16} = 2.9$, $p < 0.05$]. Escalation ratio was 39.3 ± 6.6 in msPs and 19.2 ± 3.3 in Wistars.

Effect of Ro 64-6198 on Cocaine and Saccharin Self-Administration in Wistar Rats

We next tested the effect of Ro 64-6198 on cocaine self-administration in Wistar rats. One additional LgA Wistar rat was excluded from the analysis because of the loss of catheter patency. Ro 64-6198 significantly decreased the number of infusions that were received by ShA Wistar rats [$F_{(2,8)} = 16.5$, $p < 0.01$]. Dunnett's *post hoc* test revealed that 3.0 mg/kg but not 1.0 mg/kg Ro 64-6198 significantly decreased the number of infusions compared with vehicle ($p < 0.01$; **Figure 2A**). Under LgA conditions, the ANOVA indicated no effect of treatment either during the first hour [$F_{(2,7)} = 2.8$, $p > 0.05$] or during the entire 6 h session [$F_{(2,7)} = 2.0$, $p > 0.05$; **Figures 2B,C**]. The ANOVA of the effect of Ro 64-6198 on saccharin self-administration indicated a main effect of treatment [$F_{(2,8)} = 27.7$, $p < 0.001$]. Dunnett's *post hoc* test showed that 3 mg/kg Ro 64-6198 significantly decreased saccharin self-administration ($p < 0.01$; **Figure 2D**).

Effect of Ro 64-6198 on Cocaine and Saccharin Self-Administration in msP Rats

In msP rats, Ro 64-6198 significantly decreased cocaine self-administration both in the ShA condition [$F_{(2,8)} = 15.3$, $p < 0.001$; **Figure 3A**] and in the first hour of the LgA condition [$F_{(2,8)} = 11.6$, $p < 0.01$; **Figure 3B**]. Dunnett's *post hoc* test

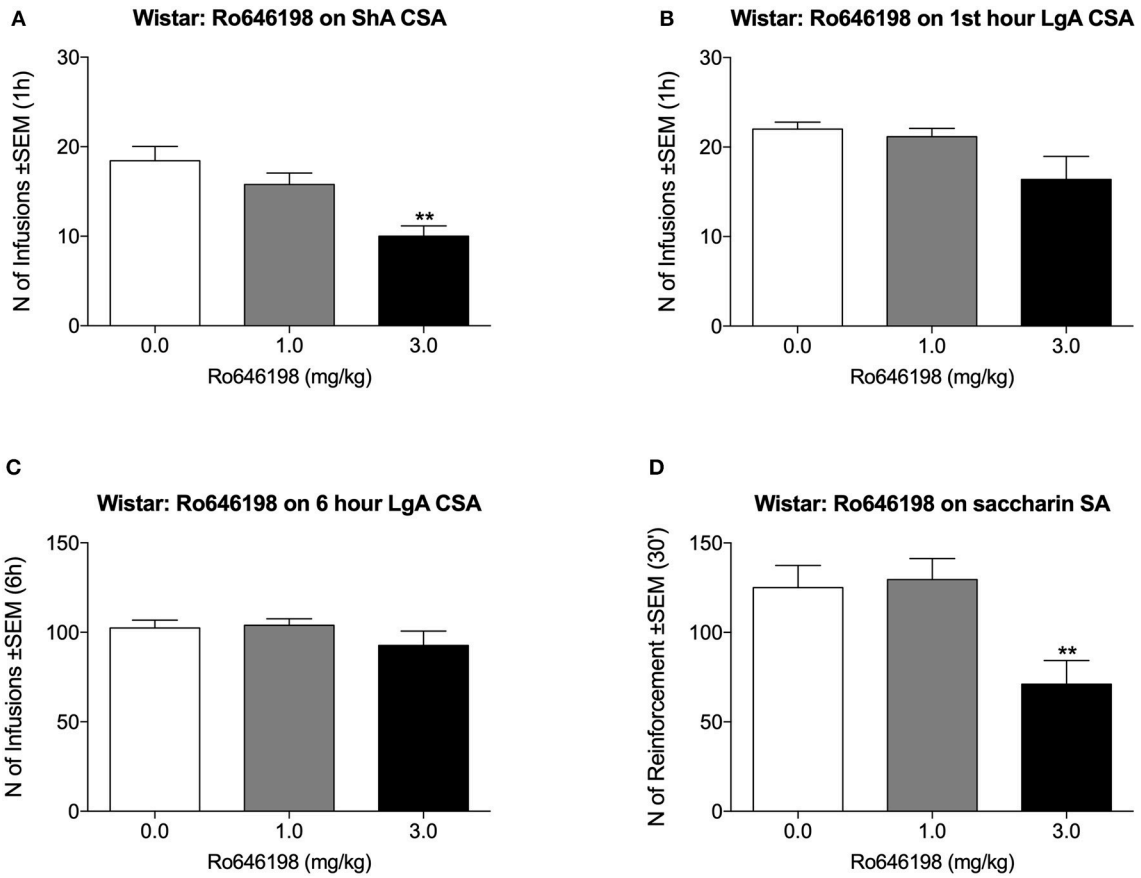

FIGURE 2 | Effect of Ro 64-6198 on cocaine and saccharin intake in Wistar rats. **(A)** Ro 64-6198 at a dose of 3.0 mg/kg decreased the number of infusions that were received by ShA rats. **(B,C)** Ro 64-6198 did not affect cocaine self-administration in LgA-trained rats either during the first hour of the session **(B)** or during the entire 6 h session **(C)**. **(D)** Ro 64-6198 at a dose of 3.0 mg/kg decreased saccharin self-administration. The data are expressed as mean ± SEM. $^{**}p < 0.01$, compared with Ro 64-6198 vehicle (0.0 mg/kg).

revealed a significant effect of 3.0 mg/kg Ro 64-6198 ($p < 0.01$). The ANOVA of the effect of Ro 64-6198 at 6 h in the LgA group revealed no effect of treatment [$F_{(2,8)} = 0.3, p > 0.05$; **Figure 3C**]. The ANOVA indicated no effect of Ro 64-6198 on saccharin self-administration [$F_{(2,8)} = 2.0, p > 0.05$; **Figure 3D**].

DISCUSSION

The present study found that cocaine intake was similar in Wistar and msP rats under ShA self-administration conditions, but Wistar rats received a slightly higher number of infusions. When the rats were exposed to LgA self-administration, msP rats exhibited greater escalation of cocaine intake. These results replicate our recent study, in which we found that msP rats exhibited greater escalation of cocaine self-administration compared with Wistar rats. Additionally, in response to an amphetamine challenge, msP rats exhibited greater locomotor stimulation, greater activation of mesolimbic circuitry, and higher extracellular levels of dopamine in the nucleus accumbens compared with Wistar rats (5). The msP rat line was originally selected for excessive alcohol drinking and preference. However, unknown is why they are also hypersensitive to psychostimulants. One possibility is that some of the genetic traits that confer greater vulnerability to psychostimulants also result in an increase in alcohol intake (i.e., common genetic factors may be responsible for increases in both alcohol consumption and the vulnerability to psychostimulants). In humans, cocaine and alcohol are often co-abused, and common genetic traits that confer vulnerability to their use have begun to emerge (2–4).

Previous studies showed that Sardinian alcohol-preferring rats, from which msP rats were derived, are characterized by the lower expression of dopamine D_1 and D_2 receptors in the striatum (29, 30). Under basal condition, these two rat lines may present a hypodopaminergic state that motivates them to take higher amounts of alcohol and cocaine. Theserat lines' ability to increase mesolimbic dopamine levels may help counteract such a deficiency in dopamine. Clinical studies have shown that human addicts have relatively low levels of dopamine receptors in the ventral striatum (31–34). Similar to msP rats, human addicts also present an increase in striatal activation, revealed by functional magnetic resonance imaging, in response to drug-related stimuli (5, 35–37). One possibility is that msP

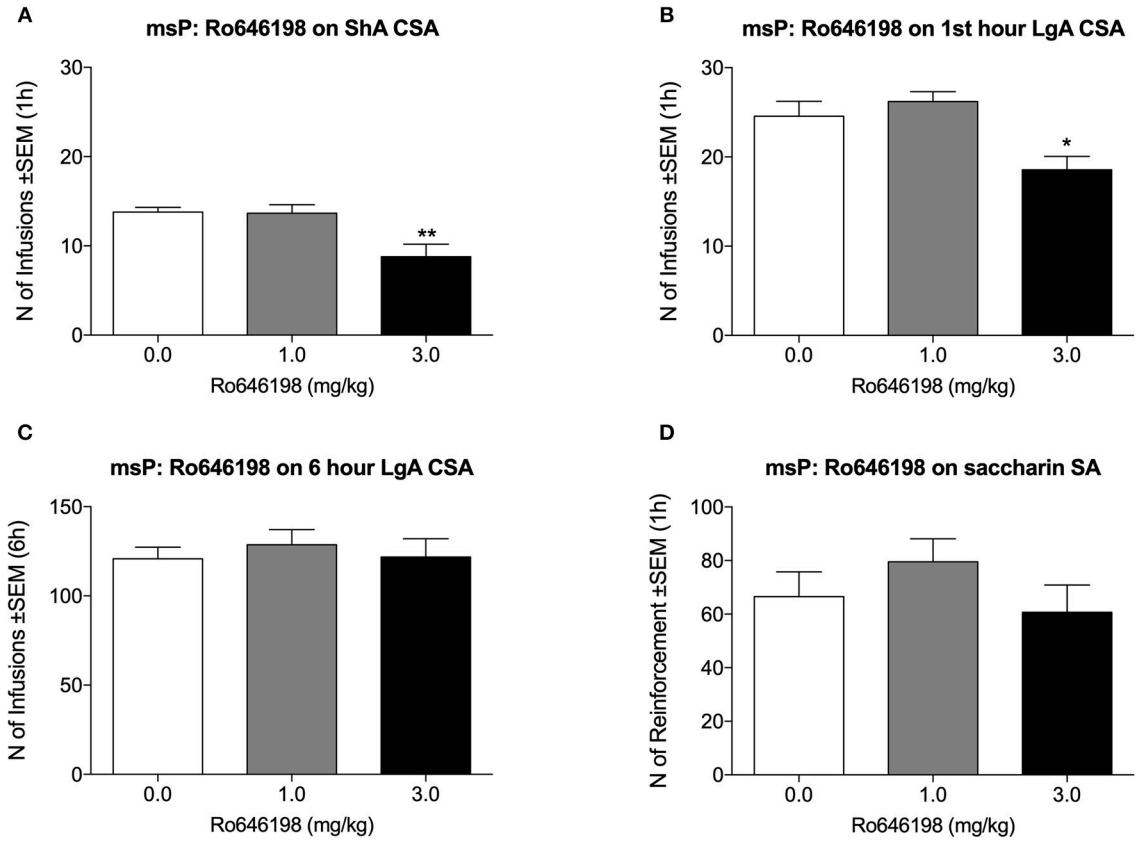

FIGURE 3 | Effect of Ro 64-6198 on cocaine and saccharin intake in msP rats. **(A)** Ro 64-6198 at a dose of 3.0 mg/kg decreased the number of infusions that were received in the ShA session. **(B,C)** Ro 64-6198 at a dose of 3.0 mg/kg decreased the number of infusions that were received by LgA rats during the first hour of the session **(B)** but not during the entire 6 h session **(C)**. **(D)** Ro 64-6198 did not affect saccharin self-administration. The data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with Ro 64-6198 vehicle (0.0 mg/kg).

rats may represent an animal model that mimics conditions that are associated with advanced stages of the addiction cycle, reflected by their greater tendency to escalate drug use. Another neurochemical alteration that has been detected in msP rats is overexpression of the N/OFQ system in various mesolimbic structures (8). The activation of NOP receptors following the administration of N/OFQ in the ventral tegmental area attenuated dopamine release in the nucleus accumbens (38). The intracerebroventricular administration of N/OFQ suppressed the morphine-induced increase in extracellular dopamine levels in the nucleus accumbens (39). Moreover, N/OFQ administration in the nucleus accumbens attenuated the ability of cocaine to enhance local extracellular dopamine levels (40). These data suggest that greater activity of the N/OFQ system may further contribute to the reduction of the basal tone of the dopamine system, thus contributing to the motivation to consume drugs of abuse. This possibility is indirectly supported by a previous study, in which NOP receptor knockout rats self-administered less cocaine, alcohol, and heroin compared with wildtype controls (41). Refuting this hypothesis, however, is evidence that the pharmacological activation of NOP receptors attenuates the motivation for several drugs of abuse, including cocaine and amphetamine (19, 20, 42–45). To clarify the role of the N/OFQ system in the modulation of drug abuse-related behaviors, we tested the effect of the selective and potent NOP receptor agonist Ro 64-6198 on cocaine self-administration in both Wistar and msP rats. As expected, Ro 64-6198 reduced ShA cocaine self-administration in both Wistar and msP rats. Under LgA conditions, the effect of Ro 64-6198 was significant only in msP rats. Moreover, the half-life of Ro 64-6198 in rodents is relatively long (5.5 h), and the significant effect of Ro 64-6198 that was observed in the first hour of the LgA session was not observed at 6 h (27).

In Wistar rats, 3 mg/kg Ro 64-6198 significantly decreased saccharin self-administration. This effect was not observed in msP rats. This may suggest that the effect of Ro 64-6198 on self-administration is secondary to the nonspecific inhibition of locomotor activity. However, this possibility is unlikely. Although Ro 64-6198 reduced saccharin self-administration in Wistar rats, it did not affect saccharin self-administration in msP rats. Previous studies reported that Ro 64-6198 doses up to 3 mg/kg exert specific effects that are not linked to motor impairment (46, 47).

Previous studies also showed that NOP receptor activation by Ro 64-6198 reduced the motivation for alcohol and morphine. Therefore, the lower motivation to pursue a reward may also extend to natural reinforcers (48–50). This hypothesis is supported by previous findings, in which the stimulation of N/OFQ transmission in NOP receptor knockout mice suppressed both basal and drug-induced increases in the hedonic state (51).

The activation of NOP receptors leads to rapid and prolonged receptor desensitization (52, 53). For example, after treatment with Ro 64-6198, NOP receptors rapidly internalized, and N/OFQ-mediated transmission remained impaired for at least 30 min (27). Based on these findings, one hypothesis is that the effect on cocaine self-administration may be mediated by NOP receptor desensitization rather than NOP receptor activation. This may explain why Ro 64-6198 was slightly more effective in msP rats than in Wistar rats because of msP rats' innate overexpression of NOP receptors that may be more sensitive to desensitization. This receptor desensitization hypothesis could also explain why the effect of Ro 64-6198 is relatively short (1 h), notwithstanding its relatively long half-life of 5.5 h. After 1 h, the desensitized NOP receptors may progressively become available again. Finally, the receptor desensitization hypothesis may reconcile recent findings that NOP-deficient rats exhibited lower motivation for cocaine and self-administered less cocaine (41) and may explain why, similar to agonist, NOP antagonists could reduce drug self-administration (54, 55).

Consistent with the desensitization hypothesis, it is tempting to hypothesize that chronic treatment with Ro 64-6198 would have led to a more pronounced effect, possibly leading to reduction of cocaine intake also in Wistar rats. Earlier studies, however, have shown that chronic (25 days) and acute treatment with Ro64-6198 produced comparable reduction of NOP binding levels due to receptor internalization (27). Based on this observation we speculate that our treatment condition was sufficient to detect an effect of Ro 64-6198. To further asses this conclusion future studies will have to investigate the effect of chronic drug treatment on cocaine self-administration. Another potential limitation is that the present study was limited to the analysis of the effect of Ro 64-6198 on cocaine intake without exploring its action of the motivation for the drug using progressive ratio, punished responding or second order schedules contingencies. The absence of these data hamper the possibility to drive clear conclusions on the effect of NOP activation on the motivation of this psychostimulant. Finally, we did not explore whether LgA exposure to cocaine may have led to a dependent-like state signaled by expression of drug withdrawal or hyper-anxiety. However, these conditions were demonstrated in earlier studies in which the same LgA schedule used here was applied (56).

In conclusion, the present study found that msP rats escalated their cocaine self-administration more rapidly than Wistar rats, and NOP receptor activation by Ro 64-6198 reduced cocaine consumption. The mechanism by which NOP receptor agonism leads to such effects is unknown, but receptor desensitization may be one possibility.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RC was responsible for the study concept and design. HL, GS, and QS performed the experiments. CN and AM assisted with the data analysis and interpretation of findings. HL, AM, NC, and RC drafted the manuscript. All of the authors critically reviewed the content and approved the final version for publication.

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REFERENCES

1. Karila L, Gorelick D, Weinstein A, Noble F, Benyamina A, Coscas S, et al. New treatments for cocaine dependence: a focused review. *Int J Neuropsychopharmacol.* (2008) 11:425–38. doi: 10.1017/S1461145707008097
2. Buhler KM, Gine E, Echeverry-Alzate V, Calleja-Conde J, de Fonseca FR, and Lopez-Moreno JA. Common single nucleotide variants underlying drug addiction: more than a decade of research. *Addict Biol.* (2015) 20:845–71. doi: 10.1111/adb.12204
3. Li MD, Burmeister M. New insights into the genetics of addiction. *Nat Rev Genet.* (2009) 10:225–31. doi: 10.1038/nrg2536
4. Palmer RH, Brick L, Nugent NR, Bidwell LC, McGeary JE, Knopik VS, et al. Examining the role of common genetic variants on alcohol, tobacco, cannabis and illicit drug dependence: genetics of vulnerability to drug dependence. *Addiction.* (2015) 110:530–7. doi: 10.1111/add.12815
5. Bifone A, Gozzi A, Cippitelli A, Matzeu A, Domi E, Li H, et al. phMRI, neurochemical and behavioral responses to psychostimulants distinguishing genetically selected alcohol-preferring from genetically heterogenous rats. *Addict Biol*. (2018). doi: 10.1111/adb.12671
6. Hansson AC, Cippitelli A, Sommer WH, Fedeli A, Bjork K, Soverchia L, et al. Variation at the rat Crhr1 locus and sensitivity to relapse into alcohol seeking induced by environmental stress. *Proc Natl Acad Sci USA*. (2006) 103:15236–41. doi: 10.1073/pnas.0604419103
7. Natividad LA, Buczynski MW, Herman MA, Kirson D, Oleata CS, Irimia C, et al. Constitutive increases in amygdalar corticotropin-releasing factor and fatty acid amide hydrolase drive an anxious phenotype. *Biol Psychiatry.* (2017) 82:500–510. doi: 10.1016/j.biopsych.2017.01.005
8. Economidou D, Hansson AC, Weiss F, Terasmaa A, Sommer WH, Cippitelli A, et al. Dysregulation of nociceptin/orphanin FQ activity in the amygdala is linked to excessive alcohol drinking in the rat. *Biol Psychiatry. (*2008) 64:211–8. doi: 10.1016/j.biopsych.2008.02.004
9. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, et al. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature.* (1995) 377:532–5. doi: 10.1038/377532a0
10. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, et al. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science.* (1995) 270:792–4. doi: 10.1126/science.270.5237.792

11. Mannelli P. Agonist-antagonist combinations in opioid dependence: a translational approach. *Dipend Patologiche.* (2010) 5:17–24.
12. Zaveri NT. The nociceptin/orphanin FQ receptor (NOP) as a target for drug abuse medications. *Curr Top Med Chem.* (2011) 11:1151–6. doi: 10.2174/156802611795371341
13. Toll L. The use of bifunctional NOP/mu and NOP receptor selective compounds for the treatment of pain, drug abuse, and psychiatric disorders. *Curr Pharm Des.* (2013) 19:7451–60. doi: 10.2174/13816128113199990354
14. Witkin JM, Statnick MA, Rorick-Kehn LM, Pintar JE, Ansonoff M, Chen Y, et al. The biology of Nociceptin/Orphanin FQ (N/OFQ) related to obesity, stress, anxiety, mood, and drug dependence. *Pharmacol Ther.* (2014) 141:283–99. doi: 10.1016/j.pharmthera.2013.10.011
15. Lutfy K, Zaveri NT. The Nociceptin receptor as an emerging molecular target for cocaine addiction. *Prog Mol Biol Transl Sci.* (2016) 137:149–81. doi: 10.1016/bs.pmbts.2015.10.003
16. Kallupi M, Shen Q, de Guglielmo G, Yasuda D, Journigan VB, Zaveri NT, et al. Buprenorphine requires concomitant activation of NOP and MOP receptors to reduce cocaine consumption. *Addict Biol.* (2018) 23:585–95. doi: 10.1111/adb.12513
17. Shen Q, Deng Y, Ciccocioppo R, Cannella N. Cebranopadol, a mixed opioid agonist, reduces cocaine self-administration through nociceptin opioid and mu opioid receptors. *Front Psychiatry*. (2017) 8:234. doi: 10.3389/fpsyt.2017.00234
18. de Guglielmo G, Matzeu A, Kononoff J, Mattioni J, Martin-Fardon R, George O. Cebranopadol blocks the escalation of cocaine intake and conditioned reinstatement of cocaine seeking in rats. *J Pharmacol Exp Ther.* (2017) 362:378–84. doi: 10.1124/jpet.117.241042
19. Kotlinska J, Rafalski P, Biala G, Dylag T, Rolka K, Silberring J. Nociceptin inhibits acquisition of amphetamine-induced place preference and sensitization to stereotypy in rats. *Eur J Pharmacol.* (2003) 474:233–9. doi: 10.1016/S0014-2999(03)02081-8
20. Kotlinska J, Wichmann J, Legowska A, Rolka K, Silberring J. Orphanin FQ/nociceptin but not Ro 65-6570 inhibits the expression of cocaine-induced conditioned place preference. *Behav Pharmacol.* (2002) 13:229–35. doi: 10.1097/00008877-200205000-00006
21. Marquez P, Nguyen AT, Hamid A, Lutfy K. The endogenous OFQ/N/ORL-1 receptor system regulates the rewarding effects of acute cocaine. *Neuropharmacology.* (2008) 54:564–8. doi: 10.1016/j.neuropharm.2007.11.003
22. Marquez P, Hamid A, Lutfy K. The role of NOP receptors in psychomotor stimulation and locomotor sensitization induced by cocaine and amphetamine in mice. *Eur J Pharmacol.* (2013) 707:41–5. doi: 10.1016/j.ejphar.2013.03.021
23. Bebawy D, Marquez P, Samboul S, Parikh D, Hamid A, Lutfy K. Orphanin FQ/nociceptin not only blocks but also reverses behavioral adaptive changes induced by repeated cocaine in mice. *Biol Psychiatry.* (2010) 68:223–30. doi: 10.1016/j.biopsych.2010.02.010
24. Devine DP, Reinscheid RK, Monsma FJ, Jr, Civelli O, Akil H. The novel neuropeptide orphanin FQ fails to produce conditioned place preference or aversion. *Brain Res.* (1996) 727:225–9. doi: 10.1016/0006-8993(96)00476-3
25. Lin AP, Ko MC. The therapeutic potential of nociceptin/orphanin FQ receptor agonists as analgesics without abuse liability. *ACS Chem Neurosci.* (2013) 4:214–24. doi: 10.1021/cn300124f
26. de Guglielmo G, Martin-Fardon R, Teshima K, Ciccocioppo R, Weiss F. MT-7716, a potent NOP receptor agonist, preferentially reduces ethanol seeking and reinforcement in post-dependent rats. *Addict Biol.* (2015) 20:643–51. doi: 10.1111/adb.12157
27. Dautzenberg FM, Wichmann J, Higelin J, Py-Lang G, Kratzeisen C, Malherbe P, et al. Pharmacological characterization of the novel nonpeptide orphanin FQ/nociceptin receptor agonist Ro 64-6198: rapid and reversible desensitization of the ORL1 receptor *in vitro* and lack of tolerance *in vivo*. *J Pharmacol Exp Ther.* (2001) 298:812–9.
28. Ahmed SH, Koob GF. Transition from moderate to excessive drug intake: change in hedonic set point. *Science.* (1998) 282:298–300. doi: 10.1126/science.282.5387.298
29. De Montis MG, Gambarana C, Gessa GL, Meloni D, Tagliamonte A, Stefanini E. Reduced [3H]SCH 23390 binding and DA-sensitive adenylyl cyclase in the limbic system of ethanol-preferring rats. *Alcohol Alcohol.* (1993) 28:397–400.
30. Stefanini E, Frau M, Garau MG, Garau B, Fadda F, Gessa GL. Alcohol-preferring rats have fewer dopamine D2 receptors in the limbic system. *Alcohol Alcohol.* (1992) 27:127–30.
31. Hietala J, West C, Syvalahti E, Nagren K, Lehikoinen P, Sonninen P, et al. Striatal D2 dopamine receptor binding characteristics *in vivo* in patients with alcohol dependence. *Psychopharmacology (Berl).* (1994) 116:285–90. doi: 10.1007/BF02245330
32. Volkow ND, Wang GJ, Maynard L, Fowler JS, Jayne B, Telang F, et al. Effects of alcohol detoxification on dopamine D2 receptors in alcoholics: a preliminary study. *Psychiatry Res.* (2002) 116:163–72. doi: 10.1016/S0925-4927(02)00087-2
33. Volkow ND, Wang GJ, Telang F, Fowler JS, Logan J, Jayne M, et al. Profound decreases in dopamine release in striatum in detoxified alcoholics: possible orbitofrontal involvement. *J Neurosci.* (2007) 27:12700–6. doi: 10.1523/JNEUROSCI.3371-07.2007
34. Martinez D, Gil R, Slifstein M, Hwang DR, Huang Y, Perez A, et al. Alcohol dependence is associated with blunted dopamine transmission in the ventral striatum. *Biol Psychiatry.* (2005) 58:779–86. doi: 10.1016/j.biopsych.2005.04.044
35. Braus DF, Wrase J, Grusser S, Hermann D, Ruf M, Flor H, et al. Alcohol-associated stimuli activate the ventral striatum in abstinent alcoholics. *J Neural Transm (Vienna).* (2001) 108:887–94. doi: 10.1007/s007020170038
36. Mann K, Vollstadt-Klein S, Reinhard I, Lemenager T, Fauth-Buhler M, Hermann D, et al. Predicting naltrexone response in alcohol-dependent patients: the contribution of functional magnetic resonance imaging. *Alcohol Clin Exp Res.* (2014) 38:2754–62. doi: 10.1111/acer.12546
37. Reinhard I, Lemenager T, Fauth-Buhler M, Hermann D, Hoffmann S, Heinz A, et al. A comparison of region-of-interest measures for extracting whole brain data using survival analysis in alcoholism as an example. *J Neurosci Methods.* (2015) 242:58–64. doi: 10.1016/j.jneumeth.2015.01.001
38. Murphy NP, Maidment NT. Orphanin FQ/nociceptin modulation of mesolimbic dopamine transmission determined by microdialysis. *J Neurochem.* (1999) 73:179–86. doi: 10.1046/j.1471-4159.1999.0730179.x
39. Di Giannuario A, Pieretti S. Nociceptin differentially affects morphine-induced dopamine release from the nucleus accumbens and nucleus caudate in rats. *Peptides.* (2000) 21:1125–30. doi: 10.1016/S0196-9781(00)00250-3
40. Vazquez-DeRose J, Stauber G, Khroyan TV, Xie XS, Zaveri NT, Toll L. Retrodialysis of N/OFQ into the nucleus accumbens shell blocks cocaine-induced increases in extracellular dopamine and locomotor activity. *Eur J Pharmacol.* (2013) 699:200–6. doi: 10.1016/j.ejphar.2012.11.050
41. Kallupi M, Scuppa G, de Guglielmo G, Calo G, Weiss F, Statnick MA, et al. Genetic Deletion of the Nociceptin/Orphanin FQ receptor in the rat confers resilience to the development of drug addiction. *Neuropsychopharmacology.* (2017) 42:695–706. doi: 10.1038/npp.2016.171
42. Rutten K, De Vry J, Bruckmann W, Tzschentke TM. Effects of the NOP receptor agonist Ro65-6570 on the acquisition of opiate- and psychostimulant-induced conditioned place preference in rats. *Eur J Pharmacol.* (2010) 645:119–26. doi: 10.1016/j.ejphar.2010.07.036
43. Zhao RJ, Woo RS, Jeong MS, Shin BS, Kim DG, Kim KW. Orphanin FQ/nociceptin blocks methamphetamine place preference in rats. *Neuroreport.* (2003) 14:2383–5. doi: 10.1097/00001756-200312190-00019
44. Lutfy K, Khaliq I, Carroll FI, Maidment NT. Orphanin FQ/nociceptin blocks cocaine-induced behavioral sensitization in rats. *Psychopharmacology (Berl).* (2002) 164:168–76. doi: 10.1007/s00213-002-1192-1
45. Zaveri NT, Marquez PV, Meyer ME, Hamid A, Lutfy K. The Nociceptin Receptor (NOP) Agonist AT-312 blocks acquisition of morphine- and cocaine-induced conditioned place preference in mice. *Front Psychiatry.* (2018) 9:638. doi: 10.3389/fpsyt.2018.00638
46. Goeldner C, Spooren W, Wichmann J, Prinssen EP. Further characterization of the prototypical nociceptin/orphanin FQ peptide receptor agonist Ro 64-6198 in rodent models of conflict anxiety and despair. *Psychopharmacology (Berl).* (2012) 222:203–14. doi: 10.1007/s00213-012-2636-x
47. Varty GB, Hyde LA, Hodgson RA, Lu SX, McCool MF, Kazdoba TM, et al. Characterization of the nociceptin receptor (ORL-1) agonist, Ro64-6198, in tests of anxiety across multiple species. *Psychopharmacology (Berl).* (2005) 182:132–43. doi: 10.1007/s00213-005-0041-4
48. Kuzmin A, Sandin J, Terenius L, Ogren SO. Acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice:

effects of opioid receptor-like 1 receptor agonists and naloxone. *J Pharmacol Exp Ther.* (2003) 304:310–8. doi: 10.1124/jpet.102.041350
49. Shoblock JR, Wichmann J, Maidment NT. The effect of a systemically active ORL-1 agonist, Ro 64-6198, on the acquisition, expression, extinction, and reinstatement of morphine conditioned place preference. *Neuropharmacology.* (2005) 49:439–46. doi: 10.1016/j.neuropharm.2005.04.008
50. Kuzmin A, Kreek MJ, Bakalkin G, Liljequist S. The nociceptin/orphanin FQ receptor agonist Ro 64-6198 reduces alcohol self-administration and prevents relapse-like alcohol drinking. *Neuropsychopharmacology.* (2007) 32:902–10. doi: 10.1038/sj.npp.1301169
51. Sakoori K, Murphy NP. Endogenous nociceptin (orphanin FQ) suppresses basal hedonic state and acute reward responses to methamphetamine and ethanol, but facilitates chronic responses. *Neuropsychopharmacology.* (2008) 33:877–91. doi: 10.1038/sj.npp.1301459
52. Spampinato S, Di Toro R, Qasem AR. Nociceptin-induced internalization of the ORL1 receptor in human neuroblastoma cells. *Neuroreport.* (2001) 12:3159–63. doi: 10.1097/00001756-200110080-00035
53. Spampinato S, Baiula M. Agonist-regulated endocytosis and desensitization of the human nociceptin receptor. *Neuroreport.* (2006) 17:173–7. doi: 10.1097/01.wnr.0000198431.21765.b5
54. Rorick-Kehn LM, Ciccocioppo R, Wong CJ, Witkin JM, Martinez-Grau MA, Stopponi S, et al. A novel, orally bioavailable nociceptin receptor antagonist, LY2940094, reduces ethanol self-administration and ethanol seeking in animal models. *Alcohol Clin Exp Res.* (2016) 40:945–54. doi: 10.1111/acer.13052
55. Cippitelli A, Schoch J, Debevec G, Brunori G, Zaveri NT, Toll L. A key role for the N/OFQ-NOP receptor system in modulating nicotine taking in a model of nicotine and alcohol co-administration. *Sci Rep.* (2016) 6:26594. doi: 10.1038/srep26594
56. Aujla H, Martin-Fardon R, Weiss F. Rats with extended access to cocaine exhibit increased stress reactivity and sensitivity to the anxiolytic-like effects of the mGluR 2/3 agonist LY379268 during abstinence. *Neuropsychopharmacology.* (2008) 33:1818–26. doi: 10.1038/sj.npp.1301588

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REVIEW
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Cutting-Edge Search for Safer Opioid Pain Relief: Retrospective Review of Salvinorin A and Its Analogs

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Over the years, pain has contributed to low life quality, poor health, and economic loss. Opioids are very effective analgesic drugs for treating mild, moderate, or severe pain. Therapeutic application of opioids has been limited by short and long-term side effects. These side effects and opioid-overuse crisis has intensified interest in the search for new molecular targets and drugs. The present review focuses on salvinorin A and its analogs with the aim of exploring their structural and pharmacological profiles as clues for the development of safer analgesics. Ethnopharmacological reports and growing preclinical data have demonstrated the antinociceptive effect of salvinorin A and some of its analogs. The pharmacology of analogs modified at C-2 dominates the literature when compared to the ones from other positions. The distinctive binding affinity of these analogs seems to correlate with their chemical structure and *in vivo* antinociceptive effects. The high susceptibility of salvinorin A to chemical modification makes it an important pharmacological tool for cellular probing and developing analogs with promising analgesic effects. Additional research is still needed to draw reliable conclusions on the therapeutic potential of salvinorin A and its analogs.

Keywords: analgesic, opioid receptors, salvinorin A, side effects, analogs

INTRODUCTION

Pain management is a challenging medical issue that requires a wide range of expertise and innovative ideas (1). Medicinal chemistry as well as extensive analysis of opioid receptors have increased the possibility of developing novel analgesics that are devoid of detrimental actions (2–6). Pain as an unpleasant sensory and emotional experience has been managed by different classes of drugs such as non-steroidal anti-inflammatory drugs, glucocorticoids, sodium channels inhibitors (local anesthetics), anti-epileptic drugs, tricyclic antidepressants, and opiates (2).

Opioid medications that mimic endogenous opioid peptides (dynorphins, endorphins, and enkephalins) typically bind to subtypes of opioid receptors (kappa-KOP, mu-MOP, and/or delta-DOP) to suppress pain (7). In addition, the activation of nociceptin/orphanin FQ peptide receptor (the fourth members of the opioid family of G protein-coupled receptors) by its endogenous peptide nociceptin/orphanin FQ (N/OFQ) modulates stress, reward and pain circuitry in several brain areas (8–11). A schematic representation of the pain and opioid sites of action, as shown in **Figure 1**, identifies important structures and pain modulatory circuits (12). The detailed account of signal transduction through opioid receptors, as illustrated by **Figure 2**, has been widely reported (13–17).

The KOP ligands are important research tools and promising molecules for safer treatment of pain (18). The antagonists or partial agonists of KOP could prevent relapse to drug dependence (19–23). The blockade of KOP on dopamine terminals could disinhibit dopamine release in the nucleus accumbens and prevent drug withdrawal-induced dysphoria (24). This receptor remains an important cellular mediator of stress, reward, abuse, emotion, perception (25, 26), sedation (27), hypothermia (28), depression (29, 30), hallucination (31), conditioned place aversion, and locomotion impairment (32). Despite the possibility of undesirable KOP-mediated effects (33, 34), evidence has shown that this receptor subtype is an alternative molecular target for the development of safer analgesics (35).

Recently, Che et al. (3) conducted research on the active-state crystal structure of the KOP complex with a high-affinity agonist to provide molecular details of KOP and overcome the therapeutic limitations of its agonist. In this study, the authors identified residues that are critical for KOP activation and illuminate key molecular determinants of subtype selectivity and signaling bias. The affinity and specificity of drugs to KOP are fundamental to the array of inducible-biological effects (**Table 1**). The development of drugs that clearly separate pain relief from unwanted side effects has remained challenging and elusive.

Natural products are important sources of new drugs (36). Several active principles from medicinal plants have been used for pain relief (37). The main active principle of *Salvia divinorum* (38), salvinorin A, had been suggested as a useful research tool toward the development of analgesic drugs (39). Salvinorin A has a distinctive mode of action and pharmacology. Unlike psilocybin and lysergic acid diethylamide (alkaloidal hallucinogens which interact with specific serotonin receptor subtypes), the hallucinogenic effect of salvinorin A has been associated with its potent and selective KOP agonism. Salvinorin A shows no significant binding to over 50 other pharmacologically important receptors, transporter proteins and ion channels (40). As a non-nitrogenous KOP agonist (40), this compound differs from typical alkaloid opioid agonists. Recently, the potent antinociceptive effect of salvinorin A was reported in the neuropathic pain model (41). Over the years, medicinal chemists have synthesized several hundred analogs of this compound including herkinorin, kurkinorin, P-37, PR-38, methoxymethyl- and ethoxymethyl ether of salvinorin B, and β-tetrahydropyranyl ether of salvinorin B (4, 6, 42). Previous preclinical reports on salvinorin A and its analogs have revealed their promising antinociceptive effects (41, 43, 44). Hence, this review explored the structural and pharmacological profiles of salvinorin A and its analogs toward the development of new analgesic drugs.

SALVINORIN A AND ITS ANALOGS: STRUCTURE-ACTIVITY RELATIONSHIP

Salvinorin A

The unique biological effects of salvinorin A (**Figure 3A**) have motivated many scientists to seek correlations between its chemical structure and pharmacological activity (4, 6, 42).

Following the determination of the salvinorin A structure through A single-crystal x-ray analysis [(38, 45), molecular modeling studies were performed to determine the interaction of this compound with KOP (40). Initially, the salvinorin A crystal structure (45) was docked by superimposition of its aromatic centroids and the carbonyl atoms with those of bound U69593 (known KOP agonist which shares structural similarity such as an aromatic ring and ester carbonyl groups separated by a short bond with salvinorin A). As a hydrogen bond acceptor, the carbonyl functionality supports the proposed role of Y139 and its interaction with the lactone carbonyl of salvinorin A (40, 46).

Previous study showed the list of residues that could form the salvinorin A-binding site of the KOP (40). The KOP models could accommodate the furan oxygen and 4-methoxycarbonyl functionality but not the 2-acetoxy group (40). The key residues in KOP that are responsible for the high binding affinity and efficacy of salvinorin A as well as important contacts between this compound and KOP have been identified through mutagenesis studies (6, 47). Potent and efficacious interactions of this compound with KOP are due to novel binding modes within a common three-dimensional space for binding and activating KOP (47).

Additional studies correlated the structure and activities of salvinorin A with the potential binding site on KOP. For instance, a change to the furan ring resulted in analogs that are more sterically demanding than a one-for-one aryl ring replacement (6). Sterically hindered environment of C-1 carbonyl of salvinorin A is not essential for activity as it is incapable of forming specific donor/acceptor contacts with residues in the receptor model, (48). In contrary, the 2-acetoxy group of salvinorin A which makes specific donor/acceptor contacts in the model is required for activity (45). Meanwhile, the lack of consensus binding model makes generalization of structure-activity relationships a challenge (6).

According to Yan (47), salvinorin A uses its flexible functional groups at C-2, C-4 and C-12 to optimize KOP interactions and stabilize itself in the binding site. Moreso, this compound also takes advantage of the conformational changes induced by G protein-coupling to facilitate active state stabilization and activation of downstream signaling events.

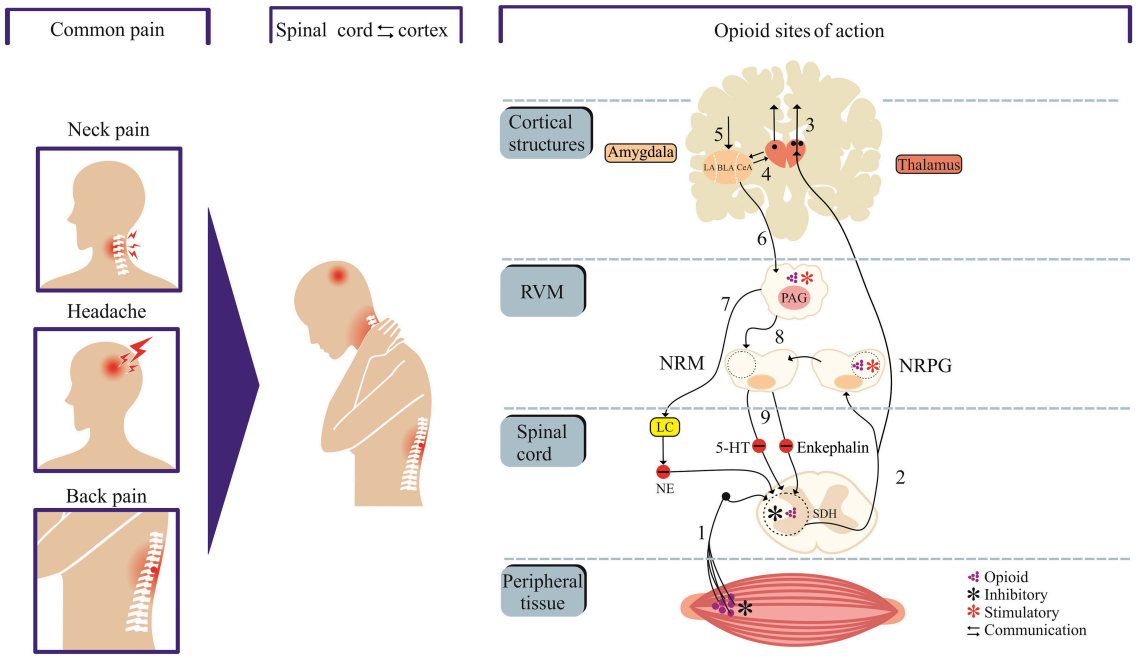


FIGURE 1 | Neural circuits of pain and opioid site of action. Cortex and spinal cord communication modulate pain perception and offer targets for opioid drugs. Neural projections from peripheral tissue transmit nociceptive inputs through primary afferent fibers (1) to the spinal dorsal horn (SDH) before reaching the thalamus (2). Neural projections from thalamus target cortical sites (centers of pain processing, cognition, perceptions and integration) and amygdala ("emotional site"). The amygdala receives nociceptive inputs from the thalamus and cortex. The descending pain control system is mediated through projections from structures such as amygdala and hypothalamus to the periaqueductal gray matter (PAG) which in turn communicates with the rostral ventromedial medulla (RVM). The neural components within RVM [the nucleus raphe magnus (NRM) and nucleus reticularis paragigantocellularis (NRPG)] project to the spinal or medullary dorsal horns to directly or indirectly enhance or attenuate nociceptive transmission. The 5-hydroxytryptamine (5-HT) and enkephalin-containing neurons in the NRM project to the substantia gelatinosa of the dorsal horn and exert an inhibitory influence on transmission. Opioids sites of action include dorsal horn and peripheral terminals of nociceptive afferent neurons where opioids inhibit transmission. Opioids stimulate PAG and NRPG (blue asterisk), which in turn project to the rostroventral medulla. The locus coeruleus (LC) which receives inputs from the PAG releases noradrenalin to the dorsal horn, which in turn inhibits nociceptive transmission. Areas labeled 2–4 in red color and 5–8 in green color represent ascending and descending tracts, respectively.

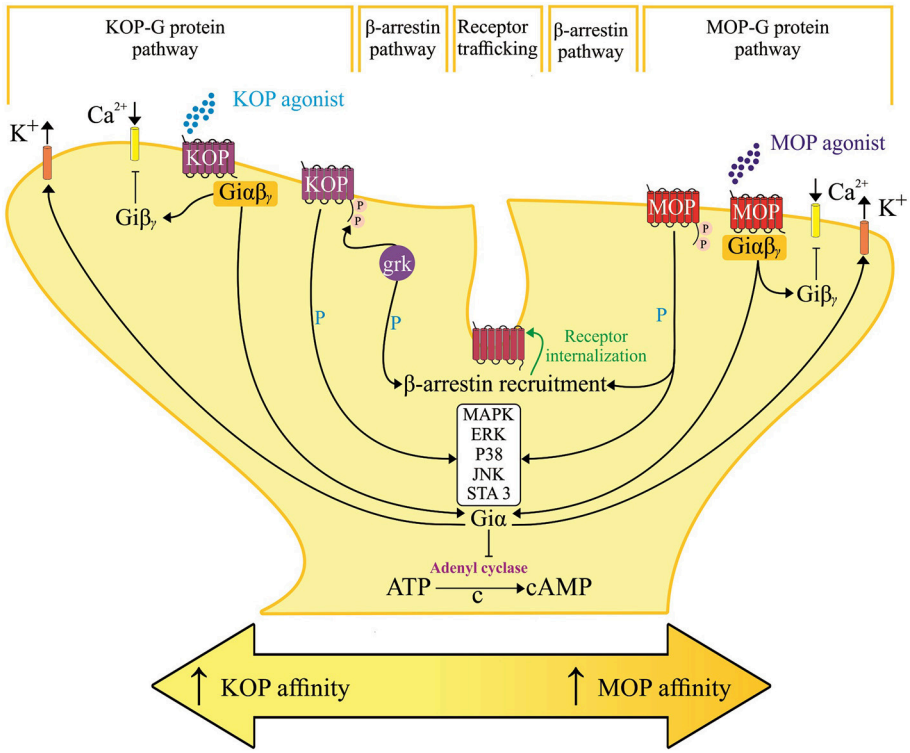
Salvinorin A, a neo-clerodane diterpenoid with seven stereogenic centers and three different types of ester functionalities, is a challenging substrate for chemical modifications because minor modifications can result in a complete loss or increase in pharmacological activity. For example, a product of hydrolysis of the C2 acetoxy side chain (salvinorin B) is totally devoid of activity, but other changes to this position actually demonstrate the highest KOP binding affinities (4, 6). Structural modifications of salvinorin A at the C1, C4, C12, and the C17-positions have been mostly associated with a reduction in KOP binding affinity (4, 6, 49). Over the years, the carbonate, carbamate, ester, ether, amine, amide, sulfonic ester, sulfonamide, thioester, halide, and other groups have been introduced to the salvinorin A molecule with a wide variety of outcomes (6, 50).

Furthermore, potential interactions of salvinorin A with other receptors have been either hypothesized or demonstrated preclinically by some researchers. Previous study showed that salvinorin A allosterically modulates MOP binding (51). The *in vitro* testing which showed the binding affinity of salvinorin A (EC50 values of 89 nM) against the D2 High receptor and blockade by 10 μM S-sulpiride (an antagonist of DRD2) has resulted into the discussion of partial agonism of salvinorin A at D2 receptor (52). Moreso, computational studies have predicted CB1, CB2, or DRD2 as a potential targets of salvinorin A (53). In an *in vivo* test, the attenuation of neuropathic pain by Salvinorin A was blocked by CB1 and KOP antagonists (41). The inhibition of the effects of salvinorin A on colonic motility by antagonists of OPRK, CB1 and CB2 *in vitro* and largely by antagonists of OPRK *in vivo* (54) suggests mechanistic complexity in the activity of salvinorin A as against widely acclaimed KOP selectivity.

ANALOGS FROM THE MODIFICATION OF SALVINORIN A AT C-2

Herkinorin

Analogs with bulky alkyl esters at C-2 resulted in a loss in affinity for KOP (55), but the replacement of the alkyl with aryl esters at C-2 results in a lower affinity and potency for KOP; and an increase in affinity for MOP (56). Herkinorin (**Figure 3B**) is the first example of salvinorin A derivative with Ki at MOP (12 ± 1.0 nM) while still retaining lower affinity at KOP (90 ± 2.0 nM).



Salvinorin A	Morphine
Methoxymethyl ether Sal B	Herkinorin
Ethoxymethyl ether Sal B	Herkamide
β-tetrahydropyranyl ether Sal B	Kurkinorin

FIGURE 2 | Hypothetical representation of signal transduction and trafficking of mu [μ] and kappa [κ] opioid receptor. Converging downstream pathways are activated by salvinorin A and its analogs with selective action and varying affinity on their respective opioid receptor subtypes. Arrows, activation; T lines, blockade of function; βγ, G protein β-γ subunit; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinases; GRK-3, G protein-receptor kinase 3; P, phosphorylation; C →, cyclization of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) through the cleavage of pyrophosphate.

Methoxymethyl and Ethoxymethyl Ether of Salvinorin B

These compounds have an alkoxyalkyl ether bond, which replaced acetoxy group at C-2. The alkoxy methyl ether substituents improved KOP affinity and potency. The methoxymethyl ether of salvinorin B (**Figure 3G**) has higher binding affinity to KOP (Ki = 0.60 ± 0.1 nM) and potency (EC_{50} = 0.40 ± 0.04 nM) than salvinorin A. The putative synergistic binding interactions of the additional oxygen in the substituent have been associated with the higher affinity and potency (57, 58). The ethoxymethyl ether of salvinorin B (**Figure 3F**) also displayed a higher KOP binding affinity (Ki = 0.32 nM) and potency (EC50 = 0.14 nM) than other salvinorin A analogs (4, 59).

Methyl Salvinorin B-2-O-Malonate and 2-O-Cinnamoylsalvinorin B

Previous studies have shown the synthesis and biological activities of Michael acceptor-type of salvinorin A analogs, such as methyl salvinorin B-2-*O*-malonate (PR-37) and 2-*O*-cinnamoylsalvinorin B (PR-38) (42, 54, 60). The addition of a second H-binding acceptor leads to the development of a malonate analog (PR-37) (**Figure 3D**) that displayed a 3-fold improvement in KOP affinity (Ki = 2.0 ± 0.9 nM) (42). However, other malonic ester substitutions with different carbonyl spacings reduced biological activity (6). The replacement of the acetate substituent with the spirolactone group caused a restriction in bond rotation and a decrease in potency (61). The analog with cinnamic ester functionality (PR-38) (**Figure 3E**) displayed not

only KOP affinity (Ki = 9.6 ± 2.0 nM) but also MOP (Ki = 52 ± 9.0 nM) with 5.4 MOP/KOP selectivity (42).

Herkamide

Tidgewell (62) showed a lower KOP affinity as a result of bioisosteric exchange of the 2-acetoxy subunit of salvinorin A (Ki = 1.9 ± 0.2) with acetamide (Ki = 30 ± 2.0 nM). Although the introduction of a phenyl ring in the herkamide analog (**Figure 3I**) decreased KOP affinity, an increase in affinity for MOP - Ki = 3.1 ± 0.4 as compared to herkinorin Ki = 12 ± 1.0 nM was reported. In addition, herkamide MOP selectivity (KOP/MOP = 0.0004) was shown to be higher than that of herkinorin (KOP/MOP = 0.13).

TABLE 1 | Varying degree of opioid receptor involvement in some pharmacological effect.

Effects	MOP	DOP	KOP
Analgesia	+++	±	++
Sedation	++	–	++
Respiratory depression	+++	++	–
Constipation	++	++	+
Euphoria	+++	–	–
Dysphoria	–	–	+++
Depressive behavior	–	–	+++
Hallucination	±	–	+++
Physical dependence	+++	–	+

MOP, mu-opioid receptor; DOP, delta-opioid receptor; KOP, kappa-opioid receptor; ±, more or less; -, no effect; +, low effect; ++, intermediate effect; +++, high.

Kurkinorin

The introduction of a double bond between C-2 and C-3 in herkinorin resulted in the new analog kurkinorin (**Figure 3C**). *In vitro* functional assay revealed that kurkinorin was more selective for MOP (>8,000-fold selectivity over KOP) than morphine (66-fold selectivity over KOP) and herkinorin (4.25-fold selectivity over KOP). Moreover, kurkinorin has similar potency when compared to MOP agonist such as DAMGO in forskolin-induced cAMP accumulation assays (63).

β-Tetrahydropyranyl Ether of Salvinorin B

The relative flexibility of the acetoxy (C-2) subunit and potential adoption of different conformations when interacting with KOP has been hypothesized and studied in β-tetrahydropyranyl ether of salvinorin B (**Figure 3H**). Prevatt-Smith et al. (59) applied the concept of conformational restriction toward the

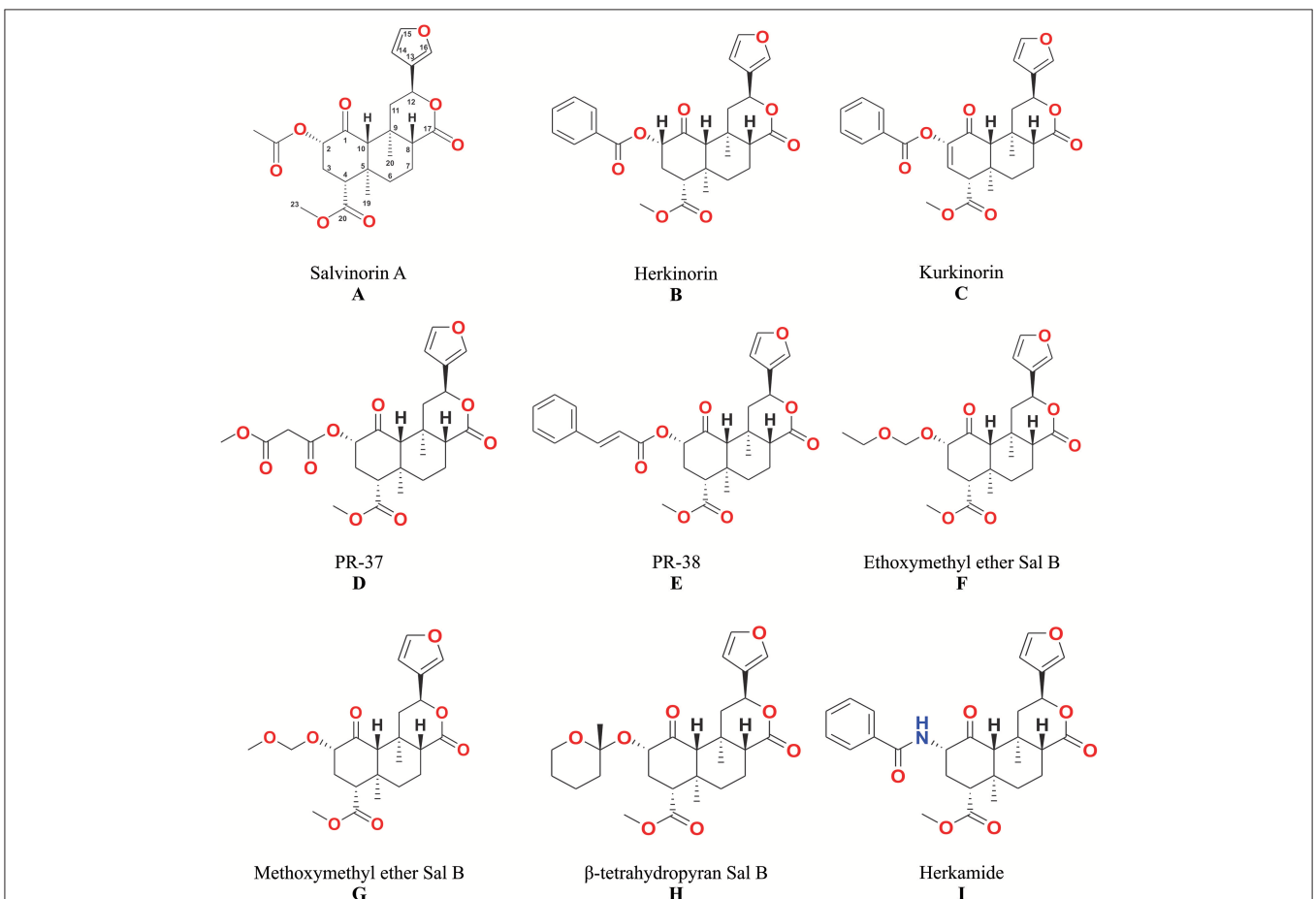


FIGURE 3 | The structures of salvinorin A **(A)** and its analogs **(B–I)**.

development of ligands as tools to elucidate KOP affinity and potency. The new analog β-tetrahydropyranyl ether of salvinorin B showed slightly higher KOP affinity (Ki = 6.21 ± 0.4) than salvinorin A (Ki = 7.40 ± 0.4). This result showed that the rotational restriction strategy as proposed by Prevatt-Smith et al. (59) only led to small changes in binding values.

ANALOGS FROM THE MODIFICATION OF SALVINORIN A AT C-4

Unlike the modification of salvinorin A at C-2, the structural modifications at the C-4 present challenges because selective hydrolysis of methyl ester requires a more drastic condition that often leads to C-8 epimerization (6). Hence, additional efforts are often needed toward the separation of diastereoisomers. This seems to be part of the reason why analogs from C-4 position are fewer than that of C-2. A total loss of KOP binding affinity (Ki > 1000 nM) was reported for methyl, propyl and methoxymethyl esters at C-4 (57, 64).

The loss of KOP binding affinity in the long-chain ester has been associated with the fact that the pocket where the methyl ester fits is small and delimited by Trp287 and Tyr320 amino acids residues (65). Reduction of ester to alcohol has also led to an 87-fold decrease in KOP binding affinity as compared to salvinorin A (58). Consistent with these data, other studies have shown 33-fold and 385-fold losses in KOP affinity (64, 66). Some esters with modified regiochemistry with the exception of cyclopropyl ester showed a 17-fold loss of affinity (64).

The replacement of methyl ester at C-4 with amides or amines resulted in a 535-fold loss or a total loss (Ki > 10,000 nM) of KOP binding affinity, respectively (66). With the exception of alanine, the introduction of amino acid at C-4 resulted in a total loss of affinity (57, 64). Some substitutions with functional groups, such as carboxylic acid and aldehyde, have also resulted in total loss of KOP binding affinity (64).

ANALOGS FROM THE MODIFICATION OF SALVINORIN A AT C-12

The study of the analogs with substitutions at C-12 (the furan ring of salvinorin A) has attracted attention as a result of their metabolic stability (67). Although the interaction between furan and KOP is not well-established, the removal of this ring in salvinorin A resulted in total loss of activity while its hydrogenation resulted in a 7-fold loss (Ki = 14 ± 1.0 vs. 1.9 ± 0.2) in KOP binding affinity (68). Perhaps the possibility of hydrogen bonding, hydrophobic interactions or even π-π stacking type is essential for a receptor's recognition. (69), the regiochemical modification of the furan resulted in a 2-fold decrease in potency (EC_{50} = 12.2 ± 4.4 nM) without significant change in efficacy (E_{max} = 97 ± 2%) when compared to salvinorin A (vs. EC_{50} = 6.11 ± 0.04 nM and vs. E_{max} = 97 ± 8%).

DISCUSSION ON ANTINOCICEPTIVE EFFECT OF SALVINORIN A AND ITS ANALOGS

Medicinal chemists have consistently modified salvinorin A structures to produce a wide range of analogs (4, 6). These efforts have helped to further understanding of salvinorin A chemistry and pharmacology as well as developing new compounds with potential therapeutic values (70). Since the identification of salvinorin A by Bücheler et al. (71), scientists have shown interests in its analgesic potential. Recently, the effectiveness of salvinorin A in a rodent model of pain showed that this compound could be beneficial for neuropathic pain relief (41). Salvinorin A is lipophilic, and it is mainly absorbed through the respiratory tract and to a lesser extent by the oral mucosa (72). Following the isolation and characterization of salvinorin A *in vitro* (receptor binding and functional assays) and *in vivo*, chemical modification of this compound led to changes in pharmacological parameters including stability, bioavailability, binding affinity, potency, functional activities, and selectivity (6, 73, 74). Hence, the activities of salvinorin A and its analogs offer clues toward the development of safer analgesics.

The pharmacological characterization of salvinorin A has been widely published (75). Salvinorin A was reported as a selective agonist of KOP through a competitive radioligand binding affinity assay (40). This pharmacological profile was subsequently replicated and confirmed by the findings of Chavkin and co-collaborators (76). As an important pharmacological target, the KOP has been implicated in the antinociceptive effect of salvinorin A (28, 77–79). Consistent with these reports, the potent antineuropathic pain of salvinorin A was blocked by the administration of a KOP antagonist (41). In addition to its selectivity to KOP, salvinorin A has a very high KOP potency. For instance, doses as low as 200 micrograms of this compound produce hallucination (31).

The pharmacology of salvinorin A is considered unique as a result of its structure and binding to the KOP (5). Despite being a potent activator of KOP-mediated G protein signaling, receptor internalization by salvinorin A is still poorly known (79). The internalization of receptor and β-arrestin recruitment are two cellular events that often accompany G protein activation (5). These cellular events have been linked to the underlying mechanism of unwanted side effects (80, 81). Hence, specific functional groups or structural features of salvinorin A that are critical to KOP interaction could be explored to repurpose analogs with only antinociceptive effect.

As highlighted above, binding affinity parameter has consistently been used for preclinical screening and as a basis for structural activity relationship studies. However, varying values of binding affinity data of salvinorin A and its analogs from different laboratories have raised questions about their reliability. Inconsistent data or lack of replicability of binding data could have resulted from the use of different radioligands to measure binding constants for the same analog (6).

Nature provides important chemical and pharmacological clues through the hydrolysis of salvinorin A at C-2 position that leads to salvinorin B and eliminates KOP activity (4,

48, 76). In this manner, the C-2 position represents a critical pharmacophore for salvinorin A and KOP interaction (57, 66, 76). In addition, the therapeutic potential of salvinorin A is limited as a result of its fast hydrolysis at the C-2 position by esterases (76, 82). Previous study which showed loss of the antinociceptive activity of salvinorin A after 20 min of intrathecal injection confirmed its short duration of action (77).

Medicinal chemists have advanced understanding of salvinorin A through its analogs (4, 6, 42). As mentioned earlier in this review, the aromatic substitution through the introduction of a phenyl group at C-2 as in herkinorin reduced KOP and increased MOP binding affinity (56). The structural change and selective activation of opioid receptors seem to be important clues to the antinociceptive effect of this compound. However, there is no data to exclude the possible hallucinations and physical dependence that are often associated with KOP and MOP agonists, respectively. Selective activation of opioid receptor-mediated beneficial pathways over deleterious signaling pathways offers an alternative therapeutic opportunity (3, 83, 84). According to some authors, the selective activation of Gi/o protein-mediated pathways over arrestin-mediated signaling could be a clue to designing safer drugs (85–87).

Some experimental data on salvinorin A analogs have shown preferential activation of G protein, β-arrestin recruitment among other molecular targets. Previous data showed that herkinorin promoted phosphorylation of MAP kinases ERK1/2 independent from β-arrestin-2 signaling and without promoting MOP recruitment of β-arrestin-2 (88). The β-arrestin-2 knockout mice with opioid treatment exhibited reduced opioid tolerance, improved the antinociceptive effect devoid of respiratory depression and constipation (89–91). Some authors have associated opioid dependence with the internalization of G protein—coupled receptors (80, 81, 92). The fact that herkinorin did not promote MOP internalization makes its potential application as an analgesic far more interesting.

Kurkinorin, which is considered to be extremely selective to MOP, showed a complete pharmacological change from salvinorin A which is known for a very high KOP binding affinity (63, 76). Moreover, kurkinorin also has greater selectivity for MOP than herkinorin. However, kurkinorin was found to recruit β-arrestin 2 (EC50 $>$ 140 nM) with an efficacy of 96% and a bias factor of 0.57 when compared to DAMGO (63). Although these data suggest that kurkinorin may produce a morphine-like antinociceptive effect, chemical changes in the structure of these compounds provide important information on the molecular features that are necessary for molecular recognition of a ligand by opioid receptors. Hence, additional modification could be sufficient to prevent potential undesirable activity of kurkinorin without compromising antinociceptive property.

Animal models of abdominal pain and pruritus have also been explored to further the study on some salvinorin A analogs and their potential antinociceptive effect (43, 93). The aromatic analogs such as PR-37 and PR-38, which displayed lower affinity for KOP, blocked nociceptive responses. The intraperitoneal administration of PR-38 (10 mg/kg) and salvinorin A (3 mg/kg) elicited a significant decrease in pain-related behaviors. The higher dose of this analog suggests that salvinorin A is more potent than PR-38. In 2015, Salaga et al. showed attenuation of compound 48/80-induced itch responses in mice by PR-37 and PR-38 (93). The antiscratch activity of PR-37 was blocked by the selective nor-binaltorphimine (KOP antagonist), and that of PR-38 by β-funaltrexamine (selective MOP antagonist). In this study, both PR-37 and PR-38 induced antiscratch activity at the same doses of 10 and 20 mg/kg.

Pharmacological evaluation of β-tetrahydropyranyl ether of salvinorin B has provided effective insight into the antinociceptive activity of this analog and salvinorin A. The non-linear regression analysis of hot water tail-withdrawal latency revealed β-tetrahydropyranyl ether of salvinorin B to be more potent (ED_{50} 1.4 mg/kg) than salvinorin A (ED_{50} 2.1 mg/kg) (44). In addition, salvinorin A and β-tetrahydropyranyl ether of salvinorin B reduced both phase 1 nociceptive pain and phase 2 inflammatory pain in formalin test. The β-tetrahydropyranyl ether of salvinorin B produced a longer duration of action in the tail-withdrawal assay when compared to the salvinorin A. An increased duration of action has been attributed to the substitutions of tetrahydropyran group at C-2 position (44).

CLINICAL AND ETHNOPHARMACOLOGICAL CONSIDERATIONS OF SALVINORIN A AND ITS ANALOGS

Currently, except for the ethnopharmacological reports, there is a dearth of clinical data to support the analgesic property of *S. divinorum* and salvinorin A (94). Salvinorin A has a long history of use as an entheogen by the shamans/healers of the Mazatec people (95). The ingestion of this plant species induces a short-lived inebriant state with intense, bizarre feelings of depersonalization (71, 96). At low infusion doses, the plant leaves have been used to treat headache, rheumatism, anemia, constipation, anuria, and diarrhea (97, 98). These pharmacological effects among others have been attributed to salvinorin A (99).

Headache is a daily painful experience that affects individuals of all ages (100, 101). Medicinal plant application to ameliorate unpleasant sensory and emotional experience that is associated with a headache is a common practice. The analgesic effect of *S. divinorum* leaf through infusion, chewing and swallowing could be attributed to its salvinorin A content. One kilogram of dried leaves or eight kilograms of fresh leaves of *S. divinorum* delivers about 1.5 g of salvinorin A when smoked, vaporized and inhaled (31, 99).

In addition to the antiheadache property of salvinorin A, its potential role in the antirheumatoid activity of *S. divinorum* leaf preparation further supports the antinociceptive property. The origins of rheumatoid arthritis (RA) remains controversial, and its origins in the New or Old World are subjects of several scientific works (102). However, several studies have shown higher prevalence of RA among the natives and women between the ages of 35 and 50 (103, 104). The treatment of RA among the Mazatec peoples is expected given its high incidence. The reports of the use of *S. divinorum* for RA treatment are plausible,

in view of the potent antinociceptive effect of salvinorin A against chronic and neuropathic pain (41).

The effect of *S. divinorum* infusion against anemia, constipation, anuria, and diarrhea could provide additional therapeutic benefits not only from its main isolate but also from some of salvinorin A analogs (105, 106). Gastrointestinal tract discomfort, constipation and diarrhea are among the therapeutic limitations of some analgesic medications including NSAIDs and opioids (107).

ADDICTIVE PROPERTIES: COMPARISON OF OPIOIDS WITH SALVINORIN A AND ITS ANALOGS

Salvinorin A as a selective KOP agonist does not elicit an addictive effect. This property has stimulated research into its semi-synthetic analogs as therapeutic agents (108). The activation of KOP produces anti-addictive effects by regulating dopamine levels in the brain (106). Unlike salvinorin A, there is dearth of scientific data on the addictive or anti-addictive tendency of its analogs. This may be connected with the fact that most of these compounds were not studied *in vivo*. However, based on the neurobiology of addiction, analogs with high affinity for MOP including kurkinorin, herkamide, and herkinorin need to be evaluated for addictive property and compared with the available drugs being used to manage pain. In addition, the potential addictive property of analogs with high KOP affinity including methoxymethyl ether of salvinorin B, β-tetrahydropyranyl ether of salvinorin B, and ethoxymethyl ether of salvinorin B needs to be evaluated since KOP often promote aversion, withdrawal and abstinence (109). There are possibilities of analog such as 2-*O*-cinnamoylsalvinorin B with moderate dual MOP/KOP agonism to retain analgesic effect without addiction. However, biased activation of different signaling pathways that are associated with KOP is key to non-addictive, addictive, or anti-addictive effect (106). According to (109), both MOP and KOP contribute to specific aspects of addiction by triggering its onset and progression.

The reports on the side effects of salvinorin A such as locomotor decreases aversion, anhedonia, memory impairment, depressant-like behaviors, hallucinations among others (73, 106, 110) may have negatively reduced its therapeutic values. In the previous report, the intraperitoneal administration of salvinorin A significantly lowered dopamine levels in the caudate putamen to elicit conditioned place aversion in rodents (32). Salvinorin A-induced potentiation of dopamine re-uptake transporter function has been reported as a plausible mechanism of the decreases in dopamine levels (106). The neurobiology of salvinorin A induced memory impairment and other side effects is still unclear. Although there are no established structural activity relationships in respect of these side effects, the analogs of salvinorin A still hold promise for the future development of analgesic drugs without addictive and other side effects. Hence, robust preclinical studies and clinical trials will ultimately reveal the therapeutic potential of these analogs.

FINAL CONSIDERATIONS AND CONCLUSIONS

Salvinorin A was the first non-nitrogenous opioid receptor agonist. Non-nitrogenous nature of this compound can be attributed to its unique biological activities. According to Cunningham et al. (4), non-nitrogenous or non-alkaloids are promising scaffolds for new drug development. Despite the reports on the opioid receptor mediated antinociceptive effect of salvinorin A, its instability, short duration of action and side effects remains sources of concern. Systematical modification has increased understanding of the important role of substitutions at different positions of the salvinorin A scaffold and increased the possibility of developing safer analgesic drugs. Currently, the data on the binding affinity of salvinorin A analogs are yet to be correlated with possible low side effects and therapeutic advantage over existing drugs. As ligands can bind well without stabilizing the receptor's active conformation, lower or higher binding affinity is not synonymous with efficacy and potency.

Renewed focus on molecular targets seems to be promising because the activation of KOP or MOP could selectively affect β-arrestin or G-protein signaling. As the arrestin signaling pathway is responsible for many adverse effects of opioids, biased agonism for the G-protein pathway could retain analgesic effects with a reduced side effect. Hence, additional research efforts are still needed toward: (i) the modification of salvinorin A, (ii) comprehensive study of opioid receptors and associated molecular targets, (iii) extensive *in vivo* assays of salvinorin A analogs, iv. optimization of structural and pharmacological clues to develop safer analgesics.

On a final note, it is clear that FDA approval of salvinorin A as an analgesic constitutes an uphill task, however, the body of work reviewed here shows that some analogs of salvinorin A could translate to valuable drugs for the management of pain.

AUTHOR CONTRIBUTIONS

JF and JZ conceived the presented idea. JF, AM, PG, EC, GP, and OS wrote the manuscript with support from JZ, SL, RM, and OF. All authors discussed the content and contributed to the final manuscript.

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REFERENCES

1. Goesling J, Lin LA, Clauw DJ. Psychiatry and pain management: at the intersection of chronic pain and mental health. *Curr Psychiatry Rep.* (2018) 20:12. doi: 10.1007/s11920-018-0872-4
2. Chan HCS, McCarthy D, Li J, Palczewski K, Yuan S. Designing safer analgesics via μ-opioid receptor pathways. *Trends Pharmacol Sci.* (2017) 38:1016–37. doi: 10.1016/j.tips.2017.08.004
3. Che T, Majumdar S, Zaidi SA, Ondachi P, McCorvy JD, Wang S, et al. Structure of the nanobody-stabilized active state of the kappa opioid receptor. *Cell.* (2018) 172:55–67. doi: 10.1016/j.cell.2017.12.011
4. Cunningham CW, Rothman RB, Prisinzano TE. Neuropharmacology of the naturally occurring -opioid hallucinogen salvinorin A. *Pharmacol Rev.* (2011) 63:316–47. doi: 10.1124/pr.110.003244
5. Riley AP, Groer CE, Young D, Ewald AW, Kivell BM, Prisinzano TE. Synthesis and κ-opioid receptor activity of furan-substituted salvinorin A analogues. *J Med Chem.* (2014) 57:10464–75. doi: 10.1021/jm501521d
6. Roach JJ, Shenvi RA. A review of salvinorin analogs and their kappa-opioid receptor activity. *Bioorganic Med Chem Lett.* (2018) 28:1436–45. doi: 10.1016/j.bmcl.2018.03.029
7. Pathan H, Williams J. Basic opioid pharmacology: an update. *Br J Pain.* (2012) 6:11–6. doi: 10.1177/2049463712438493
8. Zaveri NT, Marquez PV, Meyer ME, Hamid A, Lutfy K. The nociceptin receptor (NOP) Agonist AT-312 blocks acquisition of morphine- and cocaine-induced conditioned place preference in mice. *Front Psychiatry.* (2018) 9:638. doi: 10.3389/fpsyt.2018.00638
9. Neal CR, Mansour A, Reinscheid R, Nothacker HP, Civelli O, Watson SJ. Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. *J Comp Neurol.* (1999) 406:503–47.
10. Anand P, Yiangou Y, Anand U, Mukerji G, Sinisi M, Fox M, et al. Nociceptin/orphanin FQ receptor expression in clinical pain disorders and functional effects in cultured neurons. *Pain.* (2016) 157:1960–9. doi: 10.1097/j.pain.0000000000000597
11. Whiteside GT, Kyle DJ. A review of the NOP (ORL-1)-Nociceptin/Orphanin FQ System covering receptor structure, distribution, role in analgesia and reward and interactions with other receptors. *ACS Symp Series.* (2013) 1131:327–68. doi: 10.1021/bk-2013-1131.ch016
12. Ossipov MH, Dussor GO, Porreca F. Central modulation of pain. *J Clin Invest.* (2010) 120:3779–87. doi: 10.1172/JCI43766
13. Al-Hasani R, Bruchas MR. Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology.* (2011) 115:1363–81. doi: 10.1097/ALN.0b013e318238bba6
14. Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, et al. Structural insights into the dynamic process of beta2-adrenergic receptor signaling. *Cell.* (2015) 161:1101–11. doi: 10.1016/j.cell.2015.04.043
15. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature.* (2011) 477:549–55. doi: 10.1038/nature10361
16. Siuda ER, Carr R, Rominger DH, Violin JD. Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. *Curr Opin Pharmacol.* (2017) 32:77–84. doi: 10.1016/j.coph.2016.11.007
17. Watson H. Biological membranes. *Essays Biochem.* (2015) 59:43–69. doi: 10.1042/bse0590043
18. Le Naour M, Lunzer MM, Powers MD, Kalyuzhny AE, Benneyworth MA, Thomas MJ, et al. Putative kappa opioid heteromers as targets for developing analgesics free of adverse effects. *J Med Chem.* (2014) 57:6383–92. doi: 10.1021/jm500159d
19. Al-Hasani R, McCall JG, Bruchas MR. Exposure to chronic mild stress prevents kappa opioid-mediated reinstatement of cocaine and nicotine place preference. *Front Pharmacol.* (2013) 4:96. doi: 10.3389/fphar.2013.00096
20. Butelman ER, Yuferov V, Kreek MJ. κ-opioid receptor/dynorphin system: Genetic and pharmacotherapeutic implications for addiction. *Trends Neurosci.* (2012) 35:587–96. doi: 10.1016/j.tins.2012.05.005
21. Grosshans M, Mutschler J, Kiefer F. Treatment of cocaine craving with as-needed nalmefene, a partial κ opioid receptor agonist. *Int Clin Psychopharmacol.* (2015) 30:237–8. doi: 10.1097/YIC.0000000000000069
22. Schlosburg JE, Whitfield TW, Park PE, Crawford EF, George O, Vendruscolo LF, et al. Long-term antagonism of opioid receptors prevents escalation of and increased motivation for heroin intake. *J Neurosci.* (2013) 33:19384–92. doi: 10.1523/JNEUROSCI.1979-13.2013
23. Smith MA, Cole KT, Iordanou JC, Kerns DC, Newsom PC, Peitz GW, et al. The mu/kappa agonist nalbuphine attenuates sensitization to the behavioral effects of cocaine. *Pharmacol Biochem Behav.* (2013) 104:40–6. doi: 10.1016/j.pbb.2012.12.026
24. Volkow ND, Morales M. The brain on drugs: from reward to addiction. *Cell.* (2015) 162:712–25. doi: 10.1016/j.cell.2015.07.046
25. Bruchas MR, Land BB, Chavkin C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res.* (2010) 1314:44–55. doi: 10.1016/j.brainres.2009.08.062
26. Chavkin C, James IF, Goldstein A. Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science.* (1982) 215:413–5. doi: 10.1126/science.6120570
27. Fantegrossi WE, Kugle KM, Valdes LJ, Koreeda M, Woods JH. Kappa-opioid receptor-mediated effects of the plant-derived hallucinogen, salvinorin A, on inverted screen performance in the mouse. *Behav Pharmacol.* (2005) 16:627–33. doi: 10.1097/00008877-200512000-00005
28. Ansonoff MA, Zhang J, Czyzyk T, Rothman RB, Stewart J, Xu H, et al. Antinociceptive and hypothermic effects of Salvinorin A are abolished in a novel strain of kappa-opioid receptor-1 knockout mice. *J Pharmacol Exp Ther.* (2006) 318:641–8. doi: 10.1124/jpet.106.101998
29. Taylor GT, Manzella F. Kappa opioids, salvinorin A and major depressive disorder. *Curr Neuropharmacol.* (2016) 14:165–76. doi: 10.2174/1570159X13666150727220944
30. Harden MT, Smith SE, Niehoff JA, McCurdy CR, Taylor GT. Antidepressive effects of the κ-opioid receptor agonist salvinorin A in a rat model of anhedonia. *Behav Pharmacol.* (2012) 23:710–5. doi: 10.1097/FBP.0b013e3283586189
31. Siebert DJ. Salvia divinorum and salvinorin A: new pharmacologic findings. *J Ethnopharmacol.* (1994) 43:53–6. doi: 10.1016/0378-8741(94)90116-3
32. Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ. Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: Agonist actions at kappa opioid receptors. *Psychopharmacology.* (2005) 179:551–8. doi: 10.1007/s00213-004-2087-0
33. Laurence L, Gulebru A, Brigitte LK, Lutz PE. The kappa opioid receptor: From addiction to depression, and back. *Front Psychiatry.* (2014) 5:170. doi: 10.3389/fpsyt.2014.00170
34. Maillet EL, Milon N, Heghinian MD, Fishback J, Schürer SC, Garamszegi N, et al. Noribogaine is a G-protein biased κ-opioid receptor agonist. *Neuropharmacology.* (2015) 99:675–88. doi: 10.1016/j.neuropharm.2015.08.032
35. Bruchas MR, Roth BL. New technologies for elucidating opioid receptor function. *Trends Pharmacol Sci.* (2016) 37:279–89. doi: 10.1016/j.tips.2016.01.001
36. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981-2014. *J Nat Prod.* (2016) 79:629–61. doi: 10.1021/np200906s
37. Veeresham C. Natural products derived from plants as a source of drugs. *J Adv Pharm Technol Res.* (2012) 3:200. doi: 10.4103/2231-4040.104709
38. Ortega A, Blount JF, Manchand PS. Salvinorin, a new trans-neoclerodane diterpene from Salvia divinorum(Labiatae). *J Chem Soc Perkin Trans.* (1982) 1:2505. doi: 10.1039/p19820002505
39. Peet MM, Baker LE. Salvinorin B derivatives, EOM-Sal B and MOM-Sal B, produce stimulus generalization in male Sprague-Dawley rats trained to discriminate salvinorin A. *Behav Pharmacol.* (2011) 22:450–7. doi: 10.1097/FBP.0b013e328349fc1b
40. Roth BL, Baner K, Westkaemper R, Siebert D, Rice KC, Steinberg S, et al. Salvinorin A: A potent naturally occurring nonnitrogenous opioid selective agonist. *Proc Natl Acad Sci USA.* (2002) 99:11934–9. doi: 10.1073/pnas.182234399
41. Coffeen U, Canseco-Alba A, Simón-Arceo K, Almanza A, Mercado F, León-Olea M, et al. Salvinorin A reduces neuropathic nociception in the insular cortex of the rat. *Eur J Pain.* (2018) 22:311–8. doi: 10.1002/ejp.1120
42. Polepally PR, Huben K, Vardy E, Setola V, Mosier PD, Roth BL, et al. Michael acceptor approach to the design of new salvinorin A-based high affinity ligands for the kappa-opioid receptor. *Eur J Med Chem.* (2014) 85:818–29. doi: 10.1016/j.ejmech.2014.07.077

43. Sałaga M, Polepally PR, Zakrzewski PK, Cygankiewicz A, Sobczak M, Kordek R, et al. Novel orally available salvinorin a analog PR-38 protects against experimental colitis and reduces abdominal pain in mice by interaction with opioid and cannabinoid receptors. *Biochem Pharmacol.* (2014) 92:618–26. doi: 10.1016/j.bcp.2014.09.018
44. Paton KF, Kumar N, Crowley RS, Harper JL, Prisinzano TE, Kivell BM. The analgesic and anti-inflammatory effects of Salvinorin A analogue β-tetrahydropyran Salvinorin B in mice. *Eur J Pain.* (2017) 21:1039–50. doi: 10.1002/ejp.1002
45. Valdes LJ, Butler WM, Hatfield GM, Paul AG, Koreeda M. Divinorin A, a psychotropic terpenoid, and divinorin B from the hallucinogenic Mexican mint, Salvia divinorum. *J Org Chem.* (1984) 49:4716–20. doi: 10.1021/jo00198a026
46. Lavecchia A, Greco G, Novellino E, Vittorio F, Ronsisvalle G. Modeling of kappa-opioid receptor/agonists interactions using pharmacophore-based and docking simulations. *J Med Chem.* (2000) 43:2124–34. doi: 10.1021/jm991161k
47. Yan F. *Molecular Mechanisms by Which Salvinorin A Binds to and Activates the Kappa-Opioid Receptor*. Case Western Reserve University, ProQuest Dissertations Publishing. 3301865 (2018).
48. Valdés LJ, Chang HM, Visger DC, Koreeda M. Salvinorin C, a new neoclerodane diterpene from a bioactive fraction of the hallucinogenic Mexican mint Salvia divinorum. *Org Lett.* (2001) 3:3935–7. doi: 10.1021/ol016820d
49. Lozama A, Cunningham CW, Caspers MJ, Douglas JT, Dersch CM, Rothman RB, et al. Opioid receptor probes derived from cycloaddition of the hallucinogen natural product salvinorin A. *J Nat Prod.* (2011) 74:718–26. doi: 10.1021/np1007872
50. Tidgewell K, Harding WW, Lozama A, Cobb H, Shah K, Kannan P, et al. Synthesis of salvinorin A analogues as opioid receptor probes. *J Nat Prod.* (2006) 69:914–8. doi: 10.1021/np060094b
51. Rothman RB, Murphy DL, Xu H, Godin JA, Dersch CM, Partilla JS, et al. Salvinorin A: allosteric interactions at the mu-opioid receptor. *J Pharmacol Exp Ther.* (2007) 320:801–10. doi: 10.1124/jpet.106.113167
52. Seeman P, Guan HC, Hirbec H. Dopamine D2High receptors stimulated by phencyclidines, lysergic acid diethylamide, salvinorin A, and modafinil. *Synapse.* (2009) 63:698–704. doi: 10.1002/syn.20647
53. Xu X, Ma S, Feng Z, Hu G, Wang L, Xie XQ. Chemogenomics knowledgebase and systems pharmacology for hallucinogen target identification-Salvinorin A as a case study. *J Mol Graph Model.* (2016) 70:284–95. doi: 10.1016/j.jmgm.2016.08.001
54. Fichna J, Schicho R, Andrews C, Bashashati M, Klompus M, McKay D, et al. Salvinorin A inhibits colonic transit and neurogenic ion transport in mice by activating κ-opioid and cannabinoid receptors. *Neurogastroenterol Motil.* (2009) 21:1326–e128. doi: 10.1111/j.1365-2982.2009.01369.x
55. Béguin C, Duncan KK, Munro TA, Ho DM, Xu W, Liu-Chen LY, et al. Modification of the furan ring of salvinorin A: identification of a selective partial agonist at the kappa opioid receptor. *Bioorg Med Chem.* (2009) 17:1370–80. doi: 10.1016/j.bmc.2008.12.012
56. Harding W W, Tidgewell K, Byrd N, Cobb H, Dersch CM, Butelman ER, et al. Neoclerodane diterpenes as a novel scaffold for μ opioid receptor ligands. *J. Med. Chem.* (2005) 48:4765–71. doi: 10.1021/jm048963m
57. Lee DYW, Karnati VVR, He M, Liu-Chen LY, Kondaveti L, Ma Z, et al. Synthesis and *in vitro* pharmacological studies of new C(2) modified salvinorin A analogues. *Bioorganic Med Chem Lett.* (2005) 15:3744–7. doi: 10.1016/j.bmcl.2005.05.048
58. Munro TA, Duncan KK, Xu W, Wang Y, Liu-Chen LY, Carlezon WA, et al. Standard protecting groups create potent and selective κ opioids: salvinorin B alkoxymethyl ethers. *Bioorganic Med Chem.* (2008) 16:1279–86. doi: 10.1016/j.bmc.2007.10.067
59. Prevatt-Smith KM, Lovell KM, Simpson DS, Day VW, Douglas JT, Bosch P, et al. Potential drug abuse therapeutics derived from the hallucinogenic natural product salvinorin A. *Medchemcomm.* (2011) 2:1217–22. doi: 10.1039/c1md00192b
60. Polepally PR, White K, Vardy E, Roth BL, Ferreira D, Zjawiony JK. Kappa-opioid receptor-selective dicarboxylic ester-derived salvinorin A ligands. *Bioorg Med Chem Lett.* (2013) 23:2860–2. doi: 10.1016/j.bmcl.2013.03.111
61. Sherwood AM, Crowley RS, Paton KF, Biggerstaff A, Neuenswander B, Day VW, et al. Addressing structural flexibility at the A-ring on salvinorin A: discovery of a potent kappa-opioid agonist with enhanced metabolic stability. *J Med Chem.* (2017) 60:3866–78. doi: 10.1021/acs.jmedchem.7b00148
62. Tidgewell K, Groer CK, Harding WW, Lozama A, Schmidt M, Marquam A, et al. Herkinorin analogues with differential β-arrestin-2 interactions. *J Med Chem.* (2008) 51:2421–31. doi: 10.1021/jm701162g
63. Crowley RS, Riley AP, Sherwood AM, Groer CE, Shivaperumal N, Biscaia M, et al. Synthetic studies of neoclerodane diterpenes from salvia divinorum: identification of a potent and centrally acting μ opioid analgesic with reduced abuse liability. *J Med Chem.* (2016) 59:11027–38. doi: 10.1021/acs.jmedchem.6b01235
64. Lee DYW, He M, Liu-Chen LY, Wang Y, Li JG, Xu W, et al. Synthesis and *in vitro* pharmacological studies of new C(4)-modified salvinorin A analogues. *Bioorganic Med Chem Lett.* (2006) 16:5498–502. doi: 10.1016/j.bmcl.2006.08.051
65. Vardy E, Mosier PD, Frankowski KJ, Wu H, Katritch V, Westkaemper RB, et al. Chemotype-selective modes of action of k-opioid receptor agonists. *J Biol Chem.* (2013) 288:34470–83. doi: 10.1074/jbc.M113.515668
66. Béguin C, Richards MR, Li JG, Wang Y, Xu W, Liu-Chen LY, et al. Synthesis and *in vitro* evaluation of salvinorin A analogues: Effect of configuration at C(2) and substitution at C(18). *Bioorg Med Chem Lett.* (2006) 16:4679–85. doi: 10.1016/j.bmcl.2006.05.093
67. St Jean DJ, Fotsch C. Mitigating heterocycle metabolism in drug discovery. *J. Med. Chem.* (2012) 55:6002–6020. doi: 10.1021/jm300343m
68. Simpson DS, Katavic PL, Lozama A, Harding WW, Parrish D, Deschamps JR, et al. Synthetic studies of neoclerodane diterpenes from Salvia divinorum: Preparation and opioid receptor activity of salvinicin analogues. *J Med Chem.* (2007) 50:3596–603. doi: 10.1021/jm070393d
69. Lovell KM, Vasiljevik T, Araya JJ, Lozama A, Prevatt-Smith KM, Day VW, et al. Semisynthetic neoclerodanes as kappa opioid receptor probes. *Bioorg Med Chem.* (2012) 20:3100–10. doi: 10.1016/j.bmc.2012.02.040
70. Sherwood AM, Williamson SE, Crowley RS, Abbott LM, Day VW, Prisinzano TE. Modular approach to pseudo-neoclerodanes as designer κ-opioid ligands. *Org Lett.* (2017b) 19:5414–7. doi: 10.1021/acs.orglett.7b02684
71. Bücheler R, Gleiter CH, Schwoerer P, Gaertner I. Use of nonprohibited hallucinogenic plants: Increasing relevance for public health? A case report and literature review on the consumption of Salvia divinorum (Diviner's Sage). *Pharmacopsychiatry.* (2005) 38:1–5. doi: 10.1055/s-2005-837763
72. Orton E, Liu R. Salvinorin A: a mini review of physical and chemical properties affecting its translation from research to clinical applications in humans. *Transl Perioper Pain Med.* (2014) 1:9–11. doi: 10.31480/2330-4871/004
73. Butelman ER, Kreek MJ. Salvinorin A, a kappa-opioid receptor agonist hallucinogen: Pharmacology and potential template for novel pharmacotherapeutic agents in neuropsychiatric disorders. *Front Pharmacol.* (2015) 6:190. doi: 10.3389/fphar.2015.00190
74. Hooker JM, Munro TA, Béguin C, Alexoff D, Shea C, Xu Y, et al. Salvinorin A and derivatives: protection from metabolism does not prolong short-term, whole-brain residence. *Neuropharmacology.* (2009) 57:386–91. doi: 10.1016/j.neuropharm.2009.06.044
75. Grundmann O, Phipps SM, Zadezensky I, Butterweck V. Salvia divinorum and salvinorin A: an update on pharmacology and analytical methodology. *Planta Med.* (2007) 73:1039–46. doi: 10.1055/s-2007-981566
76. Chavkin C, Sud S, Jin W, Stewart J, Zjawiony JK, Siebert DJ, et al. Salvinorin A, an Active component of the hallucinogenic sage salvia divinorum is a highly efficacious {{}kappa{}}-opioid receptor agonist: structural and functional considerations. *J Pharmacol Exp Ther.* (2004) 308:1197–203. doi: 10.1124/jpet.103.059394
77. John TF, French LG, Erlichman JS. The antinociceptive effect of Salvinorin A in mice. *Eur J Pharmacol.* (2006) 545:129–33. doi: 10.1016/j.ejphar.2006.06.077
78. McCurdy CR, Sufka KJ, Smith GH, Warnick JE, Nieto MJ. Antinociceptive profile of salvinorin A, a structurally unique kappa opioid receptor agonist. *Pharmacol Biochem Behav.* (2006) 83:109–13. doi: 10.1016/j.pbb.2005.12.011

79. Wang Y. Comparison of pharmacological activities of three distinct ligands (Salvinorin A, TRK-820 and 3FLB) on opioid receptors *in vitro* and their antipruritic and antinociceptive activities *in vivo*. *J Pharmacol Exp Ther.* (2004) 312:220–30. doi: 10.1124/jpet.104.073668
80. Connor M, Osborne PB, Christie MJ. μ-Opioid receptor desensitization: is morphine different? *Br J Pharmacol.* (2004) 143:685–96. doi: 10.1038/sj.bjp.0705938
81. Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein–coupled receptors and neuronal functions. *Annu Rev Neurosci.* (2004) 27:107–44. doi: 10.1146/annurev.neuro.27.070203.144206
82. Teksin ZS, Lee IJ, Nemieboka NN, Othman AA, Upreti VV, Hassan HE, et al. Evaluation of the transport, *in vitro* metabolism and pharmacokinetics of Salvinorin A, a potent hallucinogen. *Eur J Pharm Biopharm.* (2009) 72:471–7. doi: 10.1016/j.ejpb.2009.01.002
83. Kenakin T, Christopoulos A. Signalling bias in new drug discovery: Detection, quantification and therapeutic impact. *Nat Rev Drug Discov.* (2013) 12:205–16. doi: 10.1038/nrd3954
84. Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, et al. Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther.* (2006) 320:1–13. doi: 10.1124/jpet.106.104463
85. Brust TF, Morgenweck J, Kim SA, Rose JH, Locke JL, Schmid CL, et al. Biased agonists of the kappa opioid receptor suppress pain and itch without causing sedation or dysphoria. *Sci Signal.* (2016) 9:ra117. doi: 10.1126/scisignal.aai8441
86. Spetea M, Eans SO, Ganno ML, Lantero A, Mairegger M, Toll L, et al. Selective κ receptor partial agonist HS666 produces potent antinociception without inducing aversion after i.c.v. administration in mice. *Br. J. Pharmacol.* (2017) 174:2444–56. doi: 10.1111/bph.13854
87. White KL, Robinson JE, Zhu H, DiBerto JF, Polepally PR, Zjawiony JK, et al. The G protein-biased -opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities *in vivo*. *J Pharmacol Exp Ther.* (2014) 352:98–109. doi: 10.1124/jpet.114.216820
88. Groer C, Tidgewell K, Moyer R. An opioid agonist that does not induce μ-opioid receptor — arrestin interactions or receptor internalization. *Mol Pharmacol.* (2007) 71:549–57. doi: 10.1124/mol.106.028258.of
89. Bohn LM, Lefkowitz RJ, Caron MG. Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. *J Neurosci.* (2002) 22:10494–500. doi: 10.1523/JNEUROSCI.22-23-10494.2002
90. Przewlocka B, Sieja A, Starowicz K, Maj M, Bilecki W, Przewlocki R. Knockdown of spinal opioid receptors by antisense targeting β-arrestin reduces morphine tolerance and allodynia in rat. *Neurosci Lett.* (2002) 325:107–10. doi: 10.1016/S0304-3940(02)00246-X
91. Raehal KM. Morphine side effects in -arrestin 2 knockout mice. *J Pharmacol Exp Ther.* (2005) 314:1195–201. doi: 10.1124/jpet.105.087254
92. Bohn LM, Gainetdinov RR, Caron MG. G protein-coupled receptor kinase/beta-arrestin systems and drugs of abuse: psychostimulant and opiate studies in knockout mice. *Neuromolecular Med.* (2004) 5:41–50. doi: 10.1385/nmm:5:1:041
93. Salaga M, Polepally PR, Zielinska M, Marynowski M, Fabisiak A, Murawska N, et al. Salvinorin A analogues PR-37 and PR-38 attenuate compound 48/80-induced itch responses in mice. *Br J Pharmacol.* (2015) 172:4331–41. doi: 10.1111/bph.13212
94. Wu L-T, Woody G, Yang C, Li J-H, Blazer DG. Recent national trends in Salvia divinorum use and substance-use disorders among recent and former Salvia divinorum users compared with nonusers. *Subst Abuse Rehabil.* (2011) 2011:53–68. doi: 10.2147/SAR.S17192
95. Valdés LJ, Díaz J, Paul AG. Ethnopharmacology of ska María Pastora (Salvia divinorum, Epling AND Játiva-M.). *J. Ethnopharmacol.* (1983) 7:287–312. doi: 10.1016/0378-8741(83)90004-1
96. Prisinzano TE. Psychopharmacology of the hallucinogenic sage Salvia divinorum. *Life Sci.* (2005) 78:527–31. doi: 10.1016/j.lfs.2005.09.008
97. Jenks AA, Walker JB, Kim SC. Evolution and origins of the Mazatec hallucinogenic sage, Salvia divinorum (Lamiaceae): A molecular phylogenetic approach. *J Plant Res.* (2011) 124:593–600. doi: 10.1007/s10265-010-0394-6
98. Valdes LJ, Hatheld GM, Koreeda M, Paul AG. Studies of salvia divinorum (Lamiaceae), an hallucinogenic mint from the Sierra Mazateca in Oaxaca, Central Mexico. *Econ Bot.* (1987) 41:283–91. doi: 10.1007/BF02858975
99. Valdés LJ. Salvia divinorum and the unique diterpene hallucinogen, salvinorin (divinorin) A. *J Psychoactive Drugs.* (1994) 26:277–83. doi: 10.1080/02791072.1994.10472441
100. Rolan PE. Understanding the pharmacology of headache. *Curr Opin Pharmacol.* (2014) 14:30–3. doi: 10.1016/j.coph.2013.10.005
101. Solomon GD. Pharmacology and use of headache medications. *Cleve Clin J Med.* (1990) 57:627–35. doi: 10.3949/ccjm.57.7.627
102. Entezami P, Fox DA, Clapham PJ, Chung KC. Historical perspective on the etiology of rheumatoid arthritis. *Hand Clin.* (2011) 27:1–10. doi: 10.1016/j.hcl.2010.09.006
103. Beasley J. Osteoarthritis and rheumatoid arthritis: conservative therapeutic management. *J Hand Ther.* (2012) 25:163–72. doi: 10.1016/j.jht.2011.11.001
104. Rothschild BM, Woods RJ, Rothschild C, Sebes JI. Geographic distribution of rheumatoid arthritis in ancient North America: implications for pathogenesis. *Semin Arthritis Rheum.* (1992) 22:181–7. doi: 10.1016/0049-0172(92)90018-9
105. Capasso R, Borrelli F, Cascio MG, Aviello G, Huben K, Zjawiony JK, et al. Inhibitory effect of salvinorin A, from Salvia divinorum, on ileitis-induced hypermotility: cross-talk between κ-opioid and cannabinoid CB 1 receptors. *Br J Pharmacol.* (2008) 155:681–9. doi: 10.1038/bjp.2008.294
106. Kivell BM, Ewald AWM, Prisinzano TE. Salvinorin A analogs and other kappa-opioid receptor compounds as treatments for cocaine abuse. *Adv Pharmacol.* (2014) 69:482–511. doi: 10.1016/B978-0-12-420118-7.00012-3
107. Raehal KM, Schmid CL, Groer CE, Bohn LM. Functional selectivity at the -opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol Rev.* (2011) 63:1001–19. doi: 10.1124/pr.111.004598
108. Cruz A, Domingos S, Gallardo E, Martinho A. A unique natural selective kappa-opioid receptor agonist, salvinorin A, and its roles in human therapeutics. *Phytochemistry.* (2017) 137:9–14. doi: 10.1016/j.phytochem.2017.02.001
109. Darcq E, Kieffer BL. Opioid receptors: drivers to addiction? *Nat Rev Neurosci.* (2018) 19:499–514. doi: 10.1038/s41583-018-0028-x
110. MacLean KA, Johnson MW, Reissig CJ, Prisinzano TE, Griffiths, RR. Dose-related effects of salvinorin A in humans: dissociative, hallucinogenic, and memory effects. *Psychopharmacol.* (2012) 226:381–92. doi: 10.1007/s00213-012-2912-9

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Binge-Like Exposure to Ethanol Enhances Morphine's Anti-nociception in B6 Mice

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Elevation of the blood ethanol concentration (BEC) to > 80 mg/dL (17.4 mM) after binge drinking enhances inflammation in brain and neuroimmune signaling pathways. Morphine abuse is frequently linked to excessive drinking. Morphine exerts its actions mainly via the seven transmembrane G-protein-coupled mu opioid receptors (MORs). Opioid use disorders (OUDs) include combination of opioids with alcohol, leading to opioid overdose-related deaths. We hypothesized that binge drinking potentiates onset and progression of OUD. Using C57BL/6J (B6) mice, we first characterized time-dependent inflammatory gene expression change as molecular markers using qRT-PCR within 24 h after binge-like exposure to high-dose, high-concentration ethanol (EtOH). The mice were given one injection of EtOH (5 g/kg, 42% v/v, i.g.) and sacrificed at 2.5 h, 5 h, 7.5 h, or 24 h later. Inflammatory cytokines interleukin (IL)-1β, IL-6, and IL-18 were elevated in both the striatum (STr) and the nucleus accumbens (NAc) of the mice. We then investigated the expression profile of MOR in the STr at 2 min, 5 h, or 24 h after the first EtOH injection and at 24 h and 48 h after the third injection. This binge-like exposure to EtOH upregulated MOR expression in the STr and NAc, an effect that could enhance morphine's anti-nociception. Therefore, we examined the impact of binge-like exposure to EtOH on morphine's anti-nociception at the behavioral level. The mice were treated with or without 3-d binge-like exposure to EtOH, and the anti-nociceptive changes were evaluated using the hot-plate test 24 h after the final (3rd) EtOH injection with or without a cumulative subcutaneous dose (0, 0.1, 0.3, 1.0, and 3.0 mg/kg) of morphine at intervals of 30 min. The response curve of the mice given EtOH was shifted to the left, showing enhanced latency to response to morphine up to 3 mg/kg. Furthermore, co-treatment with the MOR antagonist naltrexone blocked morphine's anti-nociception in animals given either EtOH or saline. This confirms that MOR is involved in binge-like exposure to EtOH-induced changes in morphine's anti-nociception. Our results suggest that EtOH enhanced latency to analgesic response to morphine, and such effect might initiate the onset and progression of OUDs.

Keywords: morphine, mu opioid receptors, high-dose ethanol, anti-nociception, striatum, nucleus accumbens

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INTRODUCTION

Alcohol (EtOH) is the most widely used addictive substance in the world. The effects of alcohol drinking depend on the volume consumed, the concentration by volume, and the drinking pattern (1–4). Alcohol drinking patterns refers to different frequencies and amounts of alcohol intake, such as casual drinking, binge drinking, continuous drinking, frequent heavy drinking, and episodic drinking. National Institute on Alcohol Abuse and Alcoholism (NIAAA) has defined "binge drinking" as drinking enough EtOH in a short time to elevate the blood EtOH concentration (BEC) to > 80 mg/dL (5); that is, 17.4 mM. Binge drinking, particularly of hard liquor (> 40% alcohol by volume [ABV]), is a popular activity among adolescents (6). Hard liquor was involved in 43.8% of the binge drinking reported by subjects aged 13 to 20 yrs, with vodka being the most popular beverage (7). Epidemiologic studies indicate that adolescence is a risky period for initiation of EtOH use, and early onset is associated with a greater risk of late dependence or alcoholism (8–12). Alcohol consumption by adolescents also can lead to other addictive behaviors, including abuse of various other substances such as opioids, as well as neurocognitive deficits and social impairment. These pathological conditions may lead to direct and indirect changes in the neuromaturational course extending into adulthood (8–11). Not only chronic EtOH consumption, but also sporadic consumption, such as excessive weekend drinking, can provoke cognitive-deficit neuropsychological effects in young adults (13).

Binge drinking is observed in individuals with alcohol use disorders (AUDs). Chronic/repeated alcohol use alters nociception, including changes in pain sensation (14). Moreover, binge drinking induces gut leakage causing elevation of the blood endotoxin concentration (15, 16). This systemic endotoxin activity can trigger activation of inflammatory cytokines and has global effects on various cell types in different organs (17, 18). Numerous investigators have shown that binge drinking in humans and binge-like exposure to EtOH in animals encourages production of inflammatory molecules such as interleukin (IL)-1α, IL-6, IL-1β, and IL-18, as well as elevated activity of neuroimmune signaling pathways via various direct and indirect mechanisms (19, 20). Dysregulated continual synthesis of IL-6 has a pathological effect on chronic inflammation and autoimmunity (21). IL-1β is induced by pro-inflammatory signaling through Toll-like receptors (TLRs) or by cytokines, such as tumor necrosis factor (TNF)-α, IL-1β itself, and the inflammasome (22).

Morphine is a powerful, highly addictive opioid drug that exerts its analgesic action mainly via mu opioid receptors (MORs) (23). The MORs are also the principal site for morphine's induction of behavioral reward (24, 25), locomotion (26), analgesia (27), tolerance (28), and physical dependence (29). Naltrexone is a long-lasting competitive opioid antagonist that has high affinity for MORs (30, 31). Oral naltrexone has been used for many years to treat opioid dependence and has been approved since 1994 by the U.S. Food and Drug Administration to treat AUDs. Tail flick latency and hot-plate analgesia tests are common assays using rodent models to examine morphine's anti-nociception (32). MORs are involved in the interaction of morphine and EtOH, which induces neuroinflammation (33, 34).

We have reported that treatment with the pro-inflammatory cytokine IL-1β significantly increases MOR expression in endothelial cells (35) and in human U87 MG cells (36). In another *in vitro* study, we reported that the upregulation of MOR induced by lipopolysaccharide (LPS) stimulation in macrophage-like TPA-HL-60 cells and conditioned medium from LPS-stimulated TPA-HL-60 cells increases MOR expression in SH-SY5Y cells, a neuronal cell model, through actions mediated by TNF-α and granulocyte–macrophage colony-stimulating factor (37). The LPS-challenged HIV-1 transgenic (HIV-1Tg) rat model with neuroinflammation demonstrates an increase in MOR expression and is more sensitive to morphine's effect in the conditioned place preference test (38, 39).

Taken together, considering the above-mentioned studies showing that (1) binge-like exposure to EtOH induces inflammation and inflammatory cytokines (19, 20) and (2) inflammatory cytokines mediate expression of MOR (35–37) and change morphine actions (38, 39), we hypothesized that binge-like exposure to EtOH increases expression of MOR and changes morphine-induced anti-nociception by inducing elevation of inflammatory molecules in the brain. In the present study, adolescent C57BL/6J mice were given binge-like exposure to high-dose, high-concentration EtOH for 3d by intragastric (i.g.) injection to mimic underage binge alcohol drinking, such as over a weekend (40). The blood EtOH concentration (BEC) after single and repeated EtOH administration was measured. Time-dependent gene expression change was investigated using qRT-PCR as molecular markers to evaluate the response to this binge-like exposure to EtOH. The nucleus accumbens (NAc) plays an important role in processing rewarding and reinforcing stimuli including drug addiction; the striatum (STr) is part of the brain's reward circuit and a key region responsible for voluntary motor control (41). Therefore, we studied expression of the pro-inflammatory cytokine genes *Il1b, Il6,* and *Il18*, as well as the MOR gene *Oprm1*, in the NAc and STr. Finally, hot-plate tests were employed to evaluate the behavioral effect of binge-like exposure to EtOH on morphine's anti-nociception. The opioid antagonist naltrexone was used to confirm morphine's action on MOR. Our results suggest that neuroinflammation induced by binge-like exposure to EtOH contributes to elevation of morphine's anti-nociception response. Such a change might be one of the fundamental mechanisms underlying encouragement of OUDs by binge-like EtOH exposure.

MATERIALS AND METHODS

Animals

C57BL/6J mice (3–4 wks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). They were housed with four animals per ventilated plastic cage (Animal Care Systems Inc., Centennial, CO) and maintained in a temperature- and humidity-controlled environment. They were kept on a 12-h light/dark cycle and fed a standard rodent diet. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ.

TABLE 1 | EtOH administration and determination of blood EtOH concentration.

Treatment	Day 1	Day 2	Day 3
Water			Blood collection
EtOH 2 min			Blood collection 2 min after injection
EtOH 1 d 5 h			Blood collection 5 h after injection
Repeated EtOH 2 d 5 h		EtOH	Blood collection 5 h after injection
Repeated EtOH 3 d 5 h	EtOH	EtOH	Blood collection 5 h after injection

As vehicle control, water injections (not shown) were given.

EtOH Treatment and BEC Determination

The mice were allowed at least one week to adapt to the facility. To minimize the non-specific stress response to i.g. injection of EtOH, the adolescent mice (at ∼ 5 wks) were given 2-day conditioning by intragastric (i.g.) injection of water. The first group of mice was then given one dose of 5 g/kg/d of 42% v/v EtOH as a bolus via i.g. injection. Tail vein blood was collected by tail clipping prior to and at 10 min, 20 min, 1 h, 2 h, 4 h, 6 h, and 8 h after treatment. A second group of mice was designated to receive the same dose of EtOH for 1, 2, or 3 d; and blood was collected 5 h after the last injection (**Table 1**). Plasma was obtained by centrifugation of whole blood at 10,000 rpm for 10 min at 4°C and stored at −80°C until analysis. The EtOH concentration was determined using an Ethanol Assay Kit (Biovision, Milpitas, CA) following the manufacturer's instructions. The BEC data were analyzed using Student's *t*-test.

EtOH Treatment and Tissue Collection

After 2-day conditioning, the B6 mice were designated to receive 5 g/kg 42% v/v EtOH as a bolus one time and sacrificed at 2 min, 2.5 h, 5 h, 7.5 h, or 24 h after treatment, after which the brains were microdissected. The STr and NAc were stored at −80°C until analysis. A second batch of B6 mice received the same dose of EtOH for 1 or 3 days (**Table 2**). These mice were sacrificed 5 h after the last injection. By adapting the EtOH treatment regimen as reported previously (42), we conducted preliminary studies using animals receiving water or EtOH for 2 min. Other than the BEC reading, there are no significant differences between the readings of various assessments on the animals sacrificed immediately (2 min) after receiving EtOH (EtOH for 2 min) and those of the animals receiving water. For example, in the STr, ΔCt of *Il1b* was 8.77 ± 0.22 in the water group and 8.51±0.58 in the EtOH for 2 min group, with a fold change of 1.20 ± 0.38 ($p = 0.38$); and in the NAc, ΔCt of *Il1b* was 9.11 ± 0.52 in the water group and 9.42±0.53 in the EtOH for 2 min group, with a fold change of 1.74 ± 0.91 ($p = 0.10$). The above data were reproduced in two additional experiments. For the time course study of gene expression changes, it is necessary to include 2-min EtOH group that was used as control for data analysis. In line with IACUC and NIH guideline to minimize use of the animals, no water group was included in the study for Oprm1 daily expression following binge-like exposure to EtOH. As reported previously (42), we have used 2-min EtOH as control throughout this research project. Brains were microdissected, and the STr and NAc were collected and stored at −80°C until use.

TABLE 2 | EtOH administration timeline for *Oprm1* response.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5
EtOH 2 min				EtOH; sacrifice 2 min after injection	
EtOH 5 h				EtOH; sacrifice 5 h after injection	
EtOH 24 h			EtOH	Sacrifice	
Repeated EtOH 24 h	EtOH	EtOH	EtOH	Sacrifice	
Repeated EtOH 48 h	EtOH	EtOH	EtOH		Sacrifice

As vehicle control, water injections (not shown) were given.

RNA Isolation and cDNA Preparation

Total RNA was extracted from the STr and NAc using the RNeasy Mini Kit (Qiagen, Germantown, MD), followed by RNase-free DNase (Qiagen) digestion to remove contaminating DNA. The RNA quality and quantity were determined using an ND1000 Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) and verified by gel electrophoresis. An equal amount of RNA (400 ng) from each sample was converted to cDNA using the RT^2 First-Strand Kit (Qiagen) according to the manufacturer's instructions.

qRT-PCR Analysis

Gene expression was quantified using RT^2 SYBR ROX qPCR Master Mix (Qiagen) as described previously (38, 40, 43). Real-time polymerase chain reaction (PCR) was performed with the ABI Prism 7900HT Fast Detection System (Applied Biosystems, Foster, CA). The thermocycler parameters were 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. ROX was used as the passive reference. Expressions of all genes were normalized to expression of β-actin (*Actb*) and splicing factor, arginine/serine-rich 4 (*Sfrs4*). The relative expression of each gene was compared with expression of that gene in the mice given EtOH for 2 min and calculated using the ΔΔCT method (44). The primer sequences for *Il1b*, *Il6*, *Oprm1*, *Actb*, and *Sfrs4* are listed in **Table 3**. The *Il18* primers were purchased from Qiagen (Cat No. PPM03112B). Data were analyzed using one-way ANOVA followed by Dunnett's post-tests in GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA).

EtOH Treatment and Hot-Plate Tests

Male 5-week-old B6 mice were designated to receive either 5 g/kg 42% v/v EtOH or water (control) daily for 3 days. Morphine sulfate (Sigma, St. Louis, MO) was freshly prepared prior to use by dissolving it in 0.9% sterile saline. A 1.0-mg/mL morphine solution was serially diluted to create

TABLE 3 | PCR array primer sequences.

Gene symbol	Primer	Sequence 5′3′
Oprm1	Forward	CCAGGGAACATCAGCGACTG
	Reverse	GTTGCCATCAACGTGGGAC
Il1b	Forward	AATGCCACCTTTTGACAGTGATG
	Reverse	GGAAGGTCCACGGGAAAGAC
Il6	Forward	CCCCAATTTCCAATGCTCTCC
	Reverse	GGATGGTCTTGGTCCTTAGCC
Actb	Forward	GGCACCACACCTTCTACAATG
	Reverse	GGGGTGTTGAAGGTCTCAAAC
Sfrs4	Forward	GATCTGAAGAACGGGTATGGCT
	Reverse	ACACAGGTCTTTGCCGTTCA

TABLE 4 | EtOH administration and hot-plate test timeline.

Treatment	Day 1—Day 3	Day 4
Water alone	Daily water injections	Hot-plate tests
Water + morphine	Daily water injections	Cumulative doses of morphine (s.c.) and hot-plate tests
EtOH alone	Daily EtOH injections	Hot-plate tests
EtOH + morphine	Daily EtOH injections	Cumulative doses of morphine (s.c.) and hot-plate tests

doses of 0.1, 0.3, 1.0, or 3.0 mg/kg. A saline solution with no morphine was the control for morphine treatment. In our preliminary studies, the animals were given cumulative doses of morphine of 0.1, 0.3, 1.0, 3.0, and 10 mg/kg to select the morphine doses to be used. On subcutaneously (s.c.) treatment with morphine at 10 mg/kg, both control and experimental animals presented abnormal behaviors that were beyond measurement using the hot-plate test. Therefore, we chose morphine doses of 0.1, 0.3, 1.0, and 3.0 mg/kg.

As shown in **Table 4**, on the day after Day 3 of binge-like exposure to high-dose, high-concentration EtOH, the mice were injected subcutaneously (s.c.) with a cumulative dose of morphine as noted above at intervals of 30 min and placed on the hot plate of the IITC Test Analgesia Meter (Woodland Hills, CA) that was set at 55°C. The latency was recorded according to hind-paw lick or jumping on the meter. A maximum 120-s cutoff was set to avoid tissue damage. The latency (s) was plotted against morphine doses (45). By adhering to the IACUC and NIH guideline, the minimum number of animals needed to obtain statistical power was discovered and used.

In a parallel experiment, naltrexone (1 mg/kg) was administrated s.c. 5 min prior to morphine injection. Hot-plate test results were analyzed using two-way repeated measures ANOVA, followed by Bonferroni post-tests in GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA) (46).

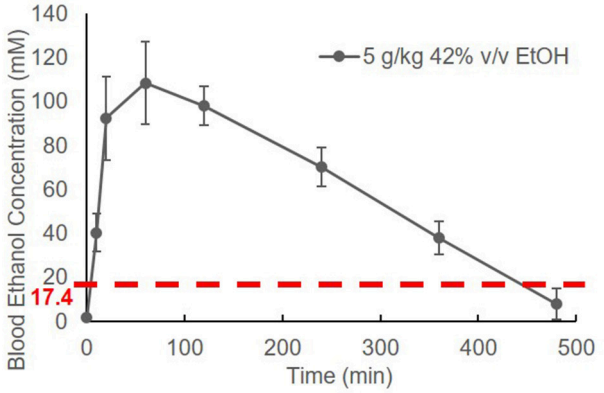

FIGURE 1 | Time course of blood EtOH concentration. Three B6 mice were given EtOH (5 g/kg, 42% v/v, i.g.). The BEC was measured prior to (0 min) and at 10 min, 20 min, 1 h, 2 h, 4 h, 6 h, and 8 h later and normalized by subtracting background (0 min BEC) and plotted against time.

RESULTS

Time Course of Blood EtOH Concentration of Mice Given Single Binge-Like Exposure to High-Dose, High-Concentration EtOH

The BEC of adolescent C57BL/6J (B6) mice was measured prior to and at 10 min, 20 min, 1 h, 2 h, 4 h, 6 h, and 8 h after EtOH administration. Striking elevation of the BEC to approximately 100 mM was observed at 20 min, and it reached a peak of 108.4 ± 18.8 mM at 1 h. The BEC then declined gradually. After a single binge-like exposure, the time required for the animal's BEC to reach < 17.4 mM was close to 8 h (**Figure 1**) .

Blood EtOH Concentration of Mice Given Repeated Binge-Like Exposure to High-Dose, High-Concentration EtOH

The BEC of the adolescent B6 mice treated with 1 d, 2d, or 3 d of high-dose. high-concentration EtOH was determined. At 2 min after EtOH treatment, the BEC had already risen to 20.25 ± 2.89 mM. The BEC at 5 h after the 1st, 2nd, and 3rd EtOH injection were compared with that of the mice given water and at 2 min after EtOH injection (**Figure 2A**). At 5 h after EtOH administration, the BEC was significantly higher than at 2 min and the basal BEC of the mice given water. There was no significant difference in the BEC at 5 h after repeated EtOH administration on different days.

Figure 2B shows that at 24 h after the 3rd EtOH delivery, the BEC had returned to the basal concentration. There was no significant difference in the BEC of these mice compared with that of the mice given water.

Elevated Inflammatory Molecule Expression After Single Binge-Like Exposure to High-Dose, High-Concentration EtOH

To explore the time-dependent response of the inflammatory genes, the gene expression change in both the STr and the NAc at

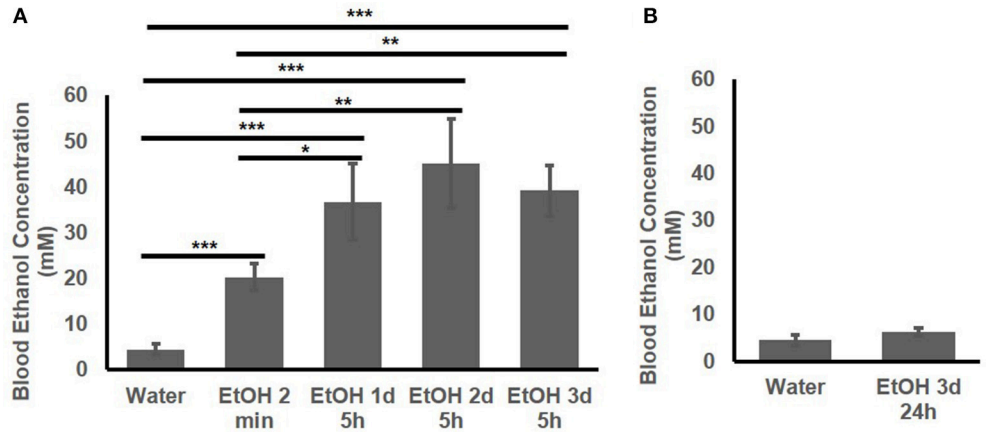

FIGURE 2 | Blood EtOH concentration after repeated EtOH administration (5 g/kg/d; 42% v/v; i.g.)**. (A)** Concentrations at 5 h after 1st, 2nd, and 3rd administration. **(B)** 24 h after 3 d of administration, BEC was back to basal concentration. Statistical analysis was performed using student's t tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $n = 4$.

2 min, 2.5 h, 5 h, 7.5 h, and 24 h after binge-like exposure to EtOH was determined. The qRT-PCR revealed that proinflammatory genes *Il18* ($F_{(4, 15)} = 9.66$, $p < 0.001$) and *Il1b* [$F_{(4,14)} = 3.21$, $p < 0.05$] showed significant changes in the STr within 24 h following EtOH exposure (**Figures 3A,B**). Expression of *Il1b* ($p < 0.05$) and *Il18* ($p < 0.01$) increased significantly at 5 h after EtOH treatment; *Il18* expression remained high until 24 h ($p < 0.01$). Within the time course of 24 h, anti-inflammatory *Il6* showed a significant change in STr [$F_{(4,14)} = 3.26$, $p < 0.05$]; *Il6* increased at 7.5 h but fell after 24 h in STr (**Figure 3C**).

Figures 3D–F shows a late response in expression of inflammatory genes *Il18* [$F_{(4,14)} = 11.10$, $p < 0.001$] and *Il1b* [$F_{(4,14)} = 5.85$, $p < 0.01$] in the NAc. At 7.5 h after binge-like exposure to high-dose, high-concentration EtOH, pro-inflammatory *Il1b* ($p < 0.01$) and *Il18* expression ($p < 0.001$) was significantly elevated at 7.5 h. Meanwhile, the extent of anti-inflammatory *Il6* decreased [$F_{(4,14)} = 0.81$, $p > 0.05$]. After 24 h, the extent of *Il1b, Il18, and Il6* expression did not show a significant difference from that in the 2-min control group.

Repeated Binge-Like Exposure to High-Dose, High-Concentration EtOH Induced Upregulation of MOR Expression

Our previous *in vitro* studies showed that MOR expression is induced by pro-inflammatory cytokines (36, 37), and therefore, we examined the time course of mRNA expression of the MOR gene *Oprm1* in the STr and NAc of brains of binge-like EtOH-treated B6 mice at 2 min, 5 h, or 24 h after the first EtOH infusion and at 24 and 48 h after the third infusion. In the STr, expression of *Oprm1* had increased significantly by 5 h after the first EtOH delivery and then gradually declined [$F_{(4,9)} = 4.25$, $p < 0.05$]; at 5 h after EtOH injection, *Oprm1* expression was significantly higher than that at 2 min ($p < 0.05$) (**Figure 4A**). **Figure 4B** shows a similar trend for *Oprm1* in the NAc [$F_{(4,9)} = 1.95$, $p > 0.05$] (**Figure 4B**).

Binge-Like Exposure to High-Dose, High-Concentration EtOH Alters Morphine's Anti-nociception

To test the behavioral consequences of the gene expression change induced by binge-like exposure to high-dose, high-concentration EtOH, hot-plate tests were performed to evaluate morphine's anti-nociception effect. 24 h after the 3rd d EtOH injection, the mice were injected s.c. with a cumulative dose (0, 0.1, 0.3, 1.0, or 3.0 mg/kg) of morphine at intervals of 30 min and then placed on a 55°C hot plate. As shown in the insert in **Figure 5A**, hot plate latency of the mice given either water or EtOH alone didn't change with no morphine injections. Of the mice given water, the latencies were 10.68 ± 3.52 s and 10.89 ± 2.79 s prior to and after injections, respectively; of the animals given EtOH, the latency readings were 10.93 ± 3.22 s and 10.88 ± 4.04 s, respectively. Morphine produced dose-dependent anti-nociception both in animals given water and in those receiving EtOH. In comparison with the animals given water (blue curve), the animals receiving EtOH showed a greater response to morphine; the response curve was shifted to the left [$F_{(4,120)} = 5.73$, $p < 0.001$] (**Figure 5A**). The latency to analgesic response was significantly enhanced in EtOH-treated animals at 3 mg/kg dose of morphine ($p < 0.001$). The response latency induced by morphine was ablated by naltrexone in animals treated with EtOH [$F_{(4,72)} = 42.78$, $p < 0.001$] or water [$F_{(4,88)} = 13.20$, $p < 0.001$], and no difference was observed between animals given EtOH and those with water [$F_{(4,55)} = 0.87$, $p > 0.05$] (**Figure 5B**).

DISCUSSION

"Binge drinking" is repeated EtOH intake causing a BEC > 80 mg/dL (17.4 mM) (5). The peak BEC of binge drinkers, from 18 to 50 years old or older, has been reported to be as high as 470 mg/dL (that is equal to 102 mM) (47). In addition to the well-characterized liver toxicity, binge drinking can cause various

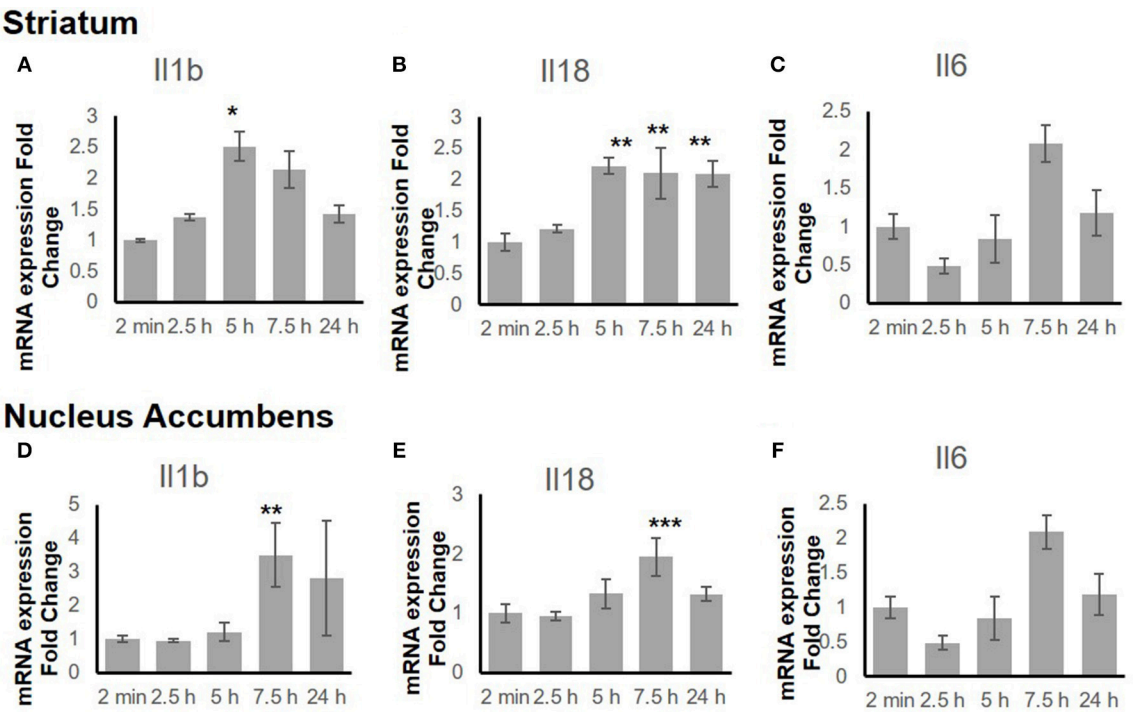

FIGURE 3 | Inflammation-related gene expression change in striatum **(A–C)** and nucleus accumbens **(D–F)** within 24 h in response to binge-like EtOH administration (42% v/v, 5 g/kg, i.g.). Data are expressed as mean ± SE. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-tests, compared with control 2-min EtOH group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $n = 4$.

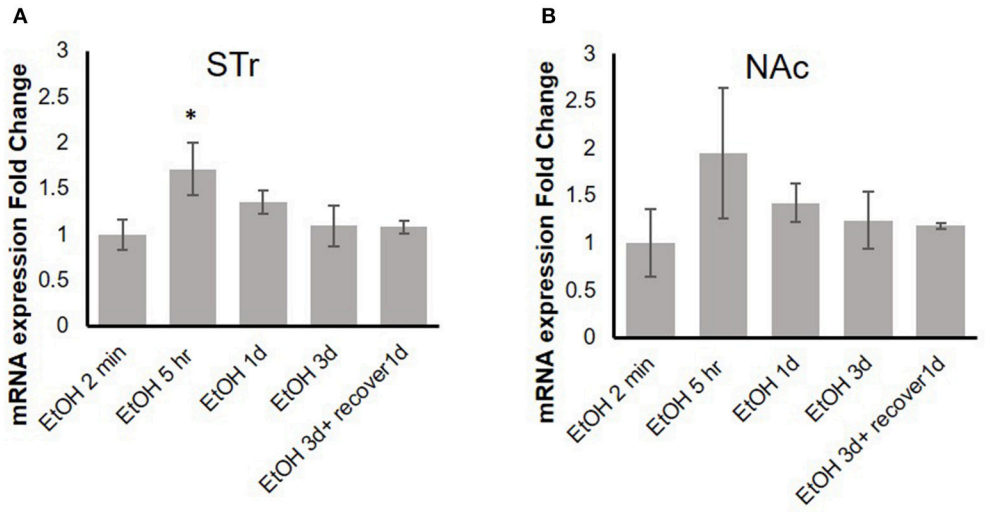

FIGURE 4 | Time-dependent expression change of MOR gene, *Oprm1*, in striatum **(A)** and nucleus accumbens **(B)** in response to binge-like EtOH administration (42% v/v, 5 g/kg/d, i.g.). Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-tests compared with control 2-min EtOH group: $^{*}p < 0.05$; $n = 4$.

neurologic disorders (48). Morphine use/abuse frequently is linked to drinking, especially excessive drinking. Combining opioids with other substances, including EtOH, increases opioid overdose deaths (49). During the last decade, an intertwined epidemic of drug abuse and addiction, EtOH addiction, and binge drinking has emerged (50).

Alcohol research investigators have commonly used rodent models to mimic human alcohol consumption, particularly

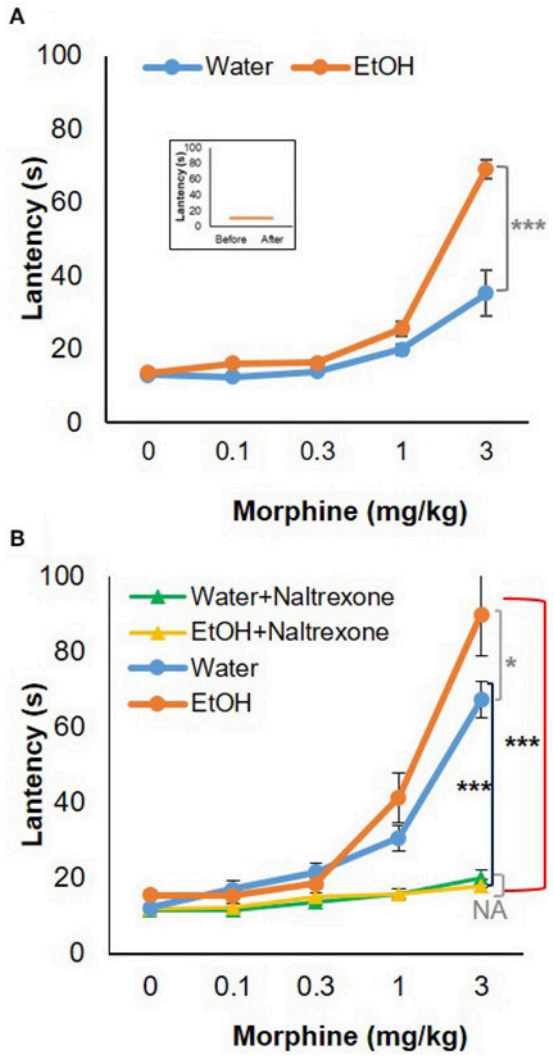

FIGURE 5 | Hot-plate analgesia test in response to morphine in 5-week-old B6 mice given 3-d binge-like EtOH treatment. **(A)** Ethanol-treated mice ($n = 4$) showed elevated response to morphine; morphine response curve was shifted to the left. (Insert) the animals given both water and EtOH didn't show change in latency without morphine injections ($n = 4$). **(B)** The response latency shift was completely ablated by opioid receptor antagonist naltrexone (1 mg/kg; s.c.) ($n = 3$). Statistical analysis was performed using two-way repeated measures ANOVA followed by Bonferroni post-tests. $^{*}p < 0.05$, $^{***}p < 0.001$.

specific drinking patterns such as binge drinking (40, 51, 52). The high dose of EtOH we chose to administer (5 g/kg; 42% v/v) is equivalent to the alcohol by volume (ABV) of the hard liquors, such as vodka. By adapting the high dose of EtOH used in many rodent studies, the specified dose of EtOH was used in our study. The dose of 5 g/kg is well-established as a binge-drinking model in mice (40, 52). To differentiate binge drinking in humans from the model in mice, we have used the term "binge-like exposure to EtOH." **Figure 1** shows that a BEC higher than 17.4 mM was detected in mice after one treatment (i.g.) with EtOH (5 g/kg). The elevation of BEC above 17.4 mM was detected within 2 min (**Figures 1**, **2A**), and the reading reached its peak, 108.4 ± 18.8 mM, at 1 h. The peak declined gradually over 7–8 h. The instant rise of BEC to the NIAAA-defined binge concentration (17.4 mM) and the prolonged high concentration of alcohol could exert significant systemic effects, including intoxication, overburden of the liver for alcohol metabolism, and early and transient pro-inflammatory states (53–56).

Underage drinking, including binge drinking over the weekend, is common (7). To mimic the underage common drinking pattern, we chose 3-d high-dose, high-concentration EtOH dose. After each of the three binge-like EtOH treatments, the instant elevation of the BEC to > 17.4 mM and the long duration of the elevated BEC followed by a reduction to below 17.4 mM on Day 1 were also observed on Day 2 and Day 3 (**Figure 2A**). At the 5 h point, the BEC was 35–40 mM on all 3 days (**Figure 2A**). The 3-d binge-like exposure to EtOH therefore gave the animals the BEC > 17.4 mM for ∼ 24 h in total. We previously reported that this 3-d high-dose, high-concentration EtOH binge-like regimen induces a stress response in the hippocampus of adolescent rats, and the downstream effects of the EtOH-induced stress response in the hippocampus appear to be involved in reduction of the spleen size (40).

In addition, there was a significantly higher plasma endotoxin concentration (200 EU/mL) in the animals given 3-d binge-like exposure to EtOH (40). Other studies have found that binge drinking in human subjects, as well as binge-like exposure to EtOH in rodents, induces gut leakage that elevates the blood endotoxin concentration (15, 16), which leads to production of inflammatory molecules, as well as greater activity of neuroimmune signaling pathways (19, 20). As noted previously, binge-like exposure to high-dose, high-concentration EtOH can trigger a severe immune response that persists even after EtOH has been metabolized (**Figures 1**, **2**) (57).

After administration of EtOH, this volatile compound distributes into the cytosol of all cells. Thus, in addition to the hippocampus in which the 3-d binge-like exposure to EtOH induces stress responses (40), this EtOH regimen is expected to affect other brain areas, including those responsible for changes in pain sensation, as it was previously reported that binge EtOH consumption increases inflammatory pain responses and mechanical and cold sensitivity (14). We focused on the STr and the NAc areas. The STr is part of the brain's reward circuit and a key region responsible for voluntary motor control (41, 58). The STr projects to the basal ganglia, a neuronal circuit necessary for voluntary movement control, and exerts neuronal activity related to movement, rewards, and the conjunction of movement and reward (41, 59). The MOR is highly expressed in the STr (60, 61). The NAc plays an important role in the generation of motivated behaviors (62) and facilitates reward seeking by integrating neurotransmitter-mediated reinforcement signals with environmental stimuli (63, 64).

Figure 3 shows the mRNA time courses of the expression of *Il1b, Il18, and Il6* genes in both the STr and the NAc after one binge-like exposure to high-dose, high-concentration EtOH.

The initial elevation of the expression these three genes in the STr appeared between 5 and 7.5 h, whereas only *Il18* remained significantly elevated at 24 h. However, in the NAc, the significant elevation of the products of these 3 genes was detected only at 7.5 h, and the elevation did not last to 24 h. The elevation time point and differential duration appeared to be brain-region dependent and suggest that EtOH-mediated effects are more intense in the STr than in the NAc because the STr projects to the NAc (65, 66).

Previous studies have demonstrated that expression of *Oprm1* is stimulated by various pro-inflammatory cytokines, including IL-1β (35–37). **Figure 4** shows that in the STr, significant upregulation of *Oprm1* was observed at 5 h after binge-like exposure to EtOH. On the other hand, there was only a trend to elevation of *Oprm1* in the NAc. Although *Oprm1* mRNA elevation disappeared by the 24-h time point, protein level of the mu opioid receptors might stay elevated; confirmation of this idea is needed. Following binge-like exposure to EtOH, inflammatory cytokines and *Oprm1* mRNA levels both change in the brain areas. However, the concurrent elevation of these genes might imply, but not confirm, the direct correlation between expression of inflammatory cytokines and *Oprm1* induced by high-dose, high-concentration EtOH. Our qPCR data showed that binge-like exposure to EtOH caused both neuroinflammation and upregulation of MOR in various brain areas. Further studies are on the way to examine the causal correlation between expression elevation of inflammatory cytokines and *Oprm1* following binge-like exposure to EtOH.

Elevation of *Oprm1* implies increased expression and activity of MOR. Anti-nociception associated with morphine use would be the behavioral outcome of this elevation. **Figure 5A** shows morphine anti-nociception in adolescent C57BL/6J mice as determined by hot-plate analgesia tests at 24 h after 3-d binge-like EtOH treatment. There was an increase in morphine-induced anti-nociception after the EtOH treatment (**Figure 5**). Co-treatment with naltrexone, the selective MOR antagonist, abolished anti-nociception of the cumulative dosage of morphine in the mice given either binge-like exposure to EtOH or saline. This suggests that MOR is involved in morphine's anti-nociception elevation by binge-like exposure to EtOH. This also confirms that the 3-d EtOH at a high dose (5 g/kg) and high concentration (42% v/v) contributed to elevation of neuroinflammation and expression of MOR.

As noted previously, morphine abuse is frequently linked to excessive drinking. A cross-tolerance could take place between EtOH intake and treatment with morphine that is the high-affinity agonist for MOR. Le et al reported that in adult male rats, chronic EtOH consumption decreases the response to treatment with morphine (67). He et al reported that repeated EtOH intake by self-administration (5–6 g/kg/24 h) decreases the anti-nociception of MOR agonists. Inhibition of MOR endocytosis is a possible mechanism underlying the cross-tolerance interaction between EtOH and MOR agonists (68). Shah et al reported that chronic EtOH consumption, but not a single injection that resulted in a BEC of approximately 15 mg/dL, decreases the analgesic potency of opioids in mice. However, the investigators were not sure of the mechanism underlying the interaction between EtOH and opioids, including morphine (69). In examining the alleviation of CRF1 receptor antagonism related to heroin and EtOH dependence, Edwards et al suggested that understanding the relations between chronic exposure to addictive substances such as EtOH and pain-related states such as nociception could reveal the mechanisms underlying the transition to addiction to various substances of abuse (70). Other than the study reported by Shah et al, all these studies suggested how treatment with EtOH changed the activity of MOR and MOR-mediated morphine-induced anti-nociception. Taking these data together with the studies showing that inflammatory cytokines mediate expression of MOR (35–37) and change morphine actions (38, 39), we have reconciled two of our previous studies in light of our current study to address how inflammation induced by various exogenous challenges such as binge drinking might change the subject's response to morphine's anti-nociception.

In one of our previous studies, we used HIV-1 transgenic (HIV-1Tg) rats, mimicking people living with HIV/AIDS and receiving combination antiretroviral therapy (cART), to demonstrate that the persistent presence of HIV-1 proteins elevates inflammation in the brain that possibly correlates with upregulation of MOR expression and the enhancement of morphine's anti-nociception (71, 72). In another study, using F344 rats, we showed that repeated treatment with LPS elevates inflammation in the brain and enhances the sensitivity to morphine's anti-nociception and morphine-induced conditioned place preference (73). With binge-like exposure to high-dose, high-concentration EtOH in adolescent mice, with the persistent presence of HIV proteins in the HIV-1Tg rats (71, 72), and with repeated treatment with LPS there was enhancement of morphine's anti-nociception secondary to upregulation of MOR expression that might be the outcome of elevation of inflammation in the brain. Taken together, our three studies appear to confirm that systemic inflammation attributable to the persistence of viral proteins, repeated treatment with LPS, or binge-like exposure to EtOH leading to elevation of plasma endotoxin, enhanced the rewarding effects of morphine, both physiologically and behaviorally, thereby increasing the potential for morphine abuse and addiction.

In summary, our research indicated that binge-like exposure to high-dose, high-concentration EtOH- enhanced morphine anti-nociception might be mediated via elevation of neuroinflammation. Because morphine is highly addictive, alteration of the animals' response to its use in the course of systemic inflammation could cause the onset and progression of OUDs in the course of inflammation following binge-like exposure to EtOH. As a result of the current study, mega-analysis using bioinformatics tools to link neuroinflammation parameters, expression of MOR, and determinants of nociception will be conducted to extend the findings of our current study.

AUTHOR CONTRIBUTIONS

SC designed the studies; participated in data collection, data analysis, and manuscript preparation; and approved the manuscript submission. WH conducted the animal treatments and PCR array analysis and participated in tissue collection and manuscript preparation. HH conducted the hot-plate tests and participated in tissue collection. IS participated in data interpretation and manuscript preparation.

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REFERENCES

1. Rehm J, Greenfield TK, Rogers JD. Average volume of alcohol consumption, patterns of drinking, and all-cause mortality: results from the US national alcohol survey. *Am J Epidemiol.* (2001) 153:64–71. doi: 10.1093/aje/153.1.64
2. Murray RP, Connett JE, Tyas SL, Bond R, Ekuma O, Silversides CK, et al. Alcohol volume, drinking pattern, and cardiovascular disease morbidity and mortality: is there a U-shaped function? *Am J Epidemiol.* (2002) 155:242–8. doi: 10.1093/aje/155.3.242
3. Mukamal KJ, Conigrave KM, Mittleman MA, Camargo CA, Stampfer MJ, Willett WC, et al. Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men. *N Engl J Med.* (2003) 348:109–18. doi: 10.1056/NEJMoa022095
4. Shield KD, Parry C, Rehm J. Chronic diseases and conditions related to alcohol use. *Alcohol Res.* (2014) 35:155–73.
5. Alcoholism NIoAAa. *NIAAA Council Approves Definition Of Binge Drinking*. NIAAA Newsletter
6. Johnston LD, O'Malley PM, Miech RA, Bachman JG, Schulenberg JE. *Monitoring the Future National Results on Drug Use: 1975-2013: Overview, Key Findings on Adolescent Drug Use*. Ann Arbor: Institute for Social Research, The University of Michigan (2014).
7. Siegel M, DeJong W, Naimi TS, Fortunato EK, Albers AB, Heeren T, et al. Brand-specific consumption of alcohol among underage youth in the United States. *Alcohol Clin Exp Res.* (2013) 37:1195–203. doi: 10.1111/acer.12084
8. Grant BF, Stinson FS, Harford TC. Age at onset of alcohol use and DSM-IV alcohol abuse and dependence: a 12-year follow-up. *J Substance Abuse* (2001) 13:493–504. doi: 10.1016/S0899-3289(01)00096-7
9. Miller JW, Naimi TS, Brewer RD, Jones SE. Binge drinking and associated health risk behaviors among high school students. *Pediatrics* (2007) 119:76–85. doi: 10.1542/peds.2006-1517
10. Brown SA, McGue M, Maggs J, Schulenberg J, Hingson R, Swartzwelder S, et al. A developmental perspective on alcohol and youths 16 to 20 years of age. *Pediatrics* (2008) 121:S290–S310. doi: 10.1542/peds.2007-2243D
11. Squeglia LM, Spadoni AD, Infante MA, Myers MG, Tapert SF. Initiating moderate to heavy alcohol use predicts changes in neuropsychological functioning for adolescent girls and boys. *Psychol Addict Behav.* (2009) 23:715. doi: 10.1037/a0016516
12. Austic E, McCabe SE, Stoddard SA, Ngo QE, Boyd C. Age and cohort patterns of medical and nonmedical use of controlled medication among adolescents. *J Addict Med.* (2015) 9:376–82. doi: 10.1097/ADM.0000000000000142
13. Sanhueza C, Garcia-Moreno LM, Exposito J. Weekend alcoholism in youth and neurocognitive aging. *Psicothema* (2011) 23:209–14.
14. Bergeson SE, Blanton H, Martinez JM, Curtis DC, Sherfey C, Seegmiller B, et al. Binge ethanol consumption increases inflammatory pain responses and mechanical and cold sensitivity: tigecycline treatment efficacy shows sex differences. *Alcoholism* (2016) 40:2506–15. doi: 10.1111/acer.13252
15. Bode C, Kugler V, Bode JC. Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *J Hepatol.* (1987) 4:8–14. doi: 10.1016/S0168-8278(87)80003-X
16. Keshavarzian A, Farhadi A, Forsyth CB, Rangan J, Jakate S, Shaikh M, et al. Evidence that chronic alcohol exposure promotes intestinal oxidative stress, intestinal hyperpermeability and endotoxemia prior to development of alcoholic steatohepatitis in rats. *J Hepatol.* (2009) 50:538–47. doi: 10.1016/j.jhep.2008.10.028
17. Szabo G, Bala S, Petrasek J, Gattu A. Gut-liver axis and sensing microbes. *Digest Dis.* (2010) 28:737–44. doi: 10.1159/000324281
18. Bala S, Marcos M, Gattu A, Catalano D, Szabo G. Acute binge drinking increases serum endotoxin and bacterial dna levels in healthy individuals. *PLoS ONE* (2014) 9:e96864. doi: 10.1371/journal.pone.0096864
19. Molina PE, Happel KI, Zhang P, Kolls JK, Nelson S. Focus on: alcohol and the immune system. *Alcohol Res Health* (2010) 33:97–108.
20. Lehnert M, Kovacs EJ, Molina PE, Relja B. Modulation of inflammation by alcohol exposure. *Media Inflammat.* (2014) 2014:283756. doi: 10.1155/2014/283756
21. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor Perspect Biol.* (2014) 6:a016295. doi: 10.1101/cshperspect.a016295
22. Wang Y, Li Y-B, Yin J-J, Wang Y, Zhu L-B, Xie G-Y, et al. Autophagy regulates inflammation following oxidative injury in diabetes. *Autophagy* (2013) 9:272–7. doi: 10.4161/auto.23628
23. Pasternak GW. Pharmacological mechanisms of opioid analgesics. *Clin Neuropharmacol.* (1993) 16:1–18.
24. Chiara GD, Alan North R. Neurobiology of opiate abuse. *Trends Pharmacol Sci.* (1992) 13:185–93. doi: 10.1016/0165-6147(92)90062-B
25. Wise RA. Neurobiology of addiction. *Curr Opin Neurobiol.* (1996) 6:243–51.
26. Stevens KE, Mickley GA, McDermott LJ. Brain areas involved in production of morphine-induced locomotor hyperactivity of the C57B1/6J mouse. *Pharmacol Biochem Behav.* (1986) 24:1739–47. doi: 10.1016/0091-3057(86)90514-9
27. Porreca F, Burks TF. Supraspinal opioid receptors in antinociception. In: Herz A, Akil H, Simon EJ, editors. *Opioids II* Berlin, Heidelberg: Springer Berlin Heidelberg (1993). p 21–51.
28. Aghajanian GK. Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. *Nature* (1978) 276:186–8. doi: 10.1038/276186a0
29. Johnson SM, Fleming WW. Mechanisms of cellular adaptive sensitivity changes: applications to opioid tolerance and dependence. *Pharmacol Rev.* (1989) 41:435–88.
30. Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, et al. Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol.* (1994) 45:330–4.
31. Steinmiller CL, Young AM. Pharmacological Selectivity of CTAP in a warm water tail-withdrawal antinociception assay in rats. *Psychopharmacology* (2008) 195:497–507.
32. Bannon AW, Malmberg AB. Models of nociception: hot-plate, tail-flick, and formalin tests in rodents. *Curr Protocols Neurosci.* (2007) Chapter 8:Unit 8.9.doi: 10.1002/0471142301.ns0809s41
33. Corrigan F, Wu Y, Tuke J, Coller JK, Rice KC, Diener KR, et al. Alcohol-induced sedation and synergistic interactions between alcohol and morphine: a key mechanistic role for Toll-Like Receptors and MyD88-dependent signalling. *Brain Behav Immunity* (2015) 45:245–52. doi: 10.1016/j.bbi.2014.12.019
34. Shrivastava P, Cabrera MA, Chastain LG, Boyadjieva NI, Jabbar S, Franklin T, et al. Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal

apoptosis in the hypothalamus: effects of neonatal alcohol. *J Neuroinflamma.* (2017) 14:83. doi: 10.1186/s12974-017-0844-3
35. Vidal EL, Patel NA, Wu G, Fiala M, Chang SL. Interleukin-1 induces the expression of mu opioid receptors in endothelial cells. *Immunopharmacology* (1998) 38:261–6. doi: 10.1016/S0162-3109(97)00085-4
36. Byrne LS, Peng J, Sarkar S, Chang SL. Interleukin-1 beta-induced up-regulation of opioid receptors in the untreated and morphine-desensitized U87 MG human astrocytoma cells. *J Neuroinflamma.* (2012) 9:252. doi: 10.1186/1742-2094-9-252
37. Langsdorf EF, Mao X, Chang SL. A role for reactive oxygen species in endotoxin-induced elevation of MOR expression in the nervous and immune systems. *J Neuroimmunol.* (2011) 236:57–64. doi: 10.1016/j.jneuroim.2011.05.009
38. Homji NF, Mao X, Langsdorf EF, Chang SL. Endotoxin-induced cytokine and chemokine expression in the HIV-1 transgenic rat. *J Neuroinflamm.* (2012) 9:3–3. doi: 10.1186/1742-2094-9-3
39. Homji NF, Vigorito M, Chang SL. Morphine-induced conditioned place preference and associated behavioural plasticity in HIV-1 transgenic rats. *Int J Clin Exp Med.* (2012) 5:105–23.
40. Liu X, Connaghan KP, Wei Y, Yang Z, Li MD, Chang SL. Involvement of the hippocampus in binge ethanol-induced spleen atrophy in adolescent rats. *Alcoholism Clin Exp Res.* (2016) 40:1489–500. doi: 10.1111/acer.13109
41. Baez-Mendoza R, Schultz W. The role of the striatum in social behavior. *Front Neurosci.* (2013) 7:233. doi: 10.3389/fnins.2013.00233
42. Chang SL, Patel NA, Romero AA. Activation and desensitization of Fos immunoreactivity in the rat brain following ethanol administration. *Brain Res.* (1995) 679:89–98. doi: 10.1016/0006-8993(95)00210-H
43. Yang Z, Nesil T, Connaghan KP, Li MD, Chang SL. Modulation effect of HIV-1 Viral proteins and nicotine on expression of the immune-related genes in brain of the hiv-1 transgenic rats. *J Neuroimmune Pharmacol.* (2016) 11:562–71. doi: 10.1007/s11481-016-9679-4
44. Winer J, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. *Anal Biochem.* (1999) 270:41–9. doi: 10.1006/abio.1999.4085
45. Nealon CM, Patel C, Worley BL, Henderson-Redmond AN, Morgan DJ, Czyzyk TA. Alterations in nociception and morphine antinociception in mice fed a high-fat diet. *Brain Res Bull.* (2018) 138:64–72. doi: 10.1016/j.brainresbull.2017.06.019
46. Masocha W, Kombian SB, Edafiogho IO. Evaluation of the antinociceptive activities of enaminone compounds on the formalin and hot plate tests in mice. *Sci Rep.* (2016) 6:21582 doi: 10.1038/srep21582
47. Cucciare MA, Darrow M, Weingardt KR. Characterizing binge drinking among US military Veterans receiving a brief alcohol intervention. *Addict Behav.* (2011) 36:362–7. doi: 10.1016/j.addbeh.2010.12.014
48. Nesic J, Duka T. Binge drinking and withdrawal: neural mechanisms in humans a2. In: *Biological Research on Addiction.* San Diego, CA: Academic Press (2013). p 333–40.
49. Kandel DB, Hu MC, Griesler P, Wall M. Increases from 2002 to 2015 in prescription opioid overdose deaths in combination with other substances. *Drug Alcohol Depend.* (2017) 178:501–11. doi: 10.1016/j.drugalcdep.2017.05.047
50. Miller JW, Gfroerer JC, Brewer RD, Naimi TS, Mokdad A, Giles WH. Prevalence of adult binge drinking: a comparison of two national surveys. *Am J Prevent Med.* (2004) 27:197–204. doi: 10.1016/j.amepre.2004.05.004
51. Brandon-Warner E, Schrum LW, Schmidt CM, McKillop IH. Rodent models of alcoholic liver disease: of mice and men. *Alcohol* (2012) 46:715–25. doi: 10.1016/j.alcohol.2012.08.004
52. Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). *Nat Protocols* (2013) 8:627. doi: 10.1038/nprot.2013.032
53. Waszkiewicz N, Szulc A. Immunity defects in acute and chronic alcohol intoxication. *Pol Merkur Lekarski* (2010) 29:269–73.
54. Hillbom M, Saloheimo P, Juvela S. Alcohol consumption, blood pressure, and the risk of stroke. *Curr Hyperten Rep.* (2011) 13:208–13. doi: 10.1007/s11906-011-0194-y
55. Yun JW, Son MJ, Abdelmegeed MA, Banerjee A, Morgan TR, Yoo SH, et al. Binge alcohol promotes hypoxic liver injury through a CYP2E1-HIF-1alpha-dependent apoptosis pathway in mice and humans. *Free Radical Biol Med.* (2014) 77:183–94. doi: 10.1016/j.freeradbiomed.2014.08.030
56. Afshar M, Richards S, Mann D, Cross A, Smith GB, Netzer G, et al. Acute immunomodulatory effects of binge alcohol ingestion. *Alcohol* (2015) 49:57–64. doi: 10.1016/j.alcohol.2014.10.002
57. Horner C, Bouchon A, Bierhaus A, Nawroth PP, Martin E, Bardenheuer HJ, et al. Role of the innate immune response in sepsis. *Der Anaesthesist* (2004) 53:10–28. doi: 10.1007/s00101-003-0626-4
58. Yager LM, Garcia AF, Wunsch AM, Ferguson SM. The ins and outs of the striatum: role in drug addiction. *Neuroscience* (2015) 301:529–41. doi: 10.1016/j.neuroscience.2015.06.033
59. Hollerman JR, Tremblay L, Schultz W. Influence of reward expectation on behavior-related neuronal activity in primate striatum. *J Neurophysiol.* (1998) 80:947–63. doi: 10.1152/jn.1998.80.2.947
60. Chaturvedi K. Opioid peptides, opioid receptors and mechanism of down regulation. *Indian J Exp Biol.* (2003) 41:5–13.
61. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev.* (2009) 89:1379–412. doi: 10.1152/physrev.00005.2009
62. Nicola SM. The nucleus accumbens as part of a basal ganglia action selection circuit. *Psychopharmacology* (2007) 191:521–50. doi: 10.1007/s00213-006-0510-4
63. Stuber GD, Klanker M, de Ridder B, Bowers MS, Joosten RN, Feenstra MG, et al. Reward-predictive cues enhance excitatory synaptic strength onto midbrain dopamine neurons. *Science* (2008) 321:1690–2. doi: 10.1126/science.1160873
64. Brown HD, McCutcheon JE, Cone JJ, Ragozzino ME, Roitman MF. Primary food reward and reward-predictive stimuli evoke different patterns of phasic dopamine signaling throughout the striatum. *Eur J Neurosci.* (2011) 34:1997–2006. doi: 10.1111/j.1460-9568.2011.07914.x
65. Quintero GC. Role of nucleus accumbens glutamatergic plasticity in drug addiction. *Neuropsychiatr Dis Treat.* (2013) 9:1499–512. doi: 10.2147/NDT.S45963
66. Navratilova E, Atcherley C, Porreca F. Brain circuits encoding reward from pain relief. *Trends Neurosci.* (2015) 38:741–50. doi: 10.1016/j.tins.2015.09.003
67. Le AD, Khanna JM, Kalant H, LeBlanc AE. Cross-tolerance between ethanol and morphine. *Adv Exp Med Biol.* (1980) 132:771–7.
68. He L, Whistler JL. Chronic ethanol consumption in rats produces opioid antinociceptive tolerance through inhibition of Mu opioid receptor endocytosis. *PLoS ONE* (2001) 6:e19372. doi: 10.1371/journal.pone.0019372
69. Shah S, Duttaroy A, Sehba F, Chen B, Philippe J, Monderson T, et al. The effect of ethanol drinking on opioid analgesia and receptors in mice. *Alcohol* (1997) 14:361–6. doi: 10.1016/S0741-8329(96)00184-X
70. Edwards S, Vendruscolo LF, Schlosburg JE, Misra KK, Wee S, Park PE, et al. Development of mechanical hypersensitivity in rats during heroin and ethanol dependence: alleviation by CRF1 receptor antagonism. *Neuropharmacology* (2012) 62:1142–51. doi: 10.1016/j.neuropharm.2011.11.006
71. Chang S, Vigorito M. Role of HIV-1 infection in addictive behavior: a study of a HIV-1 transgenic rat model. *Am J Infect Dis.* (2006) 2:98–106. doi: 10.3844/ajidsp.2006.98.106
72. Chang SL, Connaghan KP. Behavioral and molecular evidence for a feedback interaction between morphine and HIV-1 viral proteins. *J Neuroimmune Pharmacol.* (2012) 7:332–40. doi: 10.1007/s11481-011-9324-1
73. Chang S, Sarkar S, Vigorito M, Mao X. Endotoxin enhancement of morphine-induced conditioned place preference. *Int J Clin Exp Med.* (2016) 5:105–23. doi: 10.4303/jdar/235950

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MINI REVIEW
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Management of Opioid Addiction With Opioid Substitution Treatments: Beyond Methadone and Buprenorphine

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With the opioid crisis in North America, opioid addiction has come in the spotlight and reveals the weakness of the current treatments. Two main opioid substitution therapies (OST) exist: buprenorphine and methadone. These two molecules are mu opioid receptor agonists but with different pharmacodynamic and pharmacokinetic properties. In this review, we will go through these properties and see how they could explain why these medications are recognized for their efficacy in treating opioid addiction but also if they could account for the side effects especially for a long-term use. From this critical analysis, we will try to delineate some guidelines for the design of future OST.

Keywords: addiction, morphine, buprenorphine, methadone, substitution treatment

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INTRODUCTION

When people talk about opioid problems or addiction to opioids, they think of opioids that some people get on the street, such as heroin, with the idea that only a minority of persons is concerned. But the truth is very different and anyone who uses an opioid can develop addictive behaviors. This is not a specific problem for heroin users as opioids are very useful molecules and powerful medications that are generally prescribed to relieve severe pain. Thus, problematic opioid use may also include the misuse of prescription opioid medications, such as oxycodone, morphine, or codeine, or the use of a drug for which no personal prescription has been received. As a result, the number of people over-using or dependent on opioids is increasing dramatically and is a public health problem. Over the past few years, both the U.S. and Canada have seen a spectacular increase in opioid overdose rates. From 1999 to 2016, more than 350,000 people died from opioid overdose in U.S. (https://www.cdc.gov/drugoverdose/epidemic/index.html). This so-called "opioid epidemic" or "opioid crisis" started in the 1990's with the conjunction of different factors including propaganda by pharmaceutical companies claiming that their opioids had a low liability to induce addiction mainly because of the extended release formula, and a better pain management which led to a widespread use of opioid drugs for the treatment of moderate pain (1, 2).

Behind these perfectly quantified data, there is another figure that is difficult to quantify, but which is most certainly very high, people with opioid addiction. This crisis shed light on the weakness of available treatments to manage opioid addiction. Two main medications—the opioid substitution treatments (OST) are used: buprenorphine and methadone. After a rapid review of neurobiology of opoid addiction, we will review some properties of these OST that could explain why they have a certain success [for an extensive review on methadone and/or buprenorphine, see (3)]. However, this success is only relative (relapses very often occur even when patients are under

TABLE 1 | Examples of preclinical and clinical opioid drugs.

Ligand	Selectivity	Activity	
Morphine	MOPr >> KOPr	Agonist	Analgesia Respiratory depression
Heroin	*MOPr >> KOPr*	*Agonist*	Fast penetration in the brain *Acts through its mainly active metabolite, morphine*
Buprenorphine	MOPr DOPr KOPr	Partial agonist Antagonist Antagonist	Reduces withdrawal Low risk of respiratory depression
Methadone	MOPr	Agonist	Reduces withdrawal, risk of respiratory depression
TRV130	MOPr	Agonist (biased toward G protein)	
PMZ21	MOPr > KOPr > DOPr	Biased agonist Antagonist Weak agonist	 Analgesia
Cebranopadol	MOPr, KOPr, NOPr > DOPr	Agonist (partial at KOPr)	Reduced respiratory depression
AT-121	MOPr, NOPr > DOPr KOPr	Partial agonist Partial agonist	
Naloxone	MOPr, DOPr, KOPr	Antagonist	Blocks euphoric effects Reverses respiratory depression
Naltrexone	MOPr, KOPr > KOPr	Antagonist	
PL37, PL265	MOPr, DOPr (via enkephalins)	Inhibit enkephalins degradation	Analgesia Lack opioid-associated side effects

OST) and as banning opioids is not an option, it is therefore important to discuss the future of opioid research. A table with the opioids cited in the present review is included to facilitate the reading of the manuscript (**Table 1**).

THE NEUROBIOLOGICAL BASIS OF OPIOID ADDICTION

It has been known for a long time that opioids such as morphine, heroin, and derivatives induce numerous pharmacological responses, including analgesia, dependence, respiratory depression or euphoria (4, 5). From these observations, evidence that different opioid drug effects could only be explained by the existence of stereospecific receptors has emerged. In the 1970s, the endogenous opioid receptors were discovered (6–8), followed by the characterization of the endogenous opioid peptides (9). Since these identifications numerous studies have been conducted in the opioid field.

Historically, three opioid receptors have been characterized, mu (MOPr), delta (DOPr), and kappa (KOPr). Additional receptor types have been identified, but are no longer considered as "classical" opioid receptors (e.g., sigma, nociceptin/orphanin receptor, NOPr) (10). The three opioid receptors were cloned in the early nineties (11–14). Since this period, several knockout mice lines, each harboring deletions of the genes encoding a particular opioid receptor, have been used to clarify the specific role of the different receptors *in vivo* and in many physiopathological conditions (15). In this review the focus will be on reward and addiction.

It is well-known that all drugs of abuse increase extracellular dopamine levels in the nucleus accumbens (Nac), either directly (e.g., cocaine and amphetamine directly target dopamine transporters), or indirectly (e.g., opioids decrease GABA release in the ventral tegmental area, leading to an activation of dopamine neurons). Several lines of evidence indicate that MOPr play a key role in mediating the rewarding effects of opioids, while the role of DOPr remains debatable, and KOPr are considered to have opposite functions to those of MOPr in the regulation of reward and addiction. KOPr agonists have dysphoric and aversive effects in humans and rodents (16, 17), in good agreement with decreases in dopamine release in the Nac observed following injection of selective agonists in this brain structure (18).

The pharmacological responses induced by opioids (e.g., conditioned place preference, intravenous self-administration, locomotor activity, analgesia) are abolished in MOPr knockout mice, demonstrating that MOPr represent the primary *in vivo* molecular target for these ligands (15). Morphine-induced conditioned place preference in an unbiased procedure is also reduced in DOPr knockout mice (19, 20), but these animals show normal motivation to obtain morphine in intravenous self-administration paradigm (20). These results, combined with other data obtained from other experimental approaches suggest that morphine reward and motivation to obtain opioids are intact in DOPr knockout mice, however drug-context association is more certainly impaired.

Both with most clinically useful (e.g., morphine, fentanyl, oxycodone) and most largely abused (heroin) opioids, opioid-use disorder is a public health problem. The number of

opioid prescriptions sharply increased in the past two decades, increasing risks for addiction and overdoses. Addiction to prescribed opioids is associated with transition to illicit opioid use like heroin (21), and overdoses have strongly risen since the 1990s (22). As mentioned earlier the notion of "opioid crisis" or "opioid epidemic" has emerged in North America, and to a lesser extent in Australia (23). European countries appear to be less affected (24), but even if the risk in Europe appears relatively limited, vigilance is needed (25).

Opioid addiction is a brain disorder, involving alterations in neuronal circuits with complex neuroadaptative mechanisms that lead to dependence, craving, and relapse; thus contributing to the maintenance of drug use. Until now, no medication can reverse the drug-induced changes observed in the brain that are involved in the relapsing nature of opioid-use disorders, even after a protracted abstinence. Currently, the therapeutic approach using an agonist strategy with methadone and buprenorphine, has shown physical and psychosocial improvements in drug users, but these molecules possess MOPr agonist properties which limit their clinical usefulness, as described below.

CHARACTERISTICS OF THE OPIOID SUBSTITUTION TREATMENTS

The Way They Reach Their Target: Pharmacokinetic Properties

The therapeutic action of a compound strongly depends on its pharmacokinetic properties (26). The opioid users seek a rapid and intense euphoria which is obtained with heroin, which is a prodrug. Indeed, although it has a low affinity toward MOPr, its action is mainly mediated by its metabolites including morphine (27, 28). The intense and rapid euphoria following heroin administration is partly due to its high lipophilic nature, enabling the molecule to readily cross the blood-brain barrier (29). Another very important characteristic that determines the fast action of heroin is the route of administration: the intravenous route being the fastest (30). OST are both oral medications, methadone as a syrup or pills and buprenorphine as sublingual tablet or films. Methadone has a good oral bioavailability (between 40 and 95%) (31), conversely, buprenorphine has a poor oral bioavailability. In any case, both oral and sublingual routes allow the OST to diffuse slowly, thus avoiding peak effects which contribute to addiction. Therefore, after ingestion, the peak effects and peak plasma levels are reached between 1 and 6 h for methadone (average: at 4 h) (32), whereas the peak levels occur ∼1 h after buprenorphine administration (33, 34). One of the mandatory features to be a good OST is that it needs to have slow metabolism and elimination profiles which avoid patients experiencing withdrawal. Methadone and buprenorphine fulfill these criteria, with an average half-life of 22 and 32 h, respectively (31, 33), therefore these medicines are taken once a day, which favors the observance. Opioid pharmacokinetics are influenced by their interaction with enzymes that metabolize xenobiotic, such as cytochromes P450 and efflux pumps. For instance, the two diphenylpropylamine opioids loperamide and methadone, which display similar structures, have different fates once administered. Whereas, methadone transport to the brain is partly restricted by the multidrug resistance protein 1 (MDR-1) (35), loperamide is unable to cross the brain blood-brain barrier due to the presence of the same efflux pump (36) showing that loperamide is a better substrate for MDR-1 than methadone. Many pharmacogenetic studies of cytochromes P450 such as CYP450 3A4 (one of the main cytochromes involved in OST metabolism) or efflux pumps have been conducted to explain the variability in OST dosing. Overall, it appears that although some variants of these genes are associated with OST plasma levels, their influence on dose requirement is very low (37). OST pharmacokinetics is more likely to be influenced by co-prescribed drugs, which interact with their metabolism. For instance, delavirdine, an antiretroviral medication used in HIV treatment, inhibits CYP450 3A4 and thus induces an elevation of methadone plasmatic concentration and drug delayed clearance (38).

The Way They Interact With the Target: Pharmacodynamic Properties

Methadone and buprenorphine bind MOPr with a higher affinity as compared to morphine. Therefore, when a patient under OST uses heroin, its effects will be reduced, as the morphine concentration in the brain will not be high enough to displace methadone or buprenorphine from the receptor. This highlights the issue of the optimum dose of OST, so each patient must have a sufficiently high brain concentration to avoid withdrawal symptoms. In addition, buprenorphine has a very low receptor dissociation rates (39–41) conferring a long duration of action (which contributes to its long half-life) and reinforcing its inability to be displaced by other opioids. Opioid overdoses cause death by respiratory depression: indeed, whereas tolerance to analgesia develops rapidly, tolerance to respiratory depression is far weaker and slower to appear (42). Methadone is a full agonist at the MOPr (43) and its potency and efficacy increase the risk of overdose, thus requiring this drug to be administered to treat opioid dependency only in designated medical units with trained staff. Buprenorphine has a particular pharmacological profile and is described as a MOPr partial agonist (44). In pioneering studies conducted in rodents, buprenorphine displayed a ceiling effect, exerting only partial analgesia compared to morphine or more effective agonists (45). Nevertheless, more recent studies have not shown this ceiling effect in other species such as humans where buprenorphine is quite powerful (46)—probably because there is a greater MOPr reserve [i.e., more spare receptors (47)]. The ceiling effect is probably rather more specific to the target system (e.g., respiration) than to the species (48) and may be explained by differences in the receptor reserve in the different pathways (pain, respiration…), probably explaining the lack of severe respiratory depression at analgesic doses with this drug (46). As a consequence, it was allowed to prescribe buprenorphine as an ambulatory medicine in many countries including UK, France, USA. Buprenorphine is also depicted as a KOPr antagonist, which might contribute to its antidepressant effect (49).

WHY SEARCHING FOR NEW TREATMENTS FOR OPIOID ADDICTION?

It is undeniable that the actual OST, methadone and buprenorphine, have brought a substantial benefit in the opioid addiction treatments. Indeed, when associated with a risk reduction policy they substantially reduced death by overdoses and the transmission of blood-borne diseases. They help addicts to follow their recovery program and contribute to their social reintegration. OST were also shown to preserve immune (50) and memory (51) functions, have positive effects on psychopathology (52, 53) and reduce polyabuse (54).

However, like any other medications, OST are not fully effective as many patients under OST might still relapse (55, 56), and because they are MOPr agonists they may be misused (57). The promised safety of buprenorphine was challenged as soon it arrived on the market and for example in France, several death cases were reported where buprenorphine was diverted (intravenous use). Whereas, several of these cases included the concomitant use of buprenorphine with other depressants of the respiratory system (ethanol and/or benzodiazepines), some of them reported only buprenorphine use (58). More recently, when gabapentin was used with opioids a substantial increase in the risk of opioid-related death was measured (59). Beyond the high risk of fatal respiratory depression (see above), methadone is associated with prolongation of the electrocardiographic QT interval (60, 61). However, the link to cardiac dysrhythmia and sudden cardiac death remains an open question. Indeed, recent studies did not confirm the role of methadone in sudden cardiac death (62) as it was previously suspected.

Many side effects have been reported with these OST such as a decrease of cognitive performance (63) or sexual dysfunction in men (64, 65). Finally, as they remain MOPr agonists, they will contribute to maintain—very likely to a lesser extent—the allostasis generated by previously abused opioids. In rodents, a short treatment (5 days) with buprenorphine or methadone is able to induce behavioral and neurochemical modifications until 35 days after withdrawal (66, 67). It therefore appears necessary to find new MOPr agonists, or new combinations of MOPr agonists and other ligands, that would not induce the neuroadaptations responsible for the harmful effects of opioids (e.g., addiction, respiratory depression), and would therefore gradually restore homeostasis, thus allowing for instance a complete escape from addiction.

On the other hand, to avoid buprenorphine diversion, different formulations of buprenorphine are currently evaluated and usually consist of transdermal patches, subcutaneous depot injections, or subdermal implants (68). An alternative strategy to limit diversion is to combine buprenorphine with an opioid antagonist, naloxone (suboxone). Naloxone has a poor oral bioavailability, but when injected intravenously (in the case of misuse), it will precipitate withdrawal. Human studies shown that it has a reduced abuse potential (69), however recent preclinical (70) and clinical (71, 72) data questioned the lower level of rewarding properties of intravenous suboxone.

SOME LEADS ON THE FUTURE OF OPIOID RESEARCH

The "opioid crisis" dramatically exposes the need for more research in at least two main directions. One is to find better opioid analgesics with less and even virtually no addictive potential. The other direction is the discovery of new medications to treat opioid addiction. We will discuss these two directions focusing on opioid-based drugs.

Since the 1990's, studies have demonstrated that different ligands could induce (or select) different receptor conformations that could promote different signaling pathways. This concept is now known as biased agonism or functional selectivity (73). For opioid receptors, this notion combined with the pioneer work of Bohn and co-workers paved the way to design new opioids. It is now well-established that following ligand binding, MOPr activation could result in the activation of multiple downstream pathways through either G protein dependent processes (e.g., regulation of ion channels, adenylate cyclase inhibition) or G protein independent processes (e.g., beta-arrestin signaling). Beta-arrestin is a protein that binds the activated and phosphorylated receptor and is responsible for its desensitization and endocytosis (74). Bohn and co-workers found that in beta-arrestin-2 knockout mice, morphine analgesia was increased and prolonged (75, 76) with a decrease of respiratory depression and acute constipation (77). Therefore, it has been suggested that biased opioid agonists toward G protein pathway will retain analgesic effects with a reduction of side effects including tolerance mediated by beta-arrestin activation. This last point is of particular importance as tolerance, by increasing the dose required to induce the same effects, will contribute to dependence and overdose. So, recently few opioid biased agonists for the treatment of pain have been developed including TRV130, a compound recently entered in phase 3 to treat moderate and severe acute pain (78). This molecule is biased toward G proteins and shows less tolerance and respiratory depression as compared to morphine (79). Using the recent discovery of MOPr structure (80), Manglik and co-workers discovered PMZ21 a molecule that displays a protracted analgesia as compared to morphine and like the TRV130 has no rewarding effects in the conditioned place preference paradigm (81). However, this lack of rewarding effects has been recently challenged by Altarifi and colleagues who found that TRV130 reduced the threshold of intracranial self-stimulation (82). These results are not surprising as these molecules selectively target MOPr, so alternative strategies are currently considered such as targeting multiple opioid receptors to reduce some side effects and increase efficacy (83). For instance, cebranopadol a mixed MOPr/DOPr/KOPr/NOPr receptor agonist was found to be efficient in acute and chronic pain and development of tolerance was delayed as compared to equianalgesic doses of morphine (84). More recently, Ding et al. reported the discovery of AT-121, a MOPr/NOPr mixed agonist with analgesic effects in non-human primates and a lack of common opioid-associated side effects such as physical dependence, abuse potential, respiratory depression, and opioid-induced hyperalgesia (85). Finally, instead of activating

opioid receptors with synthetic compounds that could results in unwanted effects (due to overstimulation in many target systems) the use of endogenous ligands has been proposed through the blockade of the catabolism of the endogenous peptides. This approach was developed by Roques and co-workers in the 1980's who published the first study showing that blocking enzymatic degradation of enkephalins enhances their physiological effects (86). It has the advantage to target only the structures where enkephalins are expressed, thus explaining why multiple preclinical studies demonstrated that these compounds are as effective as morphine to produce analgesia but without promotion of tolerance, physical dependence, constipation or respiration depression (87). Indeed, enkephalins are highly expressed in pain-control centers (88) whereas they are found in low amount in respiratory centers (89) or locus ceruleus (90), a structure involved in the expression of opioid physical dependence (91). At the moment, two of these molecules, PL37 and PL265, are in clinical development for treating acute and chronic pain.

Regarding the treatment of opioid addiction, no real progress has been made since the introduction of methadone and buprenorphine and most of the current research consists of work related to these compounds or other marketed opioids such as modifying the formulation to obtain slow-release compounds. For instance, it has been proposed to use slow release morphine for patients who cannot tolerate methadone (92).

Recently, some opioid antagonists (e.g., naltrexone, naloxone) have been approved for opioid addiction but only for abstinent patients because of the risk of withdrawal. They have multiple benefits: lack of reinforcing effects, blockade of the euphoric effects of opioids, relative safety (no respiratory depression) (93). Even so, the adherence to these medications is generally poor, thus limiting their efficacies for the prevention of relapse in patients with opioid-use disorder. To circumvent this low treatment observance, an injectable extended-release naltrexone was developed. The first meta-analysis on its efficacy mainly revealed that, unsurprisingly, the success of extended-release naltrexone was higher in opioid detoxified patients. However, when randomization occurred after detoxification, extended-release naltrexone showed similar efficacy to buprenorphine, whereas when randomization occurred prior to detoxification, buprenorphine efficacy was superior (94). The fact remains that opioid antagonists are very efficient in emergency medicine, by preventing opioid overdose fatalities (95). Naloxone is actually the only opioid antagonist approved for treating opioid overdose. Its efficacy is based on a rapid onset of action via intravenous route (2–3 min) (96), but its shorter half-life than that of most opioid agonists, requires multiple injections or continuous administration to reverse respiratory depression. A recent study showed that it was also able to reverse buprenorphine-induced respiratory depression (97). It is noteworthy that fast opioid detoxification in opioid-dependent patients might lead to acute opioid withdrawal syndrome accompanied by catecholamine releases, responsible for cardiac and respiratory functions impairment (98).

CONCLUSION

This review was focused on opioids, but knowing whether if they will remain the gold standard in pain management is an open question considering the opioid crisis. In addition, long-term treatment with OST, more than restoring the neurobiological equilibrium disturbed by the opioid misuse, will maintain drug-induced neuroplastic changes. So, besides the short and mid-term necessary research on the discovery of safer opioids, other pharmacological strategies have to be envisioned based either on different use of existing treatments or on other neurotransmitter systems with the objectives of having painkillers devoid of any activity on the reward system.

AUTHOR CONTRIBUTIONS

NM and FN wrote the manuscript. All authors take responsibility for final content. All authors read and approved the final manuscript.

REFERENCES

1. Van Zee A. The promotion and marketing of oxycontin: commercial triumph, public health tragedy. *Am J Public Health* (2009) 99:221–7. doi: 10.2105/AJPH.2007.131714
2. Morone NE, Weiner DK. Pain as the fifth vital sign: exposing the vital need for pain education. *Clin Ther.* (2013) 35:1728–32. doi: 10.1016/j.clinthera.2013.10.001
3. Maremmani I, Gerra G. Buprenorphine-based regimens and methadone for the medical management of opioid dependence: selecting the appropriate drug for treatment. *Am J Addict.* (2010) 19:557–68. doi: 10.1111/j.1521-0391.2010.00086.x
4. Dhawan BN, Cesselin F, Raghubir R, Reisine T, Bradley PB, Portoghese PS, et al. International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol Rev.* (1996) 48:567–92.
5. Kieffer BL. Opioids: first lessons from knockout mice. *Trends Pharmacol Sci.* (1999) 20:19–26. doi: 10.1016/S0165-6147(98)01279-6
6. Terenius L. Stereospecific interaction between narcotic analgesics and a synaptic plasm a membrane fraction of rat cerebral cortex. *Acta Pharmacol Toxicol.* (1973) 32:317–20. doi: 10.1111/j.1600-0773.1973.tb01477.x
7. Simon EJ, Hiller JM, Edelman I. Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate. *Proc Natl Acad Sci USA.* (1973) 70:1947–9. doi: 10.1073/pnas.70.7.1947
8. Pert CB, Snyder SH. Opiate receptor: demonstration in nervous tissue. *Science* (1973) 179:1011–4. doi: 10.1126/science.179.4077.1011
9. Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* (1975) 258:577–80. doi: 10.1038/258577a0
10. Stein C. Opioid receptors. *Annu Rev Med.* (2016) 67:433–51. doi: 10.1146/annurev-med-062613-093100
11. Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, et al. Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. *Proc Natl Acad Sci USA.* (1993) 90:6736–40. doi: 10.1073/pnas.90.14.6736

12. Chen Y, Mestek A, Liu J, Hurley JA, Yu L. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol Pharmacol.* (1993) 44:8–12.
13. Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci USA.* (1992) 89:12048–52. doi: 10.1073/pnas.89.24.12048
14. Evans CJ, Keith DE, Morrison H, Magendzo K, Edwards RH. Cloning of a delta opioid receptor by functional expression. *Science* (1992) 258:1952–5. doi: 10.1126/science.1335167
15. Charbogne P, Kieffer BL, Befort K. 15 years of genetic approaches *in vivo* for addiction research: Opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology* (2014) 76(Pt B):204–17. doi: 10.1016/j.neuropharm.2013.08.028
16. Mucha RF, Herz A. Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology* (1985) 86:274–80. doi: 10.1007/BF00432213
17. Pfeiffer A, Brantl V, Herz A, Emrich HM. Psychotomimesis mediated by kappa opiate receptors. *Science* (1986) 233:774–6. doi: 10.1126/science.3016896
18. Spanagel R, Herz A, Shippenberg TS. Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc Natl Acad Sci USA.* (1992) 89:2046–50. doi: 10.1073/pnas.89.6.2046
19. Chefer VI, Shippenberg TS. Augmentation of morphine-induced sensitization but reduction in morphine tolerance and reward in delta-opioid receptor knockout mice. *Neuropsychopharmacology* (2009) 34:887–98. doi: 10.1038/npp.2008.128
20. Le Merrer J, Plaza-Zabala A, Del Boca C, Matifas A, Maldonado R, Kieffer BL. Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement. *Biol Psychiatry* (2011) 69:700–3. doi: 10.1016/j.biopsych.2010.10.021
21. Jones CM. Heroin use and heroin use risk behaviors among nonmedical users of prescription opioid pain relievers - United States, 2002-2004 and 2008-2010. *Drug Alcohol Depend.* (2013) 132:95–100. doi: 10.1016/j.drugalcdep.2013.01.007
22. Schiller EY. Opioid, overdose. In: Mechanic OJ, editor. *StatPearls*. Treasure Island, FL: StatPearls Publishing LLC.
23. Shipton EA, Shipton EE, Shipton AJ. A review of the opioid epidemic: what do we do about it? *Pain Ther.* (2018) 7:23–36. doi: 10.1007/s40122-018-0096-7
24. Hauser W, Schug S, Furlan AD. The opioid epidemic and national guidelines for opioid therapy for chronic noncancer pain: a perspective from different continents. *Pain Rep.* (2017) 2:e599. doi: 10.1097/PR9.0000000000000599
25. van Amsterdam J, van den Brink W. The misuse of prescription opioids: a threat for Europe? *Curr Drug Abuse Rev.* (2015) 8:3–14. doi: 10.2174/1874473708011506111842l8
26. Marie N, Canestrelli C, Noble F. Role of pharmacokinetic and pharmacodynamic parameters in neuroadaptations induced by drugs of abuse, with a focus on opioids and psychostimulants. *Neurosci Biobehav Rev.* (2018). doi: 10.1016/j.neubiorev.2018.06.006. [Epub ahead of print].
27. Inturrisi CE, Schultz M, Shin S, Umans JG, Angel L, Simon EJ. Evidence from opiate binding studies that heroin acts through its metabolites. *Life Sci.* (1983) 33(Suppl. 1):773–6. doi: 10.1016/0024-3205(83)90616-1
28. Rook EJ, Huitema AD, van den Brink W, van Ree JM, Beijnen JH. Pharmacokinetics and pharmacokinetic variability of heroin and its metabolites: review of the literature. *Curr Clin Pharmacol.* (2006) 1:109–18. doi: 10.2174/157488406775268219
29. Oldendorf WH, Hyman S, Braun L, Oldendorf SZ. Blood-brain barrier: penetration of morphine, codeine, heroin, and methadone after carotid injection. *Science* (1972) 178:984–6. doi: 10.1126/science.178.4064.984
30. Comer SD, Collins ED, MacArthur RB, Fischman MW. Comparison of intravenous and intranasal heroin self-administration by morphine-maintained humans. *Psychopharmacology* (1999) 143:327–38. doi: 10.1007/s002130050956
31. Eap CB, Buclin T, Baumann P. Interindividual variability of the clinical pharmacokinetics of methadone: implications for the treatment of opioid dependence. *Clin Pharmacokinet.* (2002) 41:1153–93. doi: 10.2165/00003088-200241140-00003
32. Inturrisi CE, Verebely K. The levels of methadone in the plasma in methadone maintenance. *Clin Pharmacol Ther.* (1972) 13:633–7. doi: 10.1002/cpt1972135part1633
33. Chiang CN, Hawks RL. Pharmacokinetics of the combination tablet of buprenorphine and naloxone. *Drug Alcohol Depend.* (2003) 70:S39–47. doi: 10.1016/S0376-8716(03)00058-9
34. Strain EC, Moody DE, Stoller KB, Walsh SL, Bigelow GE. Relative bioavailability of different buprenorphine formulations under chronic dosing conditions. *Drug Alcohol Depend.* (2004) 74:37–43. doi: 10.1016/j.drugalcdep.2003.11.008
35. Wang J-S, Ruan Y, Taylor RM, Donovan JL, Markowitz JS, DeVane CL. Brain penetration of methadone (R)- and (S)-enantiomers is greatly increased by P-glycoprotein deficiency in the blood-brain barrier of Abcb1a gene knockout mice. *Psychopharmacology* (2004) 173:132–8. doi: 10.1007/s00213-003-1718-1
36. Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest.* (1996) 97:2517–24. doi: 10.1172/JCI118699
37. Fonseca F, Torrens M. Pharmacogenetics of methadone response. *Mol Diagn Ther.* (2018) 22:57–78. doi: 10.1007/s40291-017-0311-y
38. McCance-Katz EF, Rainey PM, Smith P, Morse GD, Friedland G, Boyarsky B, et al. Drug interactions between opioids and antiretroviral medications: interaction between methadone, LAAM, and delavirdine. *Am J Addict.* (2006) 15:23–34. doi: 10.1080/10550490500419029
39. Mégarbane B, Marie N, Pirnay S, Borron SW, Gueye PN, Risède P, et al. Buprenorphine is protective against the depressive effects of norbuprenorphine on ventilation. *Toxicol Appl Pharmacol.* (2006) 212:256–67. doi: 10.1016/j.taap.2005.08.002
40. Villiger JW. Binding of buprenorphine to opiate receptors. Regulation by guanyl nucleotides and metal ions. *Neuropharmacology* (1984) 23:373–5.
41. Yassen A, Olofsen E, Romberg R, Sarton E, Teppema L, Danhof M, et al. Mechanism-based PK/PD modeling of the respiratory depressant effect of buprenorphine and fentanyl in healthy volunteers. *Clin Pharmacol Ther.* (2007) 81:50–8. doi: 10.1038/sj.clpt.6100025
42. Mohammed W, Alhaddad H, Marie N, Tardy F, Lamballais F, Risede P, et al. Comparison of tolerance to morphine-induced respiratory and analgesic effects in mice. *Toxicol Lett.* (2013) 217:251–9. doi: 10.1016/j.toxlet.2012.12.021
43. Selley DE, Liu Q, Childers SR. Signal transduction correlates of Mu opioid agonist intrinsic efficacy: receptor-stimulated [35S]GTPγS binding in mMOR-CHO cells and rat thalamus. *J Pharmacol Exp Ther.* (1998) 285:496–505.
44. Huang P, Kehner GB, Cowan A, Liu-Chen LY. Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *J Pharmacol Exp Ther.* (2001) 297:688–95.
45. Cowan A. Buprenorphine: the basic pharmacology revisited. *J Addict Med.* (2007) 1:68–72. doi: 10.1097/ADM.0b013e31806c9202
46. Dahan A, Yassen A, Romberg R, Sarton E, Teppema L, Olofsen E, et al. Buprenorphine induces ceiling in respiratory depression but not in analgesia. *Br J Anaesth.* (2006) 96:627–32. doi: 10.1093/bja/ael051
47. Kelly E. Efficacy and ligand bias at the μ-opioid receptor. *Br J Pharmacol.* (2013) 169:1430–46. doi: 10.1111/bph.12222
48. Raffa RB, Haidery M, Huang H-M, Kalladeen K, Lockstein DE, Ono H, et al. The clinical analgesic efficacy of buprenorphine. *J Clin Pharm Ther.* (2014) 39:577–83. doi: 10.1111/jcpt.12196
49. Falcon E, Browne CA, Leon RM, Fleites VC, Sweeney R, Kirby LG, et al. Antidepressant-like effects of buprenorphine are mediated by kappa opioid receptors. *Neuropsychopharmacology* (2016) 41:2344–51. doi: 10.1038/npp.2016.38
50. Sacerdote P, Franchi S, Gerra G, Leccese V, Panerai AE, Somaini L. Buprenorphine and methadone maintenance treatment of heroin addicts preserves immune function. *Brain Behav Immun.* (2008) 22:606–13. doi: 10.1016/j.bbi.2007.12.013
51. Elkana O, Adelson M, Doniger GM, Sason A, Peles E. Cognitive function is largely intact in methadone maintenance treatment patients. *World J Biol Psychiatry* (2017). doi: 10.1080/15622975.2017.1342047. [Epub ahead of print].

52. Maremmani AG, Rovai L, Pani PP, Pacini M, Lamanna F, Rugani F, et al. Do methadone and buprenorphine have the same impact on psychopathological symptoms of heroin addicts? *Ann Gen Psychiatry* (2011) 10:17. doi: 10.1186/1744-859X-10-17
53. Pani PP, Maremmani I, Pacini M, Lamanna F, Maremmani AG, Dell'osso L. Effect of psychiatric severity on the outcome of methadone maintenance treatment. *Eur Addict Res.* (2011) 17:80–9. doi: 10.1159/000321465
54. Bizzarri JV, Casetti V, Sanna L, Maremmani AGI, Rovai L, Bacciardi S, et al. The newer Opioid Agonist Treatment with lower substitutive opiate doses is associated with better toxicology outcome than the older Harm Reduction Treatment. *Ann Gen Psychiatry* (2016) 15:34. doi: 10.1186/s12991-016-0109-z
55. Kakko J, Svanborg KD, Kreek MJ, Heilig M. 1-year retention and social function after buprenorphine-assisted relapse prevention treatment for heroin dependence in Sweden: a randomised, placebo-controlled trial. *Lancet* (2003) 361:662–8. doi: 10.1016/S0140-6736(03)12600-1
56. Sees KL, Delucchi KL, Masson C, Rosen A, Clark HW, Robillard H, et al. Methadone maintenance vs 180-day psychosocially enriched detoxification for treatment of opioid dependence: a randomized controlled trial. *JAMA* (2000) 283:1303–10. doi: 10.1001/jama.283.10.1303
57. Wright N, D'Agnone O, Krajci P, Littlewood R, Alho H, Reimer J, et al. Addressing misuse and diversion of opioid substitution medication: guidance based on systematic evidence review and real-world experience. *J Public Health* (2016) 38:e368–74. doi: 10.1093/pubmed/fdv150
58. Mégarbane B, Hreiche R, Pirnay S, Marie N, Baud FJ. Does high-dose buprenorphine cause respiratory depression? Possible mechanisms and therapeutic consequences. *Toxicol Rev.* (2006) 25:79–85. doi: 10.2165/00139709-200625020-00002
59. Gomes T, Juurlink DN, Antoniou T, Mamdani MM, Paterson JM, van den Brink W. Gabapentin, opioids, and the risk of opioid-related death: A population-based nested case–control study. *PLoS Med.* (2017) 14:e1002396. doi: 10.1371/journal.pmed.1002396
60. Chou R, Weimer MB, Dana T. Methadone overdose and cardiac arrhythmia potential: findings from a review of the evidence for an American Pain Society and College on Problems of Drug Dependence clinical practice guideline. *J Pain* (2014) 15:338–65. doi: 10.1016/j.jpain.2014.01.495
61. Alinejad S, Kazemi T, Zamani N, Hoffman RS, Mehrpour O. A systematic review of the cardiotoxicity of methadone. *EXCLI J.* (2015) 14:577–600. doi: 10.17179/excli2015-553
62. Bart G, Wyman Z, Wang Q, Hodges JS, Karim R, Bart BA. Methadone and the QTc interval: paucity of clinically significant factors in a retrospective cohort. *J Addict Med.* (2017) 11:489–93. doi: 10.1097/ADM.0000000000000353
63. Pujol CN, Paasche C, Laprevote V, Trojak B, Vidailhet P, Bacon E, et al. Cognitive effects of labeled addictolytic medications. *Prog Neuropsychopharmacol Biol Psychiatry* (2018) 81:306–32. doi: 10.1016/j.pnpbp.2017.09.008
64. Gerra G, Manfredini M, Somaini L, Maremmani I, Leonardi C, Donnini C. Sexual dysfunction in men receiving methadone maintenance treatment: clinical history and psychobiological correlates. *Eur Addict Res.* (2016) 22:163–75. doi: 10.1159/000441470
65. Yee A, Loh HS, Hisham Hashim HMB, Ng CG. Clinical factors associated with sexual dysfunction among men in methadone maintenance treatment and buprenorphine maintenance treatment: a meta-analysis study. *Int J Impot Res.* (2014) 26:161–6. doi: 10.1038/ijir.2014.18
66. Allouche S, Le Marec T, Coquerel A, Noble F, Marie N. Striatal dopamine D1 and D2 receptors are differentially regulated following buprenorphine or methadone treatment. *Psychopharmacology* (2015) 232:1527–33. doi: 10.1007/s00213-014-3785-x
67. Allouche S, Le Marec T, Noble F, Marie N. Different patterns of administration modulate propensity of methadone and buprenorphine to promote locomotor sensitization in mice. *Prog Neuropsychopharmacol Biol Psychiatry* (2013) 40:286–91. doi: 10.1016/j.pnpbp.2012.10.013
68. Rosenthal RN, Goradia VV. Advances in the delivery of buprenorphine for opioid dependence. *Drug Des Dev Ther.* (2017) 11:2493–505. doi: 10.2147/DDDT.S72543
69. Mendelson J, Jones RT. Clinical and pharmacological evaluation of buprenorphine and naloxone combinations: why the 4:1 ratio for treatment? *Drug Alcohol Depend.* (2003) 70:S29–37. doi: 10.1016/S0376-8716(03)00057-7
70. Canestrelli C, Marie N, Noble F. Rewarding or aversive effects of buprenorphine/naloxone combination (Suboxone) depend on conditioning trial duration. *Int J Neuropsychopharmacol.* (2014) 17:1367–73. doi: 10.1017/S146114571400025X
71. Comer SD, Sullivan MA, Vosburg SK, Manubay J, Amass L, Cooper ZD, et al. Abuse liability of intravenous buprenorphine/naloxone and buprenorphine alone in buprenorphine-maintained intravenous heroin abusers. *Addiction* (2010) 105:709–18. doi: 10.1111/j.1360-0443.2009.02843.x
72. Monte AA, Mandell T, Wilford BB, Tennyson J, Boyer EW. Diversion of buprenorphine/naloxone coformulated tablets in a region with high prescribing prevalence. *J Addict Dis.* (2009) 28:226–31. doi: 10.1080/10550880903014767
73. Madariaga-Mazón A, Marmolejo-Valencia AF, Li Y, Toll L, Houghten RA, Martinez-Mayorga K. Mu-Opioid receptor biased ligands: a safer and painless discovery of analgesics? *Drug Discov Today* (2017) 22:1719–9. doi: 10.1016/j.drudis.2017.07.002
74. Allouche S, Noble F, Marie N. Opioid receptor desensitization: mechanisms and its link to tolerance. *Front Pharmacol.* (2014) 5:280. doi: 10.3389/fphar.2014.00280
75. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* (1999) 286:2495–8. doi: 10.1126/science.286.5449.2495
76. Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* (2000) 408:720–3. doi: 10.1038/35047086
77. Raehal KM, Walker JKL, Bohn LM. Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther.* (2005) 314:1195–201. doi: 10.1124/jpet.105.087254
78. Viscusi ER, Webster L, Kuss M, Daniels S, Bolognese JA, Zuckerman S, et al. A randomized, phase 2 study investigating TRV130, a biased ligand of the μ-opioid receptor, for the intravenous treatment of acute pain. *Pain* (2016) 157:264–72. doi: 10.1097/j.pain.0000000000000363
79. DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, et al. A G protein-biased ligand at the μ-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther.* (2013) 344:708–17. doi: 10.1124/jpet.112.201616
80. Manglik A, Kruse AC, Kobilka TS, Thian FS, Mathiesen JM, Sunahara RK, et al. Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature* (2012) 485:321–6. doi: 10.1038/nature10954
81. Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, et al. Structure-based discovery of opioid analgesics with reduced side effects. *Nature* (2016) 537:185–90.
82. Altarifi AA, David B, Muchhala KH, Blough BE, Akbarali H, Negus SS. Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. *J Psychopharmacol.* (2017) 31:730–9. doi: 10.1177/0269881116689257
83. Günther T, Dasgupta P, Mann A, Miess E, Kliewer A, Fritzwanker S, et al. Targeting multiple opioid receptors - improved analgesics with reduced side effects? *Br J Pharmacol.* (2018) 175:2857–68. doi: 10.1111/bph.13809
84. Linz K, Christoph T, Tzschentke TM, Koch T, Schiene K, Gautrois M, et al. Cebranopadol: a novel potent analgesic nociceptin/orphanin FQ peptide and opioid receptor agonist. *J Pharmacol Exp Ther.* (2014) 349:535–48. doi: 10.1124/jpet.114.213694
85. Ding H, Kiguchi N, Yasuda D, Daga PR, Polgar WE, Lu JJ, et al. A bifunctional nociceptin and mu opioid receptor agonist is analgesic without opioid side effects in nonhuman primates. *Sci Transl Med.* (2018) 10: eaar3483. doi: 10.1126/scitranslmed.aar3483
86. Roques BP, Fournié-Zaluski MC, Soroca E, Lecomte JM, Malfroy B, Llorens C, et al. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* (1980) 288:286–8.
87. Roques BP, Fournié-Zaluski M-C, Wurm M. Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nat Rev Drug Discov.* (2012) 11:292–310. doi: 10.1038/nrd3673

88. McLean S, Skirboll LR, Pert CB. Comparison of substance P and enkephalin distribution in rat brain: an overview using radioimmunocytochemistry. *Neuroscience* (1985) 14:837–52. doi: 10.1016/0306-4522(85)90147-2
89. Boudinot E, Morin-Surun M, Foutz AS, Fournié-Zaluski M, Roques BP, Denavit-Saubié M. Effects of the potent analgesic enkephalin-catabolizing enzyme inhibitors RB101 and kelatorphan on respiration. *Pain* (2001) 90:7–13. doi: 10.1016/S0304-3959(00)00382-1
90. Williams JT, Christie MJ, North RA, Roques BP. Potentiation of enkephalin action by peptidase inhibitors in rat locus ceruleus *in vitro*. *J Pharmacol Exp Ther.* (1987) 243:397–401.
91. Maldonado R, Stinus L, Gold LH, Koob GF. Role of different brain structures in the expression of the physical morphine withdrawal syndrome. *J Pharmacol Exp Ther.* (1992) 261:669–77.
92. Hämmig R, Köhler W, Bonorden-Kleij K, Weber B, Lebentrau K, Berthel T, et al. Safety and tolerability of slow-release oral morphine versus methadone in the treatment of opioid dependence. *J Subst Abuse Treat.* (2014) 47:275–81. doi: 10.1016/j.jsat.2014.05.012
93. Ling W, Mooney L, Wu L-T. Advances in opioid antagonist treatment for opioid addiction. *Psychiatr Clin North Am.* (2012) 35:297–308. doi: 10.1016/j.psc.2012.03.002
94. Jarvis BP, Holtyn AF, Subramaniam S, Tompkins DA, Oga EA, Bigelow GE, et al. Extended-release injectable naltrexone for opioid use disorder: a systematic review. *Addiction* (2018) 113:1188–209. doi: 10.1111/add.14180
95. Kerensky T, Walley AY. Opioid overdose prevention and naloxone rescue kits: what we know and what we don't know. *Addict Sci Clin Pract.* (2017) 12:4. doi: 10.1186/s13722-016-0068-3
96. Johnstone RE, Jobes DR, Kennell EM, Behar MG, Smith TC. Reversal of Morphine Anesthesia with Naloxone. *Anesthesiology* (1974) 41:361–7. doi: 10.1097/00000542-197410000-00010
97. van Dorp E, Yassen A, Sarton E, Romberg R, Olofsen E, Teppema L, et al. Naloxone reversal of buprenorphine-induced respiratory depression. *Anesthesiology* (2006) 105:51–7. doi: 10.1097/00000542-200607000-00012
98. Kim HK, Nelson LS. Reducing the harm of opioid overdose with the safe use of naloxone: a pharmacologic review. *Expert Opin Drug Saf.* (2015) 14:1137–46. doi: 10.1517/14740338.2015.1037274

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Sex Differences in Nociceptin/Orphanin FQ Peptide Receptor-Mediated Pain and Anxiety Symptoms in a Preclinical Model of Post-traumatic Stress Disorder

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Nociceptin/Orphanin FQ (N/OFQ) is a neuropeptide that modulates pain transmission, learning/memory, stress, anxiety, and fear responses via activation of the N/OFQ peptide (NOP or ORL1) receptor. Post-traumatic stress disorder (PTSD) is an anxiety disorder that may arise after exposure to a traumatic or fearful event, and often is co-morbid with chronic pain. Using an established animal model of PTSD, single-prolonged stress (SPS), we were the first to report that NOP receptor antagonist treatment reversed traumatic stress-induced allodynia, thermal hyperalgesia, and anxiety-like behaviors in male Sprague-Dawley rats. NOP antagonist treatment also reversed SPS-induced serum and CSF N/OFQ increase and circulating corticosterone decrease. The objective of this study was to examine the role of the NOP receptor in male and female rats subjected to traumatic stress using Wistar wild type (WT) and NOP receptor knockout (KO) rats. The severity of co-morbid allodynia was assessed as change in paw withdrawal threshold (PWT) to von Frey and paw withdrawal latency (PWL) to radiant heat stimuli, respectively. PWT and PWL decreased in male and female WT rats within 7 days after SPS, and remained decreased through day 28. Baseline sensitivity did not differ between genotypes. However, while male NOP receptor KO rats were protected from SPS-induced allodynia and thermal hypersensitivity, female NOP receptor KO rats exhibited tactile allodynia and thermal hypersensitivity to the same extent as WT rats. Male NOP receptor KO rats had a lower anxiety index (AI) than WT, but SPS did not increase AI in WT males. In contrast, SPS significantly increased AI in WT and NOP receptor KO female rats. SPS increased circulating N/OFQ levels in male WT, but not in male NOP receptor KO, or WT or KO female rats. These results indicate that the absence of the NOP receptor protects males from traumatic-stress-induced allodynia and hyperalgesia, consistent with our previous findings utilizing a NOP receptor antagonist. However, female NOP

receptor KO rats experience allodynia, hyperalgesia and anxiety-like symptoms to the same extent as WT females following SPS. This suggests that endogenous N/OFQ-NOP receptor signaling plays an important, but distinct, role in males and females following exposure to traumatic stress.

Keywords: allodynia, hyperalgesia, NOP receptor, PTSD, sex differences, single prolonged stress, anxiety, Nociceptin/Orphanin FQ

INTRODUCTION

Sex differences in post-traumatic stress disorder (PTSD) and the presence of co-morbid pain are well documented in the clinic, but studies of sex differences in PTSD-related behaviors using animal models has been limited primarily to fear responses/processing and hippocampal plasticity [for review see (1)]. Literature from clinical studies suggests that females exhibit a higher reported rate of PTSD and longer lasting, more severe symptoms [for review see (1–4)]. They also differ from males in the PTSD criterion/parameters noted in their response to stress (5), and in the relationship between PTSD and appearance of co-morbid conditions such as depression, schizophrenia, cognitive decline, and pain (6). Therefore, it is important to study and understand these sex differences to facilitate development of more effective treatments.

A number of preclinical PTSD models exist; one of the most frequently utilized, and well-characterized preclinical models of PTSD that possesses content and criterion validity is single-prolonged stress [SPS; (7, 8)]. The SPS model reproduces physiological and psychological symptoms that appear unpredictably over time following a short period of severe trauma. This exposure is applicable to civilians confronted with natural disasters (e.g., tornadoes, fires, and earthquakes) and other terrifying situations, as well as military populations. SPS produces sustained neuroendocrine disruption (including enhanced negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis), hyperarousal, fear extinction retention impairment, altered sleep-wake cycles, changes in the noradrenergic system, increased CRF in the brain, and reduced synaptic plasticity and BDNF that are all hallmarks of PTSD in humans (7–10). SPS also produces co-morbid depressive-like behaviors (11–13), enhanced alcohol reward (14), and cognitive impairment (15) in male rats. Ours was one of the first groups to report changes in nociceptive sensitivity in male rats as a co-morbid condition using a preclinical model of PTSD (16, 17). Since our initial report we, and other groups, confirmed and extended our findings of allodynia, hyperalgesia (18–22) and anxiety-like behaviors (11–13, 16) in males. We found only a single study that assessed changes in pain sensitivity in female rats following SPS. Female rats subjected to an enhanced SPS protocol (with foot shock) exhibited increased visceral sensitivity, but no comparisons to males were included (23).

One molecule that links the HPA axis, sensory systems and disease states is Nociceptin/Orphanin FQ (N/OFQ). N/OFQ is the endogenous ligand for the N/OFQ peptide (NOP) receptor, and also is known as ORL1 or KOR-3 (24–27). N/OFQ and the NOP receptor are widely expressed in the central nervous system (CNS), particularly in the forebrain and the descending pain pathway, which are involved in emotional and pain processing (28). N/OFQ bi-directionally modulates many key biological functions in the CNS that are impacted by PTSD and/or chronic pain (including nociceptive sensitivity, learning and memory, stress and anxiety and reward), via activation of the NOP receptor (29–32). This bidirectional modulatory pattern of N/OFQ often produces conflicting results that may vary between species, strains, time and method of drug administration, assay method and/or stress. For example, N/OFQ and other NOP agonists block stress-induced analgesia, but may produce analgesia in other instances (33–38). Serum and CSF of patients with acute and chronic pain contain elevated N/OFQ levels (39, 40). Similarly, serum and CSF from rats that exhibited allodynia and hyperalgesia following SPS contain higher N/OFQ levels than from sham-treated rats (17, 22). We subsequently reported that blockade of N/OFQ actions with a NOP antagonist prevented up-regulation of N/OFQ and anxiety-like symptoms and reversed SPS-induced allodynia and hyperalgesia, confirming a role for N/OFQ in modulation of pain sensitivity, anxiety and HPA axis modulation following traumatic stress (22).

A single nucleotide polymorphism (SNP) in the non-coding region of the NOP receptor gene was associated with PTSD symptoms in women that experienced severe traumatic stress (29), but we have no knowledge about how that SNP might alter NOP receptor expression, N/OFQ signaling or nociceptive sensitivity. While preclinical studies examining fear, hyperarousal, depression, and cognitive deficits in females exposed to traumatic stress have become more numerous (including sex differences in SPS-induced cued fear extinction retention deficits and hippocampal plasticity (41, 42), only a single study examining changes in nociceptive sensitivity in preclinical models of PTSD was found (23). Acquisition of NOP receptor gene knockout (KO) rats (ORL1-/-) (43, 44) enabled us to examine for the first time, the role of the N/OFQ-NOP receptor system in female rats following exposure to the SPS model of PTSD, and further examine its role in males.

METHODS

Animal Treatment

Wild type (WT) Wistar Han rats were purchased from Charles River Labs (Wilmington, MA). Fourteen male homozygous Oprl1-TGEM® KO (referred to herein as ORL1$^{-/-}$ or NOP "KO") rats in Wistar Han background and a set of homozygous breeders were obtained from Transposagen (Lexington, KY). The rest of the KO animals were generated from the homozygous

breeding pairs in the OUHSC animal facility; genotype was confirmed by Transnetyx (Cordova, TN). The animal protocol was approved by the University of Oklahoma Health Sciences Center's Institutional Animal Care and Use Committee. Studies conformed to the FASEB Statement of Principles for the use of animals in research and education. Research was compliant with the Animal Welfare Act Regulations and other Federal Statutes relating to animals and experiments involving animals, and adhered to the principles set forth in the Guide for Care and Use of Laboratory Animals. All experiments conformed to the guidelines of the International Association for the Study of Pain. Rats were acclimated to their environment for at least 7 days following arrival and housed in the animal facility under a 12-h light: 12-h dark cycle (lights on at 0600 h) with free access to food and water. Intact male (250–300 g) and female (175–210 g) KO and WT rats (9–10 weeks of age) were randomly divided into control or SPS groups ($N = 33$ total males and 37 total females; $N = 7\sim10$/group). **SPS** consists of complete restraint for 2 h, grouped forced swimming ($N = 2$–3 at a time) for 20 min, and exposure to diethyl ether until consciousness is lost. Once rats recovered from anesthesia, they were returned to their cages and left undisturbed for 7 days as previously described (8, 17).

Nociceptive sensitivity was assessed by measuring hind paw withdrawal threshold (PWT) from pressure and paw withdrawal latency (PWL) from radiant heat prior to SPS exposure and every 7 days thereafter through day 28. An electronic von Frey anesthesiometer (IITC Life Sciences, Inc., Woodland Hills, CA) was utilized for mechanical/tactile nociception assessment. Rats were placed in clear plastic boxes with a wire mesh floor, and acclimated for 15–20 min. PWT was obtained from the mid-plantar aspect of the left hind paw. Approximately 1.5 h after PWT assessment, the wire mesh floor was replaced with a glass floor and rats acclimated for approximately 30 min. Then, a plantar analgesia meter (IITC Life Sciences, Inc., Woodland Hills, CA) was utilized to measure PWL to an infrared light beam directed toward the left hind paw with the lamp set at 25% active intensity as previously described (17). Cutoff time was set at 30 s to prevent tissue damage. The average of three sets of scores (taken 5 min apart) was the PWT/PWL for each rat, each week. Decrease in PWT compared to control rats was termed allodynia since the intensity of the pressure applied was dynamic. Decrease in thermal sensitivity compared to control rats was termed hyperalgesia because all rats were exposed to same heat intensity and only latency was determined. All behavioral assessments were made between 0900 and 1200 h. An algesia index was determined for each treatment group by calculating the area under the PWL or PWT curve from y=0 up to the PWL/PWT at each time point, using GraphPad Prism. Sex and stress group differences were determined by two-way analysis of variance (ANOVA).

The presence of anxiety-like symptoms was assessed using the **elevated plus maze (EPM) test** on day 9 post-SPS (and again on day 30 for female rats) as previously described (17). The plus maze consisted of two open (50 × 10 cm) and two closed (50 × 10 × 40 cm) arms elevated 40 cm above floor with average light levels 40–55 lux. Each rat was placed in the center of the apparatus with its head facing a closed arm, and activity was recorded for 5 min from the center of the rat body. The apparatus was cleaned with 30% ethanol between each recording session. Recording sessions were analyzed with Any-maze software (Stoelting Co., Wood Dale, IL) for mobile/immobile time, traveled distance, arm entries, and time spent in arms. The anxiety index (AI) was calculated as described (45): 1—[(% time in open arms + % entries into open arms)/2]. Female rats were exposed to the EPM a second time on day 30 post-SPS (3 week interval between testing). Rats do not develop habituation or sensitization to the EPM when testing intervals are spaced at least 3 weeks apart (46, 47).

Collection of Fluid and Tissue Samples

Rats were euthanized by injection with Beuthanasia (i.p. 0.22 mg/kg, Schering-Plow Animal Health, Union, NJ, USA) between 1,200 and 1,600 h on day 28–30 post-SPS. Blood was withdrawn from the heart with an 18-gauge needle, and kept at room temperature for 30 min before centrifugation at 5,000 × g, 4°C for 5 min when serum was collected. CSF (150 ∼ 200 μl) from each rat was withdrawn by inserting a 26-gauge needle into the cisterna magna. Lumbar spinal cord was removed and the dorsal horn dissected and collected. All samples were stored at −80°C until biochemical analysis was performed.

Radioimmunoassay (RIA)

N/OFQ levels in 50 μL of serum or CSF were determined in duplicate by RIA (Phoenix Pharmaceuticals, Belmont, CA) according to the protocol suggested by the manufacturer. Total amount of N/OFQ immunoreactivity (IR) was calculated and expressed as pg/mL. Samples that fell outside of the range of the standard curve or that were contaminated with blood were not included; specific information is provided in the figure legend. The individual conducting each assay was blind to the grouping.

^{35}S-GTPγS Binding

Spinal cord (SC) dorsal horn membranes from male and female WT and KO rats were prepared and assayed for N/OFQ stimulation of ^{35}S-GTPγS binding as previously described to examine NOP receptor activity (22). Briefly, tissue was homogenized in 1 mL ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.4) containing 10% (w/v) sucrose and centrifuged at 1,000 × g for 10 min. The supernatant was washed twice by centrifugation at 9,000 × g for 20 min and resuspended in 1 mL of TED buffer. The suspension was kept on ice for 30 min, followed by centrifugation at 35,000 x g for 10 min. The pellet was stored at −80°C until use. Membrane protein, 10 μg as determined by BCA assay, was incubated at 25°C for 60 min in plastic tubes containing a total volume of 100 μL: 0.5% bovine serum albumin, 0.1% bacitracin, 10 μM GDP, 0.3 nM ^{35}S-GTPγS, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl and 10^{-9}—10^{-5} M N/OFQ. The reaction was terminated by rapid filtration through glass fiber filters using a Brandel cell harvester. Radioactivity was determined by liquid scintillation spectroscopy. Non-specific binding was measured in the presence of 100 μM unlabeled GTPγS, which was subtracted from total binding to define specific ^{35}S-GTPγS binding.

Data Analysis

Data Analysis and graph preparation were performed using GraphPad Prism 7.01 software (GraphPad Software, La Jolla, CA, USA). Data are expressed as mean ± SD unless indicated otherwise. Statistical comparisons of behavioral and neurochemical data were performed by two-way ANOVA with *post-hoc* analyses as automatically recommended by the software. Tukey's multiple comparisons tests were used when comparing every row (or column) mean with every other row (or column) mean. Sidak's was used to determine differences within columns or rows. Results were considered statistically significant if $P < 0.05$. All data were subjected to D'Agostino & Pearson ($N > 8$) or Shapiro-Wilk ($N < 8$) normality tests prior to analysis. Those groups that failed the normality test ($p < 0.05$) were subjected to an outlier test (ROUT; Q = 1%), as recommended (48) to determine if the outlier was responsible for the failed normality test. If exclusion of outlier(s) led to passing the normality test and altered statistical result, the exclusion was made. If it did not alter the statistical outcome, no data were excluded from that group. The 6 samples that were excluded by outlier test are listed in the appropriate figure legend. Pearson's Correlation Analysis was performed with the following data aligned from each rat: D7 and D28 PWT and PWL, D9 (and D30 in females) anxiety index and % time in open arms, serum N/OFQ and CSF N/OFQ. Correlations were made with data from Control and SPS-treated rats of each sex and genotype.

RESULTS

Nociceptive Sensitivity

Our primary goal was to determine if SPS produces tactile allodynia and thermal hyperalgesia in male and female Wistar WT and NOP receptor KO rats. Male Wistar WT rats responded to SPS (closed black circles) by developing tactile allodynia (**Figure 1A**) and thermal hyperalgesia (**Figure 1B**) at the same rate and to the same extent as previously reported in Sprague-Dawley rats. This is reflected by decreased PWT and PWL, respectively, compared to WT Control (CON) rats (black open circles). Remarkably, PWTs and PWLs of NOP KO rats subjected to SPS (solid blue circles) did not differ from those of their untreated control littermates (blue open circles), indicating that the absence of the NOP receptor protected the rats from SPS-induced allodynia and hyperalgesia. Two-way ANOVA indicated that there was a significant interaction between time and genotype-stress treatment for tactile (A: [$F_{(12, 145)} = 3.143$, $p = 0.0002$]) and thermal (B: [$F_{(12, 145)} = 4.176$, $p < 0.0001$]) stimuli. PWT and PWL in WT and KO unstressed rats did not differ from each other at any time point. Tukey's multiple comparisons test revealed WT male SPS rats differed from WT control ($^{*}p < 0.05$ and $^{**}p < 0.01$), KO Control ($^{\Delta}p < 0.05$ and $^{\Delta\Delta}$ $p < 0.01$) and KO SPS ($\#\#p < 0.05$ and $\#\#$ $p < 0.01$).

Similar to WT males, WT female rats also developed tactile allodynia (**Figure 1C**) and thermal hyperalgesia (**Figure 1D**) following SPS (solid black circles). However, unlike the males, absence of the NOP receptor did not protect female KO rats from developing allodynia or hyperalgesia following SPS (solid red circles). Female NOP KO rats developed allodynia and hyperalgesia to the same extent as WT females, but no differences in nociceptive sensitivity were noted between female WT CON (open black circles) and KO CON (open red circles) rats. Two-way ANOVA indicated significant interaction between Time x Genotype-stress treatment for tactile (C: [$F_{(12, 165)} = 2.925$, $p = 0.0011$]) and thermal stimuli (D: [$F_{(12, 165)} = 3.139$, $p = 0.0005$]). Tukey's multiple comparisons test indicated SPS-treated WT and KO rats differed from WT and KO controls. Pearson correlation analysis revealed that there was a significant correlation between D28 PWT and PWL in both WT and KO female rats (**Table 1**). The correlation just missed significance in WT males ($p = 0.05$).

To directly examine this apparent sex difference, an algesia index for each group for each stimuli was generated by calculating its area under each treatment group's time-nociceptive sensitivity curve (AUC; **Figures 1A–D**). A two-way ANOVA of the calculated AUC was performed with sex as the row effect and traumatic stress for each genotype (treatment) as the column effect (**Figures 1E–F**).There was a significant interaction between sex and treatment for tactile (E: [$F_{(3, 62)} = 4.005$; $P = 0.0114$]) and thermal (F: [$F_{(3, 62)} = 5.063$; $P = 0.0034$]) stimuli. Sidak's multiple comparison's test compared the effect of sex within each group**.** The only *post-hoc* sex difference noted was between males and females in the KO SPS groups. Females in the KO SPS group were more sensitive to tactile ($p < 0.001$) and thermal ($p < 0.001$) stimuli than males in that treatment group.

Anxiety-Like Behaviors

The secondary goal of this study was to determine if SPS produces anxiety-like symptoms in male and female Wistar WT and NOP receptor KO rats. The appearance of anxiety-like symptoms at day 9 post-SPS was assessed using the elevated plus maze (EPM) and analyzed by two-way ANOVA for genotype x traumatic stress for males (**Figures 2A–F**; **Table 2**) and females (**Figures 3A–F**; **Table 2**). Unlike the previous report in SD rats, no significant interaction or significant effect of SPS was found for any anxiety-like behavior in male Wistar rats (**Figures 2A–F**; **Table 2**). However, a significant effect of genotype was noted for % time in open Arms (**A**), % open arm entries (**B**), distance traveled in open arms (**C**), and anxiety index (AI; **D**). There was no effect of genotype on total distance traveled (**E**) or immobile time (**F**). Both groups of male KO rats appeared less "anxious" in that they spent more time, made more entries into and traveled a longer distance within the open arms, and scored a lower anxiety index than WT CON and WT SPS rats, but no differences between treatment groups were detected by *post-hoc* analysis.

Unlike their male counterparts (**Figure 2**), genotype played no role in anxiety-like behaviors in female rats (**Figures 3A–F**, **Table 2**). However, there was a significant effect of SPS in female rats at day 9 (**Table 2**), with *post-hoc* analysis indicating that female KO-SPS rats spent significantly less % of time in open arms (**A**; $p < 0.05$) and had a higher AI (**D**, $p < 0.05$) than untreated rats. Since female rats exhibited significant anxiety-like behaviors at day 9, we extended the observation of anxiety-like symptoms by EPM for 3 weeks later (Day 30). Significant

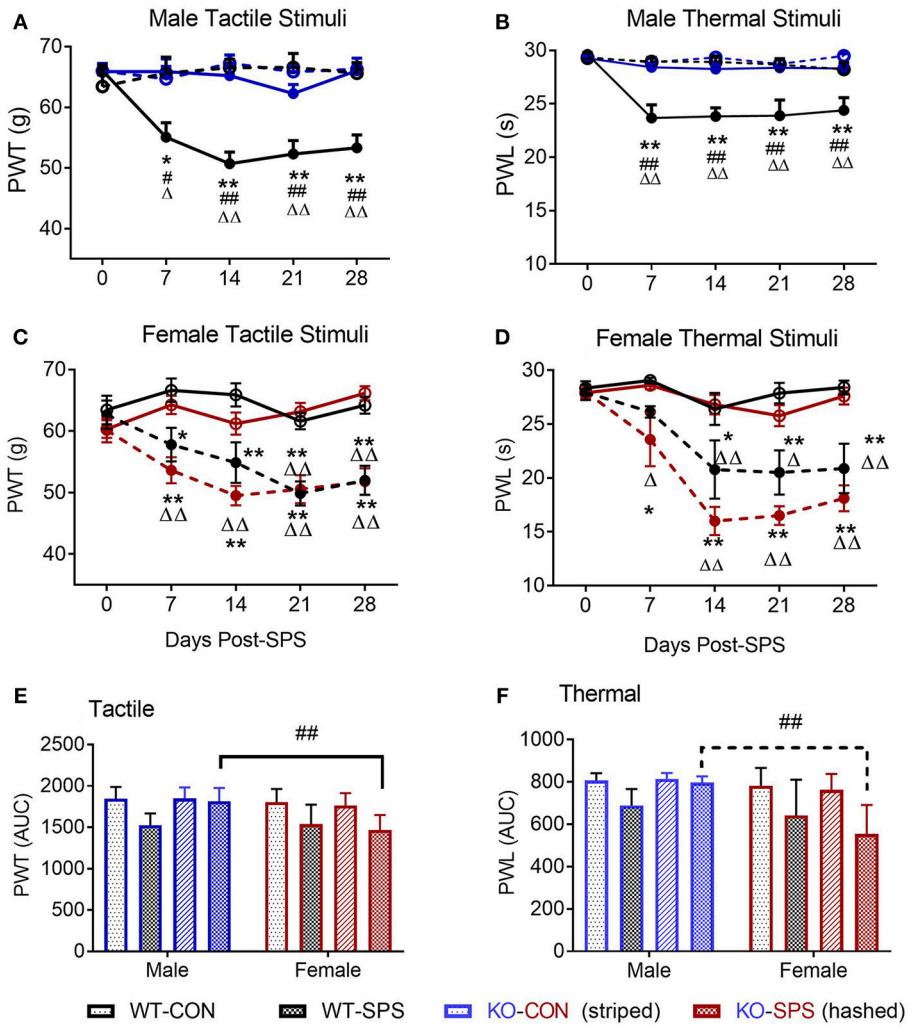

FIGURE 1 | NOP receptor KO genotype prevented development of tactile allodynia and thermal hyperalgesia in male, but not female, rats following SPS. Rats were randomly assigned into groups within their genotype and sex. For males **(A,B)** Wild type (WT)-CON ($n = 7$; black open circles), WT-SPS ($n = 7$; solid black circles) and ORL1-/- (KO): CON ($n = 9$; blue open circles), and KO-SPS ($n = 10$; solid blue circles). For Females **(C,D)** WT-CON ($n = 9$), WT-SPS ($n = 9$) and KO-CON ($n = 10$; red open circles), KO-SPS ($n = 9$; solid red circles). Mechanical **(A,C)** and Thermal **(B,D)** sensitivity was assessed prior to (day 0) and every 7 days following SPS. PWT and PWL data were analyzed by two-way ANOVA (Treatment/Time x Genotype) followed by Tukey's *post-hoc* analysis and data are presented as mean ± SEM. SPS induced allodynia and hyperalgesia in WT males as previously described, but KO male responses did not differ from WT or KO control rats (*$p < 0.05$ and **$p < 0.01$ for WT-CON vs. WT-SPS; $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ for KO-CON vs. KO-SPS; #$p < 0.05$ and ##$p < 0.01$ for WT-SPS vs. KO-SPS). In contrast, female WT and KO rats developed allodynia and hyperalgesia in response to SPS (*$p < 0.05$ and **$p < 0.01$ for WT-CON vs. WT-SPS; $^{\Delta\Delta}p < 0.01$ for KO-CON vs. KO-SPS). No differences between female WT and KO SPS or between WT and KO Control groups appeared at any time point. The area under the time-nociceptive sensitivity curves (AUC) of WT and KO males and females generated in response to tactile **(E)** and thermal **(F)** stimuli of each treatment group are presented as mean ± SD, and were analyzed by two-way ANOVA for sex × SPS treatment. Significant *post-hoc* differences between sexes within each treatment group were determined with Sidak's multiple comparison test (##$p < 0.01$).

effects of SPS were noted on all four parameters at day 30 (**Figures 3A–D**; **Table 2**). *Post-hoc* differences between groups in response to SPS became more pronounced over time as SPS-treated WT and KO rats spent a smaller fraction of time (**A**; $p < 0.01$) and traveled a shorter distance while in open arms (**C**; $p < 0.01$) compared to WT CON; open arm entries were a smaller percentage of all arm entries compared to KO CON at day 30 (**C**; $p < 0.05$ for WT SPS and $p < 0.01$ for KO-SPS). The AI increased in both SPS-treated groups at day 30 compared to WT ($p < 0.01$) and KO-CON ($p < 0.05$) rats (**Figure 3D**). There was a transient effect of genotype on total distance traveled at day 9 but not day 30 (**Figure 3E**; **Table 2**), but no individual differences were noted between groups by *post-hoc* analysis. Similar to nociceptive sensitivity in females, no effect of genotype or SPS was noted on either day of immobile time in female rats (**Figure 3F**). A direct analysis of sex differences between anxiety index at day 9 between males and females by two way ANOVA with Sidak's multiple comparisons indicates a significant interaction between

TABLE 1 | Pearson correlation analysis in male and female WT and NOP receptor KO rats.

Pairwise correlations	Male WT	Male KO	Female WT	Female KO
D28 PWT:PWL				
Pearson r	0.531	−0.082	0.76	0.90
P-value	0.05	0.739	<0.001*	<0.001*
Serum N/OFQ:D28 PWT				
Pearson r	−0.609	0.186	0.207	−0.432
P-value	0.027*	0.475	0.410	0.073
Serum N/OFQ:D28 PWL				
Pearson r	−0.102	−0.198	0.409	−0.288
P-value	0.740	0.447	0.092	0.246
CSF N/OFQ:D28 PWL				
Pearson r	0.281	−0.244	−0.801	−0.475
P-value	0.331	0.381	0.001*	0.073
CSF N/OFQ:D9 Anxiety Index				
Pearson r	−0.427	−0.305	0.736	−0.384
P-value	0.128	0.269	0.004*	0.157
CSF N/OFQ:D7 PWT				
Pearson r	0.037	−0.396	−0.651	0.003
P-value	0.900	0.144	0.016*	0.993
CSF N/OFQ:D7 PWL				
Pearson r	0.045	−0.138	−0.591	0.280
P-value	0.879	0.623	0.033*	0.311
D9 Anxiety Index: D28 PWT				
Pearson r	−0.171	0.241	−0.484	0.479
P-value	0.560	0.32	0.042*	0.071
D9 Anxiety Index: D28 PWL				
Pearson r	−0.519	0.149	−0.418	−0.322
P-value	0.057	0.543	0.084	0.117
D9 Anxiety Index: D9 % Open Arm time				
Pearson r	−0.92	−0.945	−0.86	−0.904
P-value	<0.001*	<0.001*	<0.001*	<0.001*
CSF N/OFQ: D9 % Open arms time				
Pearson r	0.583	0.303	−0.580	−0.282
P-value	0.029*	0.273	0.038*	0.242
D30 Anxiety Index: D28 PWT				
Pearson r	ND	ND	−0.576	−0.216
P-value			0.012*	0.374
D30 Anxiety Index: D28 PWL				
Pearson r	ND	ND	−0.841	−0.176
P-value			<0.001*	0.470
D30 Anxiety Index: D9 Anxiety Index				
Pearson r	ND	ND	0.318	0.467
P-value			0.199	0.044*

ND, not determined.
**Indicates a significant correlation.*

sex ([$F_{(3, 62)} = 3.687$, $P = 0.0165$]) and treatment group ([$F_{(3, 62)} = 3.19$, $P = 0.0297$]). Within each treatment group the only sex difference was found in the KO SPS group (**$p < 0.01$).

Pearson's correlation analysis of anxiety-like behaviors, PWT and PWL revealed a significant negative correlation between D9 AI and PWT in female WT rats that was absent in female KO rats (**Table 1).** A similar trend for D9 AI and PWL that just missed significance was noted for male ($p = 0.057$) and female WT ($p = 0.084$) rats; no trend was noted in KO rats. The negative correlation indicates that the anxiety index increases as PWT or PWL decrease (more sensitive to nociceptive stimuli). The same correlation between pain and anxiety was found in WT, but not KO, females with D30 AI and PWT ($p = 0.012$) and PWL ($p < 0.001$).

N/OFQ RIA

Serum and CSF samples were collected at the end of the experiment (28–30 days post-SPS) to quantify levels of N/OFQ by RIA. SPS significantly increased serum N/OFQ in male WT Wistar rats (*$p < 0.05$), but had no effect on N/OFQ levels in NOP KO rats (**Figure 4A**). There was a significant interaction between genotype and SPS ([$F_{(1, 26)} = 4.553$; $P = 0.0425$]) and a

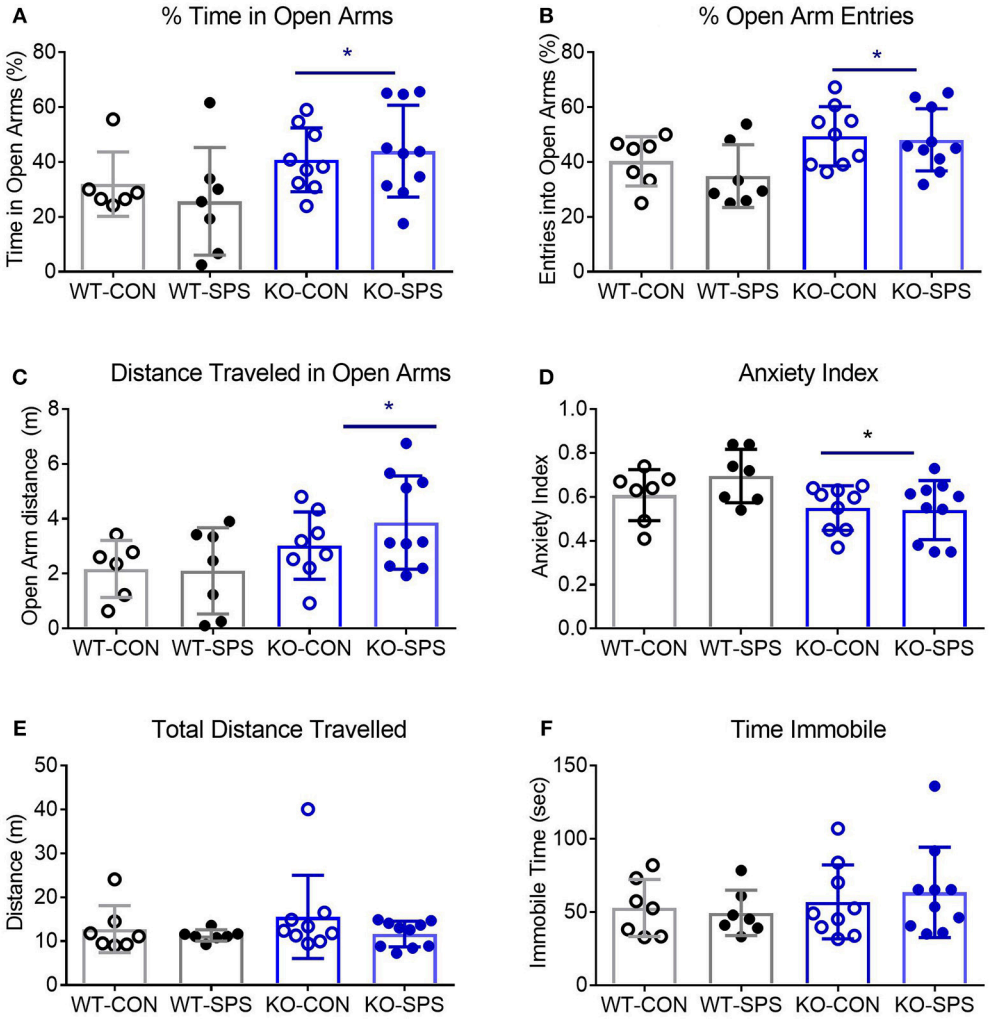

FIGURE 2 | Male NOP receptor KO rats exhibited significantly fewer anxiety-like symptoms than WT rats. Data of individual parameters **(A–F)** were analyzed by two-way ANOVA and presented as mean ± SD. WT male rats did not exhibit a significant increase in anxiety-like behaviors following exposure to SPS **(A–D)**, though the Anxiety Index **(D)** trended in that direction. However, there was a significant effect of genotype (*$p < 0.05$) on % time in open arms **(A)**, % open arm entries **(B)**, Distance traveled in open arms **(C)** and anxiety index **(D).** Rats in KO-CON and KO-SPS groups spent a greater % of time in open arms, made a greater % of entries into open arms, covered a greater distance in the open arms **(C)**, and had lower anxiety indexes **(D)** than WT rats in either treatment group. Tukey's *post-hoc* analysis revealed no *post-hoc* differences. There was no significant effect of genotype or treatment on total distance traveled **(E)** or immobile time **(F)**, confirming that rat mobility, per se, was not responsible for any differences noted. Outliers (Q = 1%): one rat each was excluded from the WT-CON group in panel A and WT-CON and KO-CON in panel C by ROUT. KO-SPS group in panel F failed the normality test, but no outliers were identified.

significant effect of SPS ($[F_{(1, 26)} = 5.209, p = 0.0309]$) by two-way ANOVA with Tukey's *post-hoc* test. Somewhat surprisingly, no significant effect of SPS or genotype was noted for CSF N/OFQ (**Figure 4B)** in males. RIA results of serum and CSF samples from female rats euthanized at day 30 post-SPS found no significant effects of genotype or stress on serum N/OFQ (**Figure 4C**). Analysis of CSF N/OFQ from female rats revealed a significant effect of genotype $[F_{(1, 24)} = 4.564, p = 0.0431]$, but no differences between groups were noted with *post-hoc* analysis (**Figure 4D**). To directly compare sex differences in levels of serum and CSF N/OFQ in the presence and absence traumatic stress, data from males and females were analyzed by two-way ANOVA for sex × treatment group, with Sidak's multiple comparisons test. We found a significant interaction between sex and treatment group $[F_{(3, 58)} = 2.85, p = 0.0451]$, and significant effects of sex $[F_{(1, 58)} = 11.53, p = 0.0014]$ and treatment group $[F_{(3, 58)} = 3.999, p = 0.0117]$. *Post-hoc* analysis indicated that male WT SPS was significantly different from female WT SPS ($p < 0.01$). The same analysis was performed with CSF but no significant interaction or effects of sex or treatment group was noted.

Sex differences also were noted in a number of pairwise correlations involving N/OFQ (**Table 1).** Serum N/OFQ negatively correlated with PWT in WT males, but not in KO males or WT females; KO females trended toward a negative correlation between serum N/OFQ and PWT ($p = 0.073$).

TABLE 2 | Elevated plus maze ANOVA values and sources of variation from comparisons of genotype and SPS treatment on anxiety-like behaviors in male and female WT and NOP receptor KO rats.

		Males		Females			
		Day 9		Day 9		Day 30	
Parameter	**Source of variation**	**F (DFn, DFd)**	***P*-value**	***F* (DFn, DFd)**	***P*-value**	***F* (DFn, DFd)**	***P*-value**
% Time in open arms	SPS		NS	(1, 33) = 8.17	0.007*	(1, 33) = 16.1	<0.001*
	Genotype	(1, 27) = 6.0	0.021*		NS		NS
% Open arm entries	SPS		NS	(1,33) = 5.96	0.02*	(1, 33) = 12.25	0.001*
	Genotype	(1, 27) = 8.67	0.006*		NS		NS
Distance traveled in open arms	SPS		NS		NS	(1, 33 = 9.05	0.005*
	Genotype	(1, 29) = 6.07	0.02*		NS		NS
Anxiety index	SPS		NS	(1, 33) = 10.3	0.003*	(1, 33) = 20.4	P < 0.001*
	Genotype	(1, 29) = 6.46	0.017*		NS		NS
Total distance traveled	Genotype		NS	(1, 33) = 5.73	0.023*		NS
Time immobile	None						

NS, not significant.

However, it was CSF N/OFQ levels that negatively correlated with PWT and PWL in WT females, with no correlation noted in males or KO females. WT males ($^*p = 0.029$) and WT females ($^*p < 0.037$) both showed significant correlations between CSF N/OFQ levels and % time in open arms (**Table 2).** However, the correlation in females was negative ($r = -0.580$), as noted with PWT and PWL while the correlation in males was positive (0.583). Thus, in females increased CSF N/OFQ levels were associated with less % of time in open arms (more anxiety), while increased CSF N/OFQ levels in males corresponded to greater % time in open arms (less anxiety). No significant correlations of CSF N/OFQ and % time in open arms were noted in KO rats.

NOP Receptor-Mediated ^{35}S-GTPγS Binding

To provide functional validation of NOP receptor loss and further examine N/OFQ efficacy following traumatic stress, N/OFQ-stimulated ^{35}S-GTPγS binding in spinal cord dorsal horn membranes from male and female WT and KO rats was assessed (**Figure 5).** No response to N/OFQ was noted in membranes from KO male or female rats, further confirming the functional loss of NOP receptors in those animals. The efficacy (E_{max}) of N/OFQ to elicit ^{35}S-GTPγS binding in the spinal cord of WT male rats subjected to SPS significantly increased ($p = 0.0285$) compared to efficacy in WT CON (**Figure 5A**). Two-way ANOVA revealed significant effects of genotype [$F_{(1, 24)} = 60.16$, $p < 0.001$] and traumatic stress [$F_{(1, 24)} = 4.673$, $p = 0.0408$]. A similar increase in E_{max} also was noted in female WT rats following SPS ($p = 0.03$). Two-way ANOVA revealed a significant interaction between genotype and traumatic stress [$F_{(1, 22)} = 6.831$, $p = 0.0159$] and a significant effect of genotype [$F_{(1, 22)} = 156.1$, $p < 0.001$]. However, N/OFQ potency for eliciting the response was 500–1000-fold less in WT females (3.1 and 2.1 μM for Control and SPS, respectively) than in WT male rats (2.1 nM Control and 5.9 nM SPS). Direct analysis of sex differences in potency (**Figure 5C)** and efficacy (**Figure 5D**) were performed using two-way ANOVA of WT data only (since there was no effect in KO). There was a significant effect of sex on potency ([$F_{(1, 23)} = 62.12$, $p < 0.001$] in both CON and SPS tissues ($p < 0.001$ by Sidak's multiple comparison test). The same type of two-way ANOVA was performed to determine sex differences in efficacy. Though significant effects of sex [$F_{(1, 23)} = 8.5$–3, $p = 0.0078$] and SPS [$F_{(1, 23)} = 10.6$, $p = 0.0035$] were noted, no sex-specific E_{max} *post-hoc* differences were found within either the CON or SPS groups (**Figure 5D**).

DISCUSSION

Stress-induced analgesia has been well-documented, however considerable evidence suggests that acute and chronic stress produce hyperalgesia, including in the SPS model (17, 19–23, 49). The goal of this study was to evaluate the role of the NOP receptor in the development of allodynia, hyperalgesia, and anxiety symptoms in male and female rats following exposure to traumatic stress using WT and NOP receptor KO rats. As anticipated, results from male NOP receptor KO rats confirm the contribution of the NOP receptor to the development of tactile allodynia and thermal hyperalgesia following SPS reported earlier with a NOP receptor antagonist (22). The most important and novel finding of the study was that unlike the KO genotype effect in males, both wild type and NOP receptor KO female rats developed allodynia and hyperalgesia to the same extent as the wild type males. Loss of the NOP receptor afforded no protection to females from SPS-induced nociceptive hypersensitivity or anxiety-like symptoms. This is the first report of traumatic stress-induced effects on tactile and thermal nociceptive sensitivity changes over time in female rats, and the first to examine the role of N/OFQ-NOP receptor system in that process.

Significant sex differences also were noted in anxiety-like behaviors following SPS in WT and NOP receptor KO rats. Unlike our previous EPM results in male Sprague-Dawley (SD) rats, we did not find a significant effect of traumatic stress on anxiety-like behaviors in WT Wistar Han males. The cause of this difference is unclear. It may result from differences in strain, age and/or housing conditions. In our previous studies, after SPS the SD rats were housed alone, with dividers between cages to

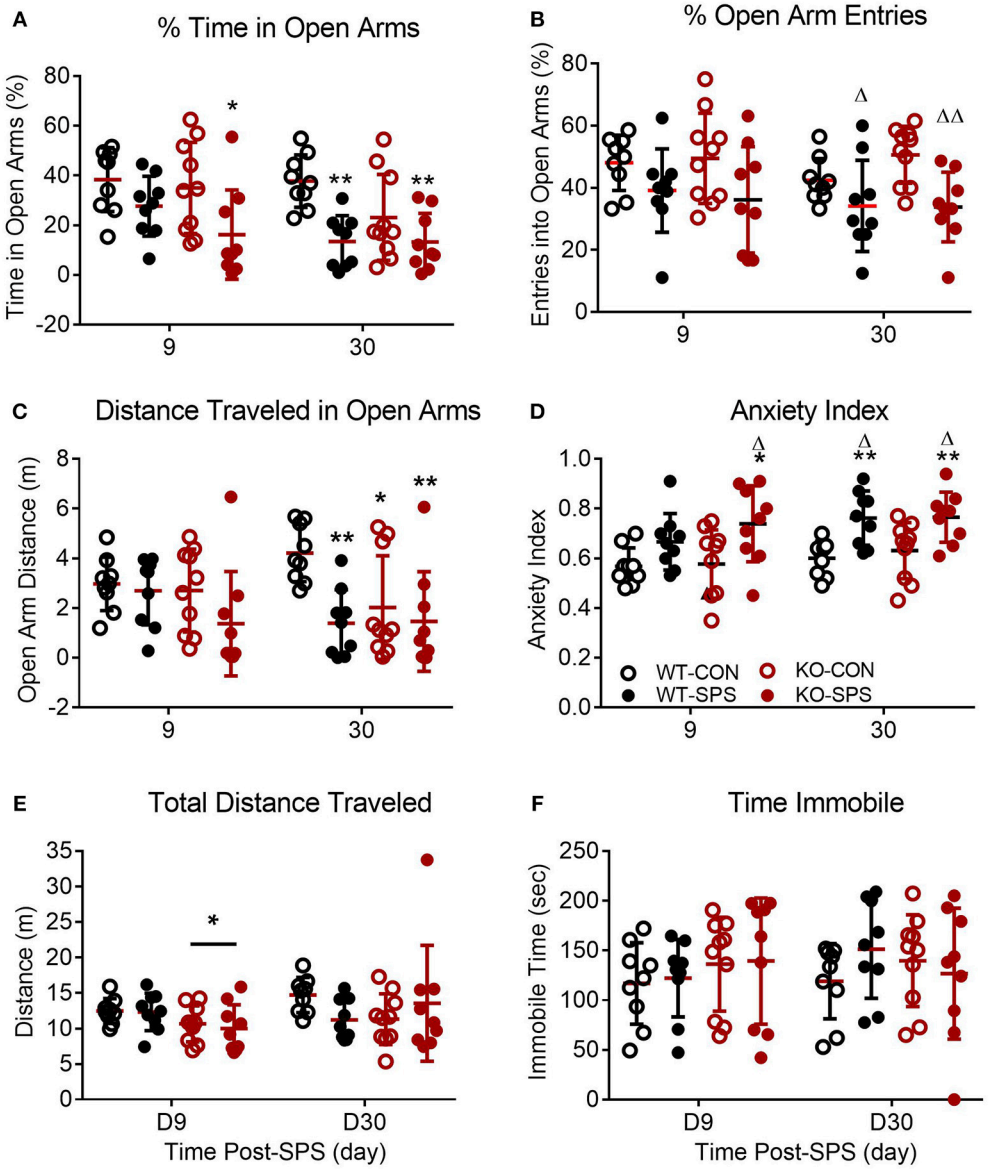

FIGURE 3 | Female WT and KO rats develop anxiety-like symptoms following SPS. Data of individual parameters **(A–F)** measured at days 9 and 30 post-SPS were analyzed by two-way ANOVA with Tukey's *post-hoc* analysis (genotype x SPS treatment) and presented as mean ± SD. Unlike male rats, there was a significant effect of SPS treatment on four parameters tested (see **Table 2** for F values). *Post-hoc* analysis confirmed that female rats exhibited a significant increase in anxiety-like behaviors at one or both time points following exposure to SPS (designated by $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. WT-CON; $^{\Delta}p < 0.05$, and $^{\Delta\Delta}p < 0.01$ vs. KO-CON) in % time in open arms **(A)**, % open arm entries **(B)**, distance traveled in open arms **(C)** and anxiety index **(D)**. Rats in SPS-treated groups spent a smaller percentage of total time in open arms, made fewer % of all arm entries into open arms, traveled a shorter distance while in open arms **(C)** and exhibited higher anxiety indexes **(D)**. However, no significant effect of SPS was noted for total distance traveled **(E)** or time immobile **(F)**. Unlike male rats, no significant effect of genotype was noted for any parameter except a transient effect on total distance traveled at day 9 (**E;** $^{*}p < 0.05$).

prevent visual contact. Changes in our cage racks precludes the use of dividers now, though unpublished studies with Sprague-Dawley rats housed in the new cages continue to exhibit elevated anxiety-like behaviors. The rats in this study were 2 weeks older when SPS was initiated compared to previous studies, due to extended quarantine upon initial receipt of the KO rats. Therefore, that age was used for all rats in this study. Instead of finding SPS-induced effects on anxiety-like behaviors in the male rats, we consistently found an effect of genotype on anxiety-like behaviors. Male KO rats in CON and SPS-treated groups showed fewer anxiety-like behaviors, including anxiety index. This did not result from sedative effects or impaired mobility as there was no difference in total traveled distance by stress or by genotype. Our results were not consistent with previous findings in untreated NOP receptor KO rats (44), where loss of the NOP receptor was anxiogenic compared to WT. Differences between

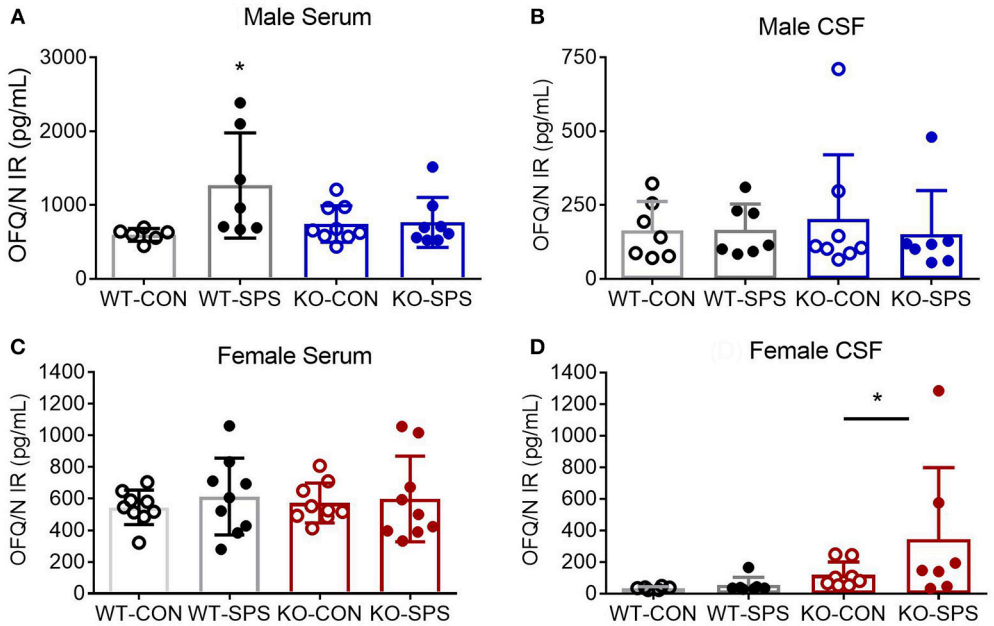

FIGURE 4 | Knockout of NOP receptor prevented SPS-induced increase in serum N/OFQ levels in male rats, but SPS did not change serum N/OFQ levels in female rats or CSF N/OFQ levels in males or females. Serum **(A,C)** and CSF **(B,D)** samples collected at day 28 (males: **A,B**) or 30 (females: **C,D**) after SPS were assessed for N/OFQ content using RIA; analysis was by two-way ANOVA with Tukey's *post-hoc* analysis and error bars represent mean ± SD. SPS increased serum N/OFQ in male WT (*$p < 0.05$), but not KO, rats. There was no significant effect of SPS on CSF N/OFQ levels in male WT or KO rats **(B)**. Sample sizes were smaller than expected because several samples were excluded. The value of one serum sample from a male WT-CON was found to be an outlier by ROUT (Q = 1%) and values from two male KO-SPS rats fell outside the range of the standard curve and could not be included. For CSF **(B)**, two values were excluded for being out of range (1 each in KO-CON and KO-SPS), and two were excluded from KO-SPS for being contaminated with blood. Both male CSF KO groups failed normality tests. No significant differences in serum N/OFQ between the four groups of female rats was found **(C)**. However, a significant effect of genotype for CSF N/OFQ levels was revealed (**D**, *$p < 0.05$). Exclusions for female rats in serum samples included one each in WT-CON and WT-SPS for being out of range and one from KO-CON for blood. CSF exclusions for female rats include: WT-CON one out of range and one by ROUT (Q = 1%); WT-SPS one out of range and one by ROUT (Q = 1%), and two female rats each in KO-CON and KO-SPS groups for being out of range of the standard curve (2) or the presence of blood in the samples (2). Both female CSF KO groups failed normality tests.

control groups in the two studies may reflect the inherent variability of EPM from one lab to the next or from differences in the number of animals housed per cage (50). Rizzi et al. housed 3-4 NOP receptor KO rats per cage, while ours were housed 1-2 per cage. N/OFQ KO mice (another model that lacks a functional N/OFQ-NOP receptor system) housed with multiple mice/cage were anxiogenic compared to WT mice, but individually housed N/OFQ KO mice were anxiolytic compared to WT (50).

Unlike the male rats that exhibited no significant effect of traumatic stress on any anxiety-like behavior, both WT and NOP receptor KO female rats developed anxiety-like behaviors by day 9 following SPS (significant effects of traumatic stress). While the clinical data indicate that PTSD symptoms in human females tend to be more pronounced and last longer (4), the limited studies using PTSD models with adult female rats (including SPS) suggests that females are more resilient to PTSD symptoms (41, 51). To determine if female anxiety-like symptoms change over time with SPS, female rats also were assessed for the appearance of anxiety-like symptoms on day 30 post-SPS (**Figure 3).** Previous work suggested that a 3 week interval in combination with a different environment prevented the decrease in open arm exploration that often is noted in a second EPM test (46). In our study the WT females exhibited identical activities in open arms and anxiety index between day 9 and 30, indicating that open arm exploration was not affected in the repeated test after 3 weeks. The significant effect of traumatic stress was underscored by day 30 post-SPS when more group differences because apparent by *post-hoc* analyses than noted at day 9. Besides females rats developing significant SPS-induced anxiety-like symptoms and males not exhibiting increased anxiety, a direct comparison of sex differences in anxiety index at day 9 indicated that significant sex differences were found only within the SPS KO treatment group, with female AI greater than males.

The role of N/OFQ and the NOP receptor in modulation of anxiety-like symptoms in males and females remains an open question. Numerous studies cite anxiolytic and anxiogenic actions of N/OFQ and other NOP agonists (for review see (32) in male rats. However, administration of two different NOP antagonists decreased anxiety-like symptoms in male SD rats exposed to two different models of traumatic stress (22, 52), without altering spontaneous locomotion. Similar gender differences in anxiety and fear-related behaviors also were reported in N/OFQ KO mice (50).

Assessment of serum and CSF N/OFQ levels in male and female rats 28 and 30 days post-SPS, respectively, revealed several interesting findings. First, serum N/OFQ also was significantly elevated in WT male rats following SPS as noted previously,

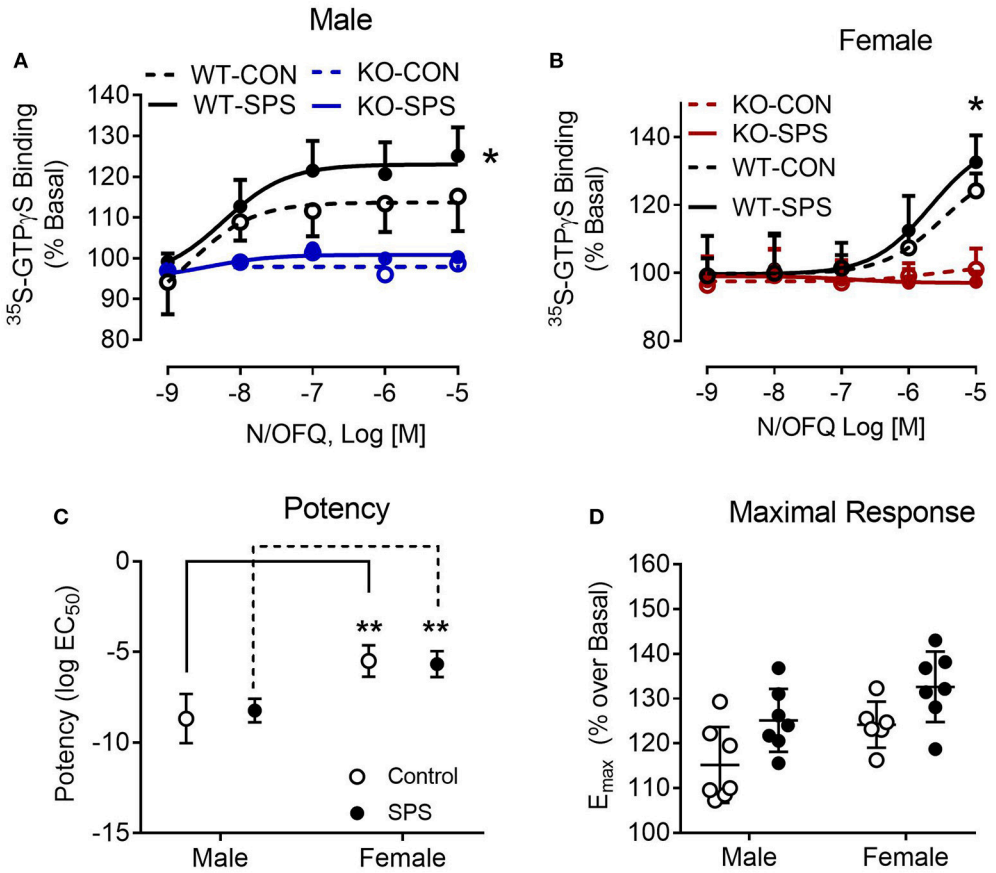

FIGURE 5 | N/OFQ efficacy increases with SPS in dorsal spinal cord (SC) membranes from Wistar Han WT male **(A)** and female **(B)** rats; no efficacy found in SC membranes from KO rats. ^{35}S-GTPγS binding was performed in the presence and absence of increasing concentrations of N/OFQ in spinal cord membranes from WT and KO male and female rats from sham control and SPS-treated groups; data are presented as mean ± SD. N/OFQ efficacy significantly increased following SPS in membranes from WT male (**A**: $^{*}p = 0.0285$; $n = 7$ per group) and female (**B**: $^{*}p = 0.0354$; $n = 6$–7) rats, but no measurable increase in basal ^{35}S-GTPγS binding was noted with N/OFQ in membranes from male or female KO rats as determined by two-way ANOVA (genotype x stress) with a *post-hoc* Sidak's multiple comparison test (effect of SPS within each genotype). These results are consistent with the genotyping results and extend that analysis to confirm that no functional NOP receptors are expressed in the KO rats. Direct analysis of sex differences in potency **(C)** and efficacy **(D)** were performed using two-way ANOVA with Sidak's multiple comparison test. There was a significant effect of sex on potency [$F_{(1,23)} = 62.12$, $p < 0.001$] in both CON and SPS tissues ($p < 0.001$). Though significant effects of sex [$F_{(1,23)} = 8.5$–3, $p = 0.0078$] and SPS [$F_{(1,23)} = 10.6$, $p = 0.0035$] were noted for efficacy, no sex-specific post-hoc differences were found between CON or SPS groups **(D)**.

but not in the SPS KO males. However, no significant increase in CSF N/OFQ following SPS in WT or KO male Wistar rats was detected, which differs from previous studies in WT SD rats (17, 22). These results are consistent with increased levels of circulating N/OFQ following traumatic stress that acts on the NOP receptor to increase nociceptive sensitivity; the absence of the NOP receptor protects male rats from allodynia and hyperalgesia.

Interestingly, serum N/OFQ levels were not significantly increased by SPS in female WT or KO rats; serum N/OFQ levels in WT SPS male rats were significantly higher than levels in female WT SPS rats (**Figure 4E).** Similar to males, no significant differences in CSF N/OFQ levels were noted between treatment groups in females, but there was a significant effect of genotype (**Figure 4D)**. Expression of the N/OFQ-NOP receptor system is modulated by female sex hormones, glucocorticoids, inflammatory mediators, and activity of the N/OFQ-NOP receptor system (53–57). This modulation is supported by the interaction between genotype and SPS on serum N/OFQ levels in males and significant effects of genotype on CSF N/OFQ levels in females. Interpretation of the CSF N/OFQ RIA results in this study was limited by greater than usual reductions in sample sizes per group due to difficulty obtaining clear CSF samples from the Wistar rats, and numerous samples being outside of the range of the standard curve of the RIA. Unfortunately, limited amounts of CSF obtained per rat precludes having enough sample left to re-assay if dilution or increased sample volume is required. There may have been subtle differences between groups that were masked by the large variability and smaller sample sizes.

We previously reported that N/OFQ efficacy at the NOP receptor in the dorsal lumbar spinal cord was increased in SPS-treated male Sprague-Dawley rats compared to controls (58), and similar results were noted with WT male and female Wistar rats in this study. However, we found significant sex differences

in potency of N/OFQ for activation of GTPγS; N/OFQ was 1000-fold more potent in males than in females in this study. This suggests that NOP receptors in the dorsal spinal cord of female rats were desensitized. Spinal N/OFQ antinociception is gender specific: female rat sensitivity to spinal N/OFQ varies depending on estrogen state (59–62). For instance, while i.t. N/OFQ alleviated mustard oil-induced hyperalgesia in male, ovariectomized (OVX) and diestrous female rats, it did not reverse hyperalgesia in OVX + estradiol or proestrous female rats (60). We did not screen the females for stage of the estrous cycle in this study. However, a previous study of fear extinction and retention in female SD rats following SPS may provide some insight. In that study, even though all females were in diestrus when they were exposed to SPS (42), by day 9 post-SPS there were equivalent numbers of rats in each stage of the estrus cycle in each treatment group. It is clear that the overall effect of traumatic stress in the current study was to produce hyperalgesia and allodynia in WT and KO females regardless of estrus cycle stage, and in KO rats independent of the NOP receptor.

N/OFQ modulates nociceptive sensitivity through supraspinal (33, 63), spinal (59–64), and peripheral nerve sites (65, 66). Supraspinal N/OFQ inhibits stress-induced analgesia and produces hyperalgesia (33, 35, 63), consistent with SPS-induced allodynia and hyperalgesia. N/OFQ activates the HPA axis following acute administration or mild stress (67–69): HPA activation by N/OFQ resembled acute stress and was blocked by NOP antagonist treatment. Though acute stress reduced N/OFQ content in the brain, N/OFQ levels were restored within 24 h (67), suggesting that stress causes release and synthesis of endogenous N/OFQ. Acute or repeated social defeat stress also elevates NOP receptor mRNA in the brain, supporting the hypothesis that dysfunction of the N/OFQ system contributes to behavioral and hormonal dysregulation following stress (69).

In mice, i.c.v. N/OFQ blocked stress-induced antinociception (SIA) equally in males and females (33). Though levels of N/OFQ did not significantly increase in CSF from WT male or female rats subjected to SPS in this study, it is possible that the lack of NOP receptor in the male KO rats ensured that stress-induced analgesia remained intact, and that could explain the protective effect of the NOP receptor KO in males. This was clearly not the case with female rats following SPS. CSF N/OFQ levels negatively correlate with PWT, PWL and % open arms time, and positively correlate with anxiety index in WT females. This is consistent with increased sensitivity to tactile and thermal stimuli, less time in open arms and a higher anxiety index in those WT females with the highest levels of CSF N/OFQ. The correlation was absent in KO females, as one might expect since no NOP receptors were available to interact with N/OFQ. However, the KO females experienced the same extent of allodynia, hyperalgesia and anxiety like behaviors as the WT females, indicating that activation of NOP receptors is not the only mechanism by which those behaviors are mediated.

Though spinal N/OFQ has generally been found to produce analgesia, very low levels of N/OFQ (femtomole) administered i.t. produced thermal hyperalgesia and tactile allodynia in male mice (70, 71). Thus, low levels of N/OFQ in the CSF following SPS also may contribute to hyperalgesia in males, and this hyperalgesia would be lost in the NOP receptor KO rats. The potency of N/OFQ for spinal NOP receptors in WT females was likely too weak to activate NOP receptors to modulate spinal nociceptive sensitivity, which is consistent with the lack of protection afforded NOP receptor KO females.

The third site at which N/OFQ has been found to alter nociceptive sensitivity is in the periphery at primary afferent nerve endings, where it increases nociceptive sensitivity via PLC/IP3-mediated release of Substance P (66). Levels of N/OFQ in the serum arise from white blood cells (65, 72, 73). Elevated serum N/OFQ in WT-SPS rats may contribute to hyperalgesia and allodynia through actions on peripheral nerve endings. While loss of the NOP receptor is sufficient to block hyperalgesia and allodynia in male rats, it is not sufficient to alleviate hyperalgesia and allodynia in female rats. There is strong evidence that sex differences in the immune system and in hormonal modulation of immune cells account for differences in chronic pain or pain sensitivity between males and females (74). This is likely the case for traumatic stress-induced allodynia, and future studies will address that possibility.

Our results confirm that circulating N/OFQ-mediated NOP receptor signaling in male rats plays an important role in modulating nociceptive sensitivity and serum N/OFQ levels following traumatic stress. Absence of NOP receptor expression prevented the development of tactile allodynia, thermal hyperalgesia and increased serum N/OFQ in male, but not female, rats following traumatic stress. However, loss of the NOP receptor in females did not alter behavioral or biochemical changes in response to SPS compared to WT controls. SPS-induced significant anxiety-like behavior in female, but not male, NOP receptor KO rats that persisted for at least 30 days. It occurred concomitantly with hyperalgesia and allodynia, and was correlated with CSF N/OFQ levels. Such dramatic differences in males and females in response to NOP receptor loss requires additional study to better understand the role of the N/OFQ-NOP receptor system in stress-induced pain modulation and the development of co-morbid PTSD symptoms such as allodynia, hyperalgesia and anxiety-like behaviors.

AUTHOR CONTRIBUTIONS

The study was written and conceived by KS and YZ. YZ, IS, and CD conducted the research. YZ and KS analyzed the data. The manuscript received input and was edited by all authors.

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REFERENCES

1. Kornfield SL, Hantsoo L, Epperson CN. What does sex have to do with it? The role of sex as a biological variable in the development of posttraumatic stress disorder. *Curr Psychiatry Rep.* (2018) 20:39. doi: 10.1007/s11920-018-0907-x
2. Bangasser DA, Eck SR, Telenson AM, Salvatore M. Sex differences in stress regulation of arousal and cognition. *Physiol Behav.* (2018) 187:42–50. doi: 10.1016/j.physbeh.2017.09.025
3. Mendoza C, Barreto GE, Avila-Rodriguez M, Echeverria V. Role of neuroinflammation and sex hormones in war-related PTSD. *Mol Cell Endocrinol.* (2016) 434:266–77. doi: 10.1016/j.mce.2016.05.016
4. Ramikie TS, Ressler KJ. Mechanisms of sex differences in fear and posttraumatic stress disorder. *Biol Psychiatry* (2018) 83:876–85. doi: 10.1016/j.biopsych.2017.11.016
5. Bangasser DA, Wicks B. Sex-specific mechanisms for responding to stress. *J Neurosci Res.* (2017) 95:75–82. doi: 10.1002/jnr.23812
6. Buttner MM, Godfrey KM, Floto E, Pittman J, Lindamer L, Afari N. Combat exposure and pain in male and female Afghanistan and Iraq veterans: the role of mediators and moderators. *Psychiatry Res.* (2017) 257:7–13. doi: 10.1016/j.psychres.2017.07.001
7. Lisieski MJ, Eagle AL, Conti AC, Liberzon I, Perrine SA. Single-prolonged stress: a review of two decades of progress in a rodent model of post-traumatic stress disorder. *Front Psychiatry* (2018) 9:196. doi: 10.3389/fpsyt.2018.00196
8. Yamamoto S, Morinobu S, Takei S, Fuchikami M, Matsuki A, Yamawaki S, et al. Single prolonged stress: toward an animal model of posttraumatic stress disorder. *Depress Anxiety* (2009) 26:1110–7. doi: 10.1002/da.20629
9. Nedelcovych MT, Gould RW, Zhan X, Bubser M, Gong X, Grannan M, et al. A rodent model of traumatic stress induces lasting sleep and quantitative electroencephalographic disturbances. *ACS Chem Neurosci.* (2015) 6:485–93. doi: 10.1021/cn500342u
10. Vanderheyden WM, Poe GR, Liberzon I. Trauma exposure and sleep: using a rodent model to understand sleep function in PTSD. *Exp Brain Res.* (2014) 232:1575–84. doi: 10.1007/s00221-014-3890-4
11. Patki G, Li L, Allam F, Solanki N, Dao AT, Alkadhi K, et al. Moderate treadmill exercise rescues anxiety and depression-like behavior as well as memory impairment in a rat model of posttraumatic stress disorder. *Physiol Behav.* (2014) 130:47–53. doi: 10.1016/j.physbeh.2014.03.016
12. Peng Z, Zhang R, Wang H, Chen Y, Xue F, Wang L, et al. Ziprasidone ameliorates anxiety-like behaviors in a rat model of PTSD and up-regulates neurogenesis in the hippocampus and hippocampus-derived neural stem cells. *Behav Brain Res.* (2013) 244:1–8. doi: 10.1016/j.bbr.2013.01.032
13. Serova LI, Laukova M, Alaluf LG, Pucillo L, Sabban EL. Intranasal neuropeptide Y reverses anxiety and depressive-like behavior impaired by single prolonged stress PTSD model. *Eur Neuropsychopharmacol.* (2014) 24:142–7. doi: 10.1016/j.euroneuro.2013.11.007
14. Yu L, Wang L, Zhao X, Song M, Wang X. Role of single prolonged stress in acquisition of alcohol conditioned place preference in rats. *Life Sci.* (2016) 151:259–63. doi: 10.1016/j.lfs.2016.03.004
15. Eagle AL, Fitzpatrick CJ, Perrine SA. Single prolonged stress impairs social and object novelty recognition in rats. *Behav Brain Res.* (2013) 256:591–7. doi: 10.1016/j.bbr.2013.09.014
16. Imanaka A, Morinobu S, Toki S, Yamawaki S. Importance of early environment in the development of post-traumatic stress disorder-like behaviors. *Behav Brain Res.* (2006) 173:129–37. doi: 10.1016/j.bbr.2006.06.012
17. Zhang Y, Gandhi PR, Standifer KM. Increased nociceptive sensitivity and nociceptin/orphanin FQ levels in a rat model of PTSD. *Mol Pain* (2012) 8:76. doi: 10.1186/1744-8069-8-76
18. He YQ, Chen Q, Ji L, Wang ZG, Bai ZH, Stephens RL Jr. PKCγ receptor mediates visceral nociception and hyperalgesia following exposure to PTSD-like stress in the spinal cord of rats. *Mol Pain* (2013) 9:35. doi: 10.1186/1744-8069-9-35
19. Qi J, Chen C, Lu YC, Zhang T, Xu H, Cui YY, et al. Activation of extracellular signal-regulated kinase1/2 in the medial prefrontal cortex contributes to stress-induced hyperalgesia. *Mol Neurobiol.* (2014) 50:1013–23. doi: 10.1007/s12035-014-8707-8
20. Sun R, Zhang Z, Lei Y, Liu Y, Lu C, Rong H, et al. Hippocampal activation of microglia may underlie the shared neurobiology of comorbid posttraumatic stress disorder and chronic pain. *Mol Pain* (2016) 12:1744806916679166. doi: 10.1177/1744806916679166
21. Sun R, Zhao Z, Feng J, Bo J, Rong H, Lei Y, et al. Glucocorticoid-potentiated spinal microglia activation contributes to preoperative anxiety-induced postoperative hyperalgesia. *Mol Neurobiol.* (2017) 54:4316–28. doi: 10.1007/s12035-016-9976-1
22. Zhang Y, Simpson-Durand CD, Standifer KM. Nociceptin/orphanin FQ peptide receptor antagonist JTC-801 reverses pain and anxiety symptoms in a rat model of post-traumatic stress disorder. *Br J Pharmacol.* (2015) 172:571–82. doi: 10.1111/bph.12701
23. He YQ, Lang XQ, Lin L, Ji L, Yuan XY, Chen Q, et al. P2X3 receptor-mediated visceral hyperalgesia and neuronal sensitization following exposure to PTSD-like stress in the dorsal root ganglia of rats. *Neurogastroenterol Motil.* (2017) 29:e12976. doi: 10.1111/nmo.12976
24. Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, Low M, et al. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. *FEBS Lett.* (1994) 347:284–8. doi: 10.1016/0014-5793(94)00561-3
25. Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, et al. ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* (1994) 341:33–8. doi: 10.1016/0014-5793(94)80235-1
26. Pan YX, Cheng J, Xu J, Rossi G, Jacobson E, Ryan-Moro J, et al. Cloning and functional characterization through antisense mapping of a kappa 3-related opioid receptor. *Mol Pharmacol.* (1995) 47:1180–8.
27. Wick MJ, Minnerath SR, Lin X, Elde R, Law PY, Loh HH. Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors. *Brain Res Mol Brain Res.* (1994) 27:37–44. doi: 10.1016/0169-328X(94)90181-3
28. Neal CR Jr, Mansour A, Reinscheid R, Nothacker HP, Civelli O, Akil H, et al. Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. *J Comp Neurol.* (1999) 412:563–605.
29. Andero R, Brothers SP, Jovanovic T, Chen YT, Salah-Uddin H, Cameron M, et al. Amygdala-dependent fear is regulated by Oprl1 in mice and humans with PTSD. *Sci Translat Med.* (2013) 5:188ra173. doi: 10.1126/scitranslmed.3005656
30. Lambert DG. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat Rev Drug Discov.* (2008) 7:694–710. doi: 10.1038/nrd2572
31. Pan Z, Hirakawa N, Fields HL. A cellular mechanism for the bidirectional pain-modulating actions of orphanin FQ/nociceptin. *Neuron* (2000) 26:515–22. doi: 10.1016/S0896-6273(00)81183-6
32. Witkin JM, Statnick MA, Rorick-Kehn LM, Pintar JE, Ansonoff M, Chen Y, et al. The biology of Nociceptin/Orphanin FQ (N/OFQ) related to obesity, stress, anxiety, mood, and drug dependence. *Pharmacol Ther.* (2014) 141:283–99. doi: 10.1016/j.pharmthera.2013.10.011
33. Mogil JS, Grisel JE, Reinscheid RK, Civelli O, Belknap JK, Grandy DK. Orphanin FQ is a functional anti-opioid peptide. *Neuroscience* (1996) 75:333–7. doi: 10.1016/0306-4522(96)00338-7
34. Mogil JS, Nessim LA, Wilson SG. Strain-dependent effects of supraspinal orphanin FQ/nociceptin on thermal nociceptive sensitivity in mice. *Neurosci Lett.* (1999) 261:147–50. doi: 10.1016/S0304-3940(99)00012-9
35. Mogil JS, Pasternak GW. The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol Rev.* (2001) 53:381–415.
36. Mogil JS, Sternberg WF, Balian H, Liebeskind JC, Sadowski B. Opioid and nonopioid swim stress-induced analgesia: a parametric analysis in mice. *Physiol Behav.* (1996) 59:123–32. doi: 10.1016/0031-9384(95)02073-X
37. Morgan MM, Grisel JE, Robbins CS, Grandy DK. Antinociception mediated by the periaqueductal gray is attenuated by orphanin FQ. *Neuroreport* (1997) 8:3431–4. doi: 10.1097/00001756-199711100-00003
38. Rizzi A, Marzola G, Bigoni R, Guerrini R, Salvadori S, Mogil JS, et al. Endogenous nociceptin signaling and stress-induced analgesia. *Neuroreport* (2001) 12:3009–13. doi: 10.1097/00001756-200110080-00006
39. Ko MH, Kim YH, Woo RS, Kim KW. Quantitative analysis of nociceptin in blood of patients with acute and chronic pain. *Neuroreport* (2002) 13:1631–3. doi: 10.1097/00001756-200209160-00012
40. Raffaeli W, Samolsky Dekel BG, Landuzzi D, Caminiti A, Righetti D, Balestri M, et al. Nociceptin levels in the cerebrospinal fluid of chronic pain patients with or without intrathecal administration of morphine. *J Pain Symptom Manage.* (2006) 32:372–7. doi: 10.1016/j.jpainsymman.2006.05.013

41. Keller SM, Schreiber WB, Staib JM, Knox D. Sex differences in the single prolonged stress model. *Behav Brain Res.* (2015) 286:29–32. doi: 10.1016/j.bbr.2015.02.034
42. Zer-Aviv TM, Akirav I. Sex differences in hippocampal response to endocannabinoids after exposure to severe stress. *Hippocampus* (2016) 26:947–57. doi: 10.1002/hipo.22577
43. Homberg JR, Mul JD, de Wit E, Cuppen E. Complete knockout of the nociceptin/orphanin FQ receptor in the rat does not induce compensatory changes in mu, delta and kappa opioid receptors. *Neuroscience* (2009) 163:308–15. doi: 10.1016/j.neuroscience.2009.06.021
44. Rizzi A, Molinari S, Marti M, Marzola G, Calo G. Nociceptin/orphanin FQ receptor knockout rats: *in vitro* and *in vivo* studies. *Neuropharmacology* (2011) 60:572–9. doi: 10.1016/j.neuropharm.2010.12.010
45. Cohen H, Matar MA, Buskila D, Kaplan Z, Zohar J. Early post-stressor intervention with high-dose corticosterone attenuates posttraumatic stress response in an animal model of posttraumatic stress disorder. *Biol Psychiatry* (2008) 64:708–17. doi: 10.1016/j.biopsych.2008.05.025
46. Adamec R, Shallow T, Burton P. Anxiolytic and anxiogenic effects of kindling–role of baseline anxiety and anatomical location of the kindling electrode in response to kindling of the right and left basolateral amygdala. *Behav Brain Res.* (2005) 159:73–88. doi: 10.1016/j.bbr.2004.10.004
47. Treit D, Menard J, Royan C. Anxiogenic stimuli in the elevated plus-maze. *Pharmacol Biochem Behav.* (1993) 44:463–9. doi: 10.1016/0091-3057(93)90492-C
48. Motulsky HJ. Interpreting results: Normality tests, in GraphPad Statistics Guide. Available online at: https://www.graphpad.com/guides/prism/7/statistics/index.htm?stat_interpreting_results_normality.htm (Accessed November 29, 2018).
49. Imbe H, Iwai-Liao Y, Senba E. Stress-induced hyperalgesia: animal models and putative mechanisms. *Front Biosci J Virt Library* (2006) 11:2179–92. doi: 10.2741/1960
50. Ouagazzal AM, Moreau JL, Pauly-Evers M, Jenck F. Impact of environmental housing conditions on the emotional responses of mice deficient for nociceptin/orphanin FQ peptide precursor gene. *Behav Brain Res.* (2003) 144:111–7. doi: 10.1016/S0166-4328(03)00066-4
51. Cohen H, Yehuda R. Gender differences in animal models of posttraumatic stress disorder. *Dis Markers* (2011) 30:141–50. doi: 10.1155/2011/734372
52. Genovese RF, Dobre S. Mitigation of adverse behavioral impact from predator exposure by the nociceptin/orphanin FQ peptide antagonist J-113397 in rats. *Behav Pharmacol.* (2017) 28:521–30. doi: 10.1097/FBP.0000000000000329
53. Buzas B, Rosenberger J, Kim KW, Cox BM. Inflammatory mediators increase the expression of nociceptin/orphanin FQ in rat astrocytes in culture. *Glia* (2002) 39:237–46. doi: 10.1002/glia.10106
54. Buzas B, Symes AJ, Cox BM. Regulation of nociceptin/orphanin FQ gene expression by neuropoietic cytokines and neurotrophic factors in neurons and astrocytes. *J Neurochem.* (1999) 72:1882–9. doi: 10.1046/j.1471-4159.1999.0721882.x
55. Clarke S, Chen Z, Hsu MS, Hill RG, Pintar JE, Kitchen I. Nociceptin/orphanin FQ knockout mice display up-regulation of the opioid receptor-like 1 receptor and alterations in opioid receptor expression in the brain. *Neuroscience* (2003) 117:157–68. doi: 10.1016/S0306-4522(02)00750-9
56. Sinchak K, Romeo HE, Micevych PE. Site-specific estrogen and progestin regulation of orphanin FQ/nociceptin and nociceptin opioid receptor mRNA expression in the female rat limbic hypothalamic system. *J Comp Neurol.* (2006) 496:252–68. doi: 10.1002/cne.20949
57. Xie GX, Ito E, Maruyama K, Suzuki Y, Sugano S, Sharma M, et al. The promoter region of human prepro-nociceptin gene and its regulation by cyclic AMP and steroid hormones. *Gene* (1999) 238:427–36. doi: 10.1016/S0378-1119(99)00350-9
58. Zhang Y, Gandhi PR, Standifer KM. Effects of single-prolonged stress on nociceptive sensitivity and the nociceptin/orphanin FQ (N/OFQ) system in spinal cord of rats. In: *International Narcotics Research Conference.* Kansas, MO (2012).
59. Claiborne J, Nag S, Mokha SS. Activation of opioid receptor like-1 receptor in the spinal cord produces sex-specific antinociception in the rat: estrogen attenuates antinociception in the female, whereas testosterone is required for the expression of antinociception in the male. *J Neurosci.* (2006) 26:13048–53. doi: 10.1523/JNEUROSCI.4783-06.2006
60. Claiborne JA, Nag S, Mokha SS. Estrogen-dependent, sex-specific modulation of mustard oil-induced secondary thermal hyperalgesia by orphanin FQ in the rat. *Neurosci Lett.* (2009) 456:59–63. doi: 10.1016/j.neulet.2009.03.106
61. Flores CA, Shughrue P, Petersen SL, Mokha SS. Sex-related differences in the distribution of opioid receptor-like 1 receptor mRNA and colocalization with estrogen receptor mRNA in neurons of the spinal trigeminal nucleus caudalis in the rat. *Neuroscience* (2003) 118:769–78. doi: 10.1016/S0306-4522(02)01000-X
62. Flores CA, Wang XM, Zhang KM, Mokha SS. Orphanin FQ produces gender-specific modulation of trigeminal nociception: behavioral and electrophysiological observations. *Neuroscience* (2001) 105:489–98. doi: 10.1016/S0306-4522(01)00179-8
63. Xie X, Wisor JP, Hara J, Crowder TL, LeWinter R, Khroyan TV, et al. Hypocretin/orexin and nociceptin/orphanin FQ coordinately regulate analgesia in a mouse model of stress-induced analgesia. *J Clin Investig.* (2008) 118:2471–81. doi: 10.1172/JCI35115
64. Yamamoto T, Nozaki-Taguchi N, Kimura S. Analgesic effect of intrathecally administered nociceptin, an opioid receptor-like1 receptor agonist, in the rat formalin test. *Neuroscience* (1997) 81:249–54. doi: 10.1016/S0306-4522(97)00166-8
65. Fiset ME, Gilbert C, Poubelle PE, Pouliot M. Human neutrophils as a source of nociceptin: a novel link between pain and inflammation. *Biochemistry* (2003) 42:10498–505. doi: 10.1021/bi0300635
66. Inoue M, Kobayashi M, Kozaki S, Zimmer A, Ueda H. Nociceptin/orphanin FQ-induced nociceptive responses through substance P release from peripheral nerve endings in mice. *Proc Natl Acad Sci USA.* (1998) 95:10949–53. doi: 10.1073/pnas.95.18.10949
67. Devine DP, Hoversten MT, Ueda Y, Akil H. Nociceptin/orphanin FQ content is decreased in forebrain neurones during acute stress. *J Neuroendocrinol.* (2003) 15:69–74. doi: 10.1046/j.1365-2826.2003.00868.x
68. Devine DP, Watson SJ, Akil H. Nociceptin/orphanin FQ regulates neuroendocrine function of the limbic-hypothalamic-pituitary-adrenal axis. *Neuroscience* (2001) 102:541–53. doi: 10.1016/S0306-4522(00)00517-0
69. Green MK, Devine DP. Nociceptin/orphanin FQ and NOP receptor gene regulation after acute or repeated social defeat stress. *Neuropeptides* (2009) 43:507–14. doi: 10.1016/j.npep.2009.08.003
70. Minami T, Okuda-Ashitaka E, Mori H, Sakimura K, Watanabe M, Mishina M, et al. Characterization of nociceptin/orphanin FQ-induced pain responses in conscious mice: neonatal capsaicin treatment and N-methyl-D-aspartate receptor GluRepsilon subunit knockout mice. *Neuroscience* (2000) 97:133–42. doi: 10.1016/S0306-4522(00)00010-5
71. Sakurada C, Sakurada S, Katsuyama S, Sasaki J, Tan-No K, Sakurada T. Involvement of tachykinin NK1 receptors in nociceptin-induced hyperalgesia in mice. *Brain Res.* (1999) 841:85–92. doi: 10.1016/S0006-8993(99)01800-4
72. Williams JP, Thompson JP, McDonald J, Barnes TA, Cote T, Rowbotham DJ, et al. Human peripheral blood mononuclear cells express nociceptin/orphanin FQ, but not mu, delta, or kappa opioid receptors. *Anesth Aanalg.* (2007) 105:998–1005. doi: 10.1213/01.ane.0000278865.11991.9d
73. Williams JP, Thompson JP, Rowbotham DJ, Lambert DG. Human peripheral blood mononuclear cells produce pre-pro-nociceptin/orphanin FQ mRNA. *Anesth Analg.* (2008) 106:865–6. doi: 10.1213/ane.0b013e3181617646
74. Sorge RE, Totsch SK. Sex differences in pain. *J Neurosci Res.* (2017) 95:1271–81. doi: 10.1002/jnr.23841

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Microglia Express Mu Opioid Receptor: Insights From Transcriptomics and Fluorescent Reporter Mice

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Background: Microglia activation contributes to chronic pain and to the adverse effects of opiate use such as analgesic tolerance and opioid-induced hyperalgesia. Both mu opioid receptor (MOR) encoded by *Oprm1/OPRM1* gene and toll like receptor 4 (TLR4) have been reported to mediate these morphine effects and a current question is whether microglia express the Oprm1 transcript and MOR protein. The aim of this study was to characterize *Oprm1*-MOR expression in naive murine and human microglia, combining transcriptomics datasets previously published by other groups with our own imaging study using the Cx3cr1-eGFP-MOR-mCherry reporter mouse line.

Methods: We analyzed microglial *Oprm1/OPRM1* expression obtained from transcriptomics datasets, focusing on *ex vivo* studies from adult wild-type animals and adult post-mortem human cerebral cortex. *Oprm1*, as well as co-regulated gene sets were examined. The expression of MOR in microglia was also investigated using our novel fluorescent Cx3cr1-eGFP-MOR-mcherry reporter mouse line. We determined whether CX3cR1-eGFP positive microglial cells expressed MOR-mCherry protein by imaging various brain areas including the Frontal Cortex, Nucleus Accumbens, Ventral Tegmental Area, Central Amygdala, and Periaqueductal Gray matter, as well as spinal cord.

Results: *Oprm1* expression was found in all 12 microglia datasets from mouse whole brain, in 7 out of 8 from cerebral cortex, 3 out of 4 from hippocampus, 1 out of 1 from striatum, and 4 out of 5 from mouse or rat spinal cord. *OPRM1* was expressed in 16 out of 17 microglia transcriptomes from human cerebral cortex. In Cx3cr1-eGFP-MOR-mCherry mice, the percentage of MOR-positive microglial cells ranged between 35.4 and 51.6% in the different brain areas, and between 36.8 and 42.4% in the spinal cord.

Conclusion: The comparative analysis of the microglia transcriptomes indicates that *Oprm1/OPRM1* transcripts are expressed in microglia. The investigation of Cx3cr1-eGFP-MOR-mCherry mice also shows microglial expression of MOR proteinin the brain and spine. These results corroborate functional studies showing the actions of MOR agonists on microglia and suppression of these effects by MOR-selective antagonists or MOR knockdown.

Keywords: microglia, opioid receptor, mu, transcriptomics, gene clusters, fluorescent reporter mice, analgesic tolerance, opioid-induced hyperalgesia

INTRODUCTION

Activation of the mu opioid receptor (MOR), encoded by the Oprm1/OPRM1 gene in rodents and humans, respectively (1, 2), mediates opioid analgesia and the adverse consequences of opioid use (3, 4). Glial cells and in particular microglia are known to contribute to chronic pain (5) as well as to opioid tolerance and opioid-induced hyperalgesia (OIH) (6–8). However, whether microglia express *Oprm1* and whether microglial *Oprm1* would have a role in chronic pain and other opioid effects remains to be demonstrated. Most studies reporting MOR expression or function in microglia have been performed on cultured microglia (9–18). It has been shown however that gene expression profiles differ between microglia in culture and adult mouse microglia *in vivo* (19) and therefore the demonstration of MOR expression in cultured microglia does not allow to conclude for MOR expression in adult microglia *in vivo*. Horvath et al. (20) have shown MOR expression in rat spinal microglia *in vivo* by immunohistochemistry. However, two other studies have contradicted these findings by showing a lack of MOR expression in spinal cord microglia (21, 22). Thus, whether microglia in adults express MOR still remains an unsolved question. Specifically, Corder et al. present considerable evidence that argues against the expression of MOR messenger and protein in mouse spinal cord microglia. Their findings are further strengthened by transcriptomic analyses which show a lack in co-expression of Oprm1 mRNA with microglial markers (21). However, critical analysis and commentary on these interesting results is not possible yet due to the limited access of the datasets used for transcriptomic analysis. Therefore, whether *Oprm1/OPRM1* are expressed by microglia remains a matter of debate that should be further investigated. To date, there are no published studies focusing on the analysis of a large series of transcriptomic datasets for Oprm1 expression in microglia that would allow assessing, unambiguously, *Oprm1* expression in microglia *in vivo*. In addition, *OPRM1*/MOR expression in human microglia is yet to be fully characterized. For this purpose, we have used novel approaches to characterize *Oprm1/OPRM1* expression in microglia based on transcriptomics and have used fluorescent reporter mice to characterize MOR expression *in vivo*.

A number of laboratories have generated transcriptomics datasets for microglia that can be used for analyzing gene expression profiles (23, 24) as well as for investigating microglia physiology and their responses in disease (25, 26). We have analyzed published datasets from microarray (MA) and RNA-sequencing (RNA-seq) studies performed on rodent and human microglia for Oprm1 and OPRM1 gene expression. We have completed this analysis by imaging microglial MOR using a novel double fluorescent Cx3cr1-eGFP-MOR-mCherry mouse line. The chemokine receptor CX3CR1 is a specific marker for phagocytic cells and labels specifically microglia and macrophages in the nervous system of naive animals (27–29). The Cx3cr1-eGFP mouse line was originally used to explore CX3CR1 function (30) then later to map the fate of tissue macrophages including microglia (28, 29). A reporter knock-in mouse line for MOR, the MOR-mCherry line, allows to map the distribution of MOR-expressing cells in mice using fluorescence imaging (31). In order to localize MOR protein in microglia, we have bred these two lines together to generate the Cx3cr1-eGFP-MOR-mCherry mouse line. Thus, we have analyzed MOR expression in various brain regions implicated in chronic pain or chronic opiate effects as well as the spinal cord in control non-pathological conditions. In addition, as sex differences are an important factor for chronic pain (32–34) and greatly impact the microglial contribution to pain (35, 36), we investigated Oprm1/OPRM1-MOR presence in microglia from both females and males.

MATERIALS AND METHODS

Transcriptomics Analyses

As microglia develop after birth until post-natal day 15, followed by stabilization of microglial numbers (37–39), we focused the Oprm1 expression analysis on microglia from juvenile-adult wild-type naïve rodent and juvenile-adult humans with no reported pain phenotype. The datasets used and related information including the pathology of patients from whom microglia were collected, are indicated in **Supplementary Tables 1–4**. The selection criteria for which datasets to include in the present study were as follows: normalized mouse datasets included in the database recently published by Friedman et al. (40) (see Data S2 in Friedman et al.) and additional normalized datasets containing *Oprm1*

Abbreviations: BDNF, brain-derived neurotrophic factor; CeA, central amygdala; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; eGFP, enhanced green fluorescent protein; FCx, frontal cortex; KO, knockout; MA, microarray; Mars-seq, massively parallel Single-cell RNA-seq; MOR, mu opioid receptor; NAcc, nucleus accumbens; OIH, opioid-induced hyperalgesia; PAG, periaqueductal gray; PBS, phosphate buffer baline; PFA, paraformaldehyde; RNA-seq, RNA-sequencing; TLR, toll like receptor; VTA, ventral tegmental area.

in their gene list; from wild-type mice aged 0.5 months and older. Microglia datasets from mouse whole brain were from the studies by Wang et al. (41), Verheijden et al. (42), Poliani et al. (43), Erny et al. (44), Szulzewsky et al. (45), Pyonteck et al. (46), Bruttger et al. (47), Lavin et al. (48), Bennett et al. (49), Gosselin et al. (50), Krasemann et al. (51), and Zhao et al. (52). Microglia datasets from mouse brain areas were from Orre et al. (53), Arumugan et al. (54), Grabert et al. (55), Friedman et al. (40), Srinivasan et al. (56), Zhang et al. 2014 (57), and Matcovitch-Natan et al. (58). Microglia datasets from rodent spinal cord were from Chiu et al. (59), Denk et al. (60), Noristani et al. (61), Matcovitch-Natan et al. (58), and Jokinen et al. (62). Information on these datasets including publication authors and year, accession number, mouse or rat strain, sex, age, dissociation, and microglia isolation methods, and genomics assay are given in **Supplementary Tables 1**–**4**.

Human datasets comprised those published by Zhang et al. (63) and contained in Friedman's **Supplementary Table 2** as well as normalized datasets recently reported by Gosselin et al. (64) and Galatro et al. (65) as described in **Supplementary Table 4**. Normal cortical regions were resected from patients with diseases described in column F. Among the 19 datasets by Gosselin et al. we selected 10 datasets derived from individuals aged 13 and older. Among the 39 datasets from adult individuals by Galatro et al. we selected 8 datasets derived from samples collected at a maximum delay of 10 h post-mortem. Information on these datasets including publication authors and year, accession number, sex, age, cortex area, dissociation, and microglia isolation methods, and genomics assay are given in **Supplementary Table 4**.

In addition to our main focus set, (A) showing only the expression levels of *Oprm1* gene transcript from purified microglia, three other groups of gene clusters were formed: (B) Myeloid cells, (C) Activation patterns and (D) Neurons and Astrocytes. The list of genes contained in each of the gene clusters of B, C, and D was formed using the gene lists defined in Friedman et al. (40) and filtered to exclude the few genes expressed by any other cell type. The Myeloid gene clusters (B) include the Microglia, the Macrophage, and the Neutrophil-Monocytes gene clusters, respectively. The activation pattern clusters (C) comprise the Interferon-related, Proliferation-related, LPS-related and Neurodegeneration-related gene clusters. The Neurons and Astrocyte gene clusters (D) are composed of the Neuron-associated, Excitatory neuron-associated, GABAergic neuron-associated, and Astrocyte-associated genes clusters.

The logarithm scale two (log2) transformation was applied to each data sample values and 1 was added to expression values to avoid indetermination for zero count reads. For each data sample, the first (25th percentile), the second (median), and the third quantile (75th percentile) values were computed. The log2 values for the Oprm1 transcript were also obtained for each dataset. The log2 values associated with each gene subset of B, C, and D categories were isolated for each sample and an average value was calculated for each subset. The R environment (version 3.5.0) was used to create the basic figures. The vioplot R package was used to obtain a violin plot like contour for the log2 values of each data sample. The log2 values and the 25th, 75th percentile and median values were superimposed on the violin plots using different colors as shown in **Figures 1**–**4**.

For determining correlations between *Oprm1/OPRM1* expression and the expression of the gene clusters described above, z-scores transformations (1+log2 values) were calculated from the mean expression level of each cluster for each dataset included in the study. The Orre et al. (53) mouse cortex dataset and the S037 dataset of the Gosselin et al. study (64) were removed from the analysis as they varied substantially from all other datasets within their group. The correlation analysis for human datasets included all dataset except for S037 set.

Cx3cr1-eGFP-MOR-mCherry Mouse Line

Animals and Ethics Statement

The animals were housed under standard light, temperature, and humidity conditions (12 h light-dark cycle, 21 ± 1 °C, 55 ± 10% humidity) with food and water *ad libitum*. Brains and spinal cords were collected from male and female mice aged between 5 and 15 weeks. All experiments were conducted respecting the European Communities Council Directives of 22 September 2010 (directive 2010/63/UE) under the guidelines of the Committee for Research and Ethical issues of IASP published in PAIN, 1983; 16:109-110, and were approved by the local ethical committee (Com'Eth d'Ethique pour l'Expérimentation Animale IGBMC-ICS, license N°17) with the agreement number 00876-02.

Cx3cr1-eGFP-MOR-mCherry Mice

The Cx3cr1-eGFP-MOR-mCherry mouse line was generated by crossing Cx3Cr1-eGFP mice (29) and MOR-mCherry mice (31) to obtain viable heterozygous animals. These were intercrossed to generate homozygous Cx3Cr1-eGFP-MOR-mCherry mice that are fertile and develop normally. Genotyping was performed by PCR to detect both Cx3Cr1-GFP sequence (35 cycles at 94°C for 30 s, 65.5°C for 30 s, and 72°C for 2 min) using the following primer sequences: Cx3Cr1-Fwd: 5′-TTCACGTTCGGTCTGGTGGGAAATC-3′, Cx3Cr1- Rev:

5′-TTCCTAGTGGAGCTAGGGTCGGGG-3′, eGFP-Fwd:

5′-GATCACTCTCGGCATGGACG-3′, and MOR-mCherry sequence (35 cycles at 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min) using a forward primer located on exon four of Oprm1 gene, -Fwd: 5′-TGACGTGACATGCAGTTGAGATTT-3′and a reverse primer located in the 3' UTR region, Rev: 5′-TCCCACAAACCCTGACAGCAAC-3′. Both female and male Cx3cr1-eGFP-MOR-mCherry mice were analyzed for MOR expression in microglia (3 animals per sex).

Tissue Preparation

Mice were deeply anesthetized with intraperitoneal administration of 100/5 mg/kg ketamine/xylazine (Virbac, Carros, France; Rompun, Bayer, La Garenne Colombes, France) and were intracardially perfused with 4% Paraformaldehyde (PFA) in Phosphate Buffer Saline (PBS) solution (4%PFA/PBS). The whole brain and spinal cord were isolated and post-fixed overnight at 4°C in 4%PFA/PBS. Samples were rinsed three times in PBS and cryoprotected in a sucrose/PBS gradient (10, 20, and 30%) for 24 h each. Tissues were embedded in OCT

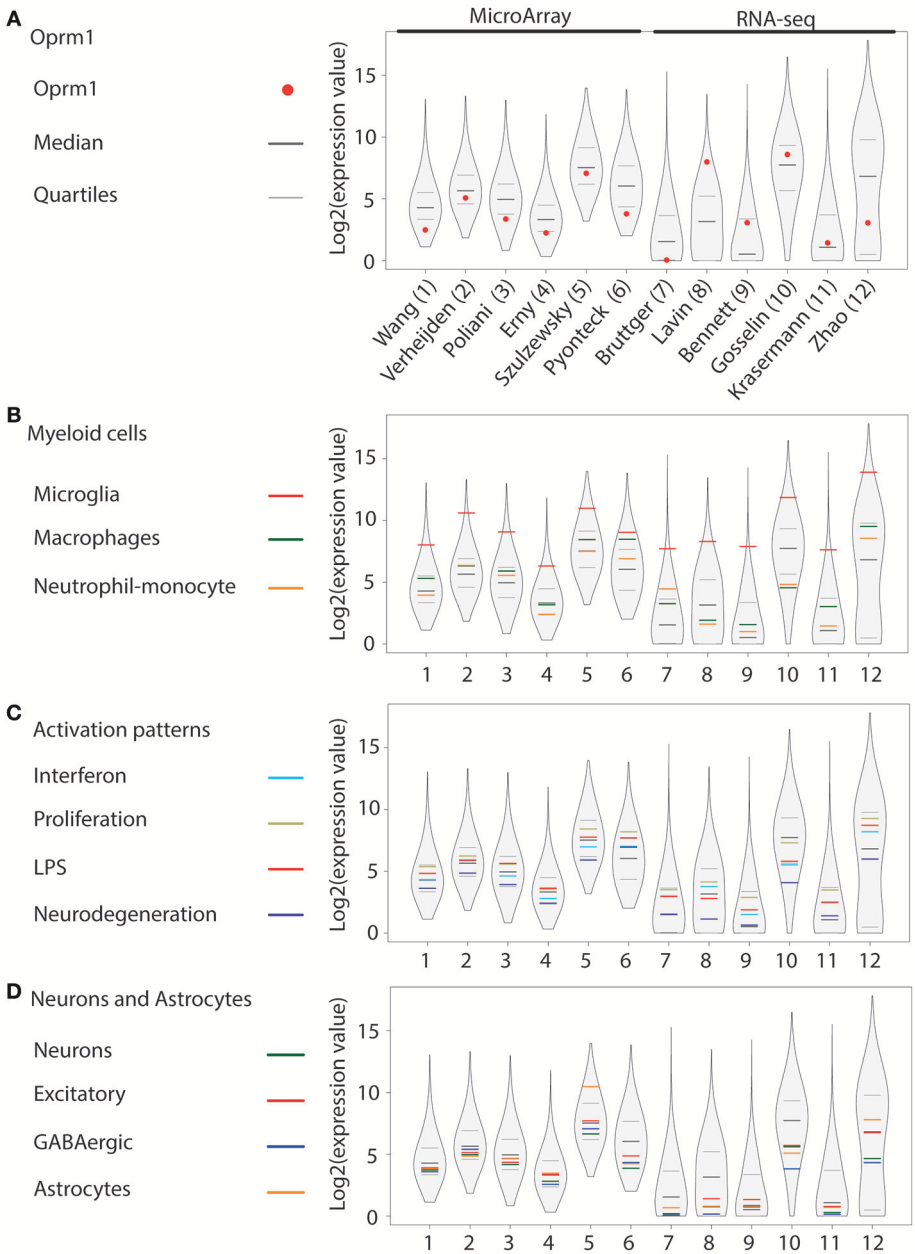

FIGURE 1 | *Oprm1* and gene clusters expression in mouse whole brain microglia datasets. Expression levels of the genes identified in each dataset are represented in violin plots demonstrating the median, as well as the 25th and 75th quartiles. Datasets are represented by name of the first author in **(A)**, which are denoted numerically in **(B–D)**. **(A)** Expression of *Oprm1* is below the median in purified microglia assayed with MA, and fluctuates above and below the median in purified microglia assayed with RNA-seq. **(B)** Expression values of the Myeloid cell clusters demonstrate a high expression of Microglia-related genes which are in the 75th quartile in all the datasets analyzed. **(C)** Expression values of the Activation pattern clusters are below the 75th quartile in all but one dataset (dataset #6). **(D)** Expression values of the Neurons and Astrocytes clusters are below the median in 5 out 6 of the datasets derived from MA assays, whereby the Astrocyte-related genes have expression values above the 75th quartile in dataset #5. When assayed with RNA-seq, the Astrocyte-related genes are below the median, except for dataset #12, where the values are above the median.

(Tissue Tek, Sakura Fine Technical, Torrance) and coronally (brain) or transversally (spinal cord) sectioned at 30 µm on Superfrost microscope slides using a cryostat (Leica CM3050S). Cryosections were dried at room temperature and then stored at −20°C before imaging.

Immunohistochemistry and Quantifications

For immunohistochemistry, cryosections were rehydrated with PBS and were then incubated in a blocking solution (10% horse serum/0.1% Triton X-100/PBS) for 30 min at room temperature. The sections were incubated with the primary antibodies diluted

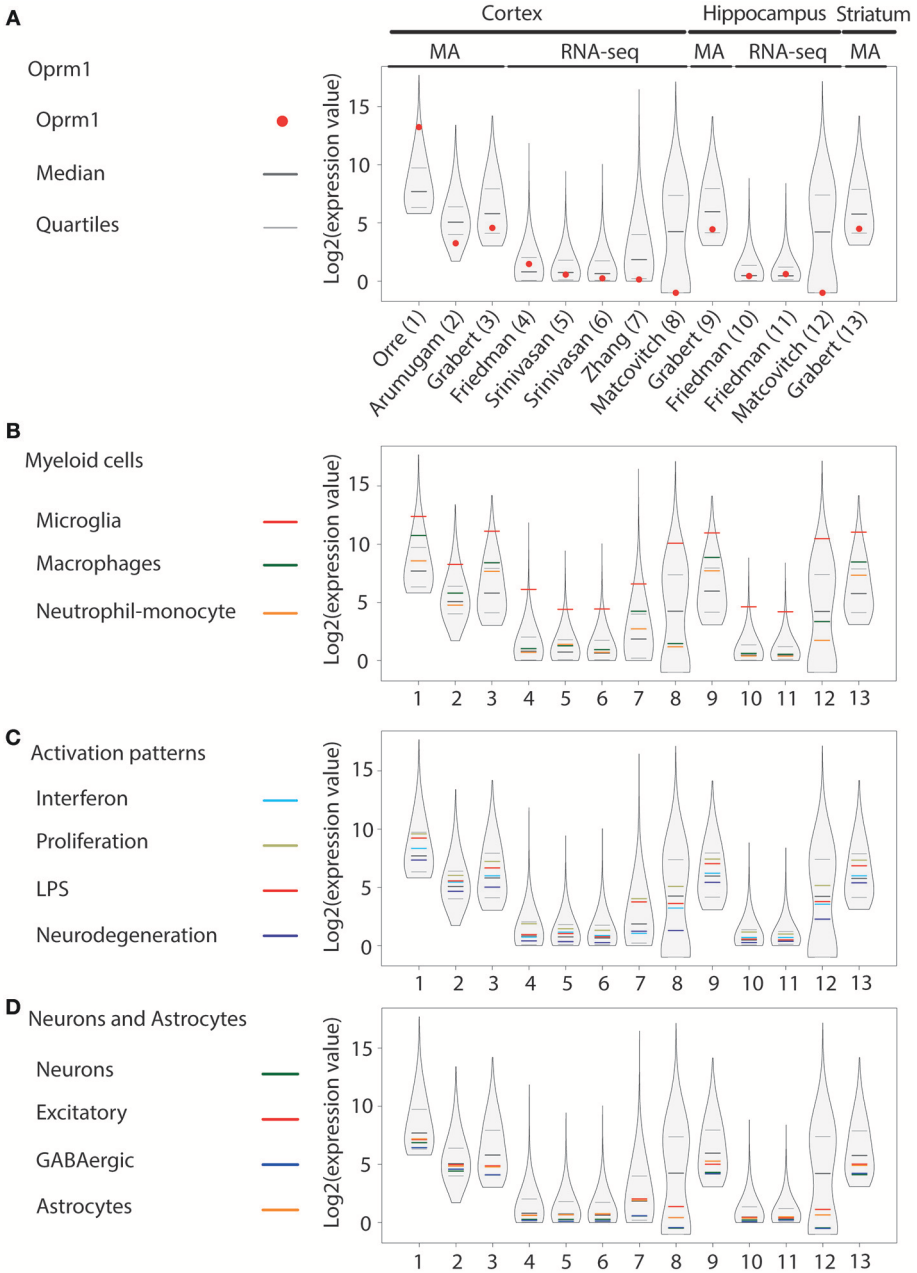

FIGURE 2 | *Oprm1* and gene clusters expression in mouse cortex, hippocampus and striatum microglia datasets. Expression levels of the genes identified in each dataset are represented in violin plots demonstrating the median, as well as the 25th and 75th quartiles. Datasets are represented by name of the first author in **(A)** which are denoted numerically in **(B–D)**. In mouse cortex and hippocampus, microglia were assayed with either microarrays (MA) or RNA-sequencing (RNA-seq) as underlined on **(A)** and assayed with MA only in the striatum (dataset #13). **(A)** Oprm1 expression values were distributed below and above the median independent of the assay used to measure gene expression. RNA-seq datasets (#8 and 12) yielded to no expression of *Oprm1* in microglia. The highest Oprm1 expression value was yielded by dataset #1 in cortical microglia. **(B)** Among the Myeloid gene clusters, the Microglia-related gene module was the mostly highly expressed in all datasets analyzed, with expression values distributed about the 75th quantile. **(C)** Proliferation-related genes were the most highly expressed within the Activation pattern clusters, distributed within the 75th quantile. **(D)** Neuron-, GABAergic-, and Astrocyte-related genes had expression values that were below the median in most of the datasets analyzed. In the cortex and hippocampus, the Excitatory neuron- and Astrocyte-related genes are distributed along the median for datasets #5–7 and #10, 11.

in 10% normal horse serum/0.1% Triton X-100/PBS overnight at 4°C. The sections were then washed 3 times in 0.1% Triton X-100/PBS and incubated with secondary antibodies and Hoechst for 1 h at room temperature, in the same solution as the primary antibodies. After washing 3 times in 0.1% Triton/PBS and once in PBS alone, slides were mounted with the S3023 aqueous

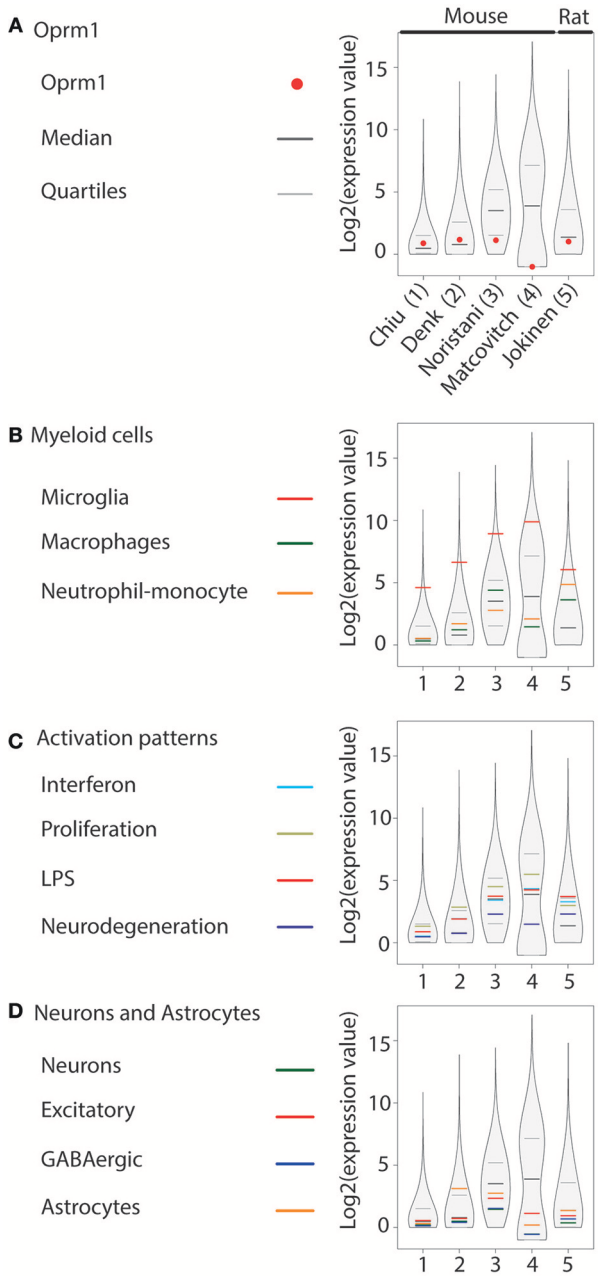

FIGURE 3 | *Oprm1* and gene clusters expression in rodent spinal cord microglia datasets. Expression levels of the genes identified in each dataset are represented in violin plots demonstrating the median, as well as the 25th and 75th quartiles. Datasets are represented by name of the first author in **(A)** which are denoted numerically in **(B–D)**. Gene expression in the spinal cord was assayed with RNA-seq exclusively. **(A)** Oprm1 expression values ranged from above the median to undetected level (#4). **(B)** In the Myeloid gene cluster, the Microglia- related genes were the mostly highly expressed in all datasets analyzed, with expression values distributed about the 75th quantile. **(C)** Proliferation-related genes were the most highly expressed within the Activation pattern clusters, except in dataset#5, which had higher expression values for the LPS module in microglia purified from rat. Overall, the expression values of Activation patterns gene clusters were low compared to the Microglia-related genes. **(D)** Neuron-, GABAergic- and Astrocyte-related genes had expression values that were low compared to the Microglia-related genes except for dataset #2 that show high Astrocyte-related module expression **(B)**.

mounting medium (DAKO). For staining cis-Golgi, trans-Glogi, and lysosomes, the rabbit polyclonal anti-GM130 antibody (11308-1-AP, Proteintech), the rabbit polyclonal anti-TGN38 antibody (NBP1-03495, Interchim), and the mouse monoclonal anti-Lamp1 antibody (H4A3, Developmental Studies Hybridoma Bank DSHB) were used. The optimal dilutions were set up in preliminary experiments. Anti-GM130, anti-TGN38, and anti-Lamp1 were used at dilutions 1:500, 1:200, and 1:200 (0.6, 5, 0.1 μg/ml), respectively. The secondary antibodies used were the Cy5 conjugated donkey anti-mouse (1:1,000, 715-176-150, Jackson) and the Cy5 conjugated donkey anti-rabbit (1:500, 711-176-152, Jackson) Ig antibodies. Images were acquired with a confocal microscope (8UV, Leica Microsystem) using a 40x objective. The FIJI package for ImageJ software was used for image analysis (66). The percent of MOR-positive microglia among the CX3CR1-eGFP-positive microglia in the different brain and spinal cord regions were quantified on multiple microscopic fields from 3 female and 3 male adult animals. The number of fields counted and of cells examined are indicated in the **Supplementary Table 5**.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 6.01 to investigate the correlations between Oprm1 expression and the different clusters mentioned above, as well as to compare MOR expression in CX3CR1-eGFP-positive microglia between male and female mice. *Oprm1* and gene module z-scores were all non-normally distributed and correlations were assessed with the Spearman's correlation test. The mean percentage of MOR-positive and CX3CR1-eGFP-positive microglia were tested for normality as well and compared using the Unpaired Student's *t*-test or Mann Whitney test where applicable. The results are presented as the mean ± SEM for microglia cell counts (percentages). A *p*-value of 0.05 or less was considered to be statistical significant.

RESULTS

Oprm1/*OPRM1* Expression in Rodent and Human Microglia Transcriptomics Datasets

Microglia From Mouse Whole Brain

Oprm1 was expressed in the twelve datasets analyzed (**Figure 1A**). Among those, six were issued from MA, see references (41–46) and six from RNA-seq, see references (47–52), as described in **Supplementary Table 1**. *Oprm1* levels ranged from above the median ($n = 4$) to between the median and first quartile ($n = 4$) and below the first quartile ($n = 4$). *Oprm1* expression tended to be more homogenous in MA than in RNA-seq datasets. The Myeloid gene modules defined by Friedman et al. (40) included Microglia, Macrophage, and Monocyte-neutrophil gene clusters. The Microglia gene cluster was highly expressed in all datasets, at the highest level of all gene clusters (**Figure 1B**). Macrophage and Neutrophil-monocyte cluster expression was distributed over and under the median among datasets (**Figure 1B**). The Proliferation cluster stood out as the most expressed among the Activation pattern gene sets, followed by the LPS, or Interferon and then Neurodegeneration

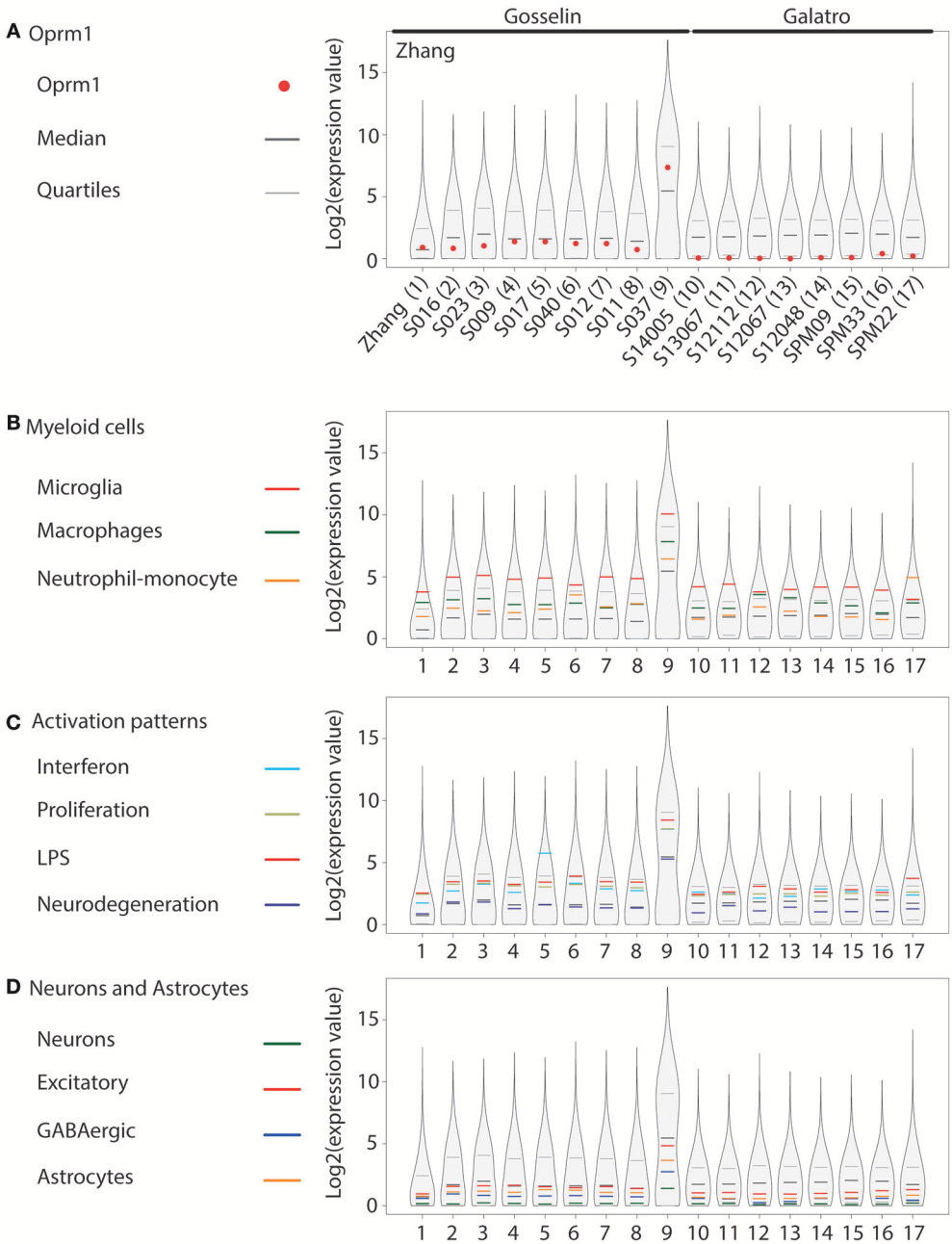

FIGURE 4 | *Oprm1* and gene clusters expression in human cortex microglia datasets. Expression levels of the genes identified in each dataset are represented in violin plots demonstrating the median, as well as the 25th and 75th quartiles. In all datasets, microglia were assayed with RNA-seq. Gosselin datasets (#2–9) comprised of microglia isolated from young males and females, while the other human datasets were from adult and aged postmortem samples of unknown gender (#1) or exclusively males (10–17). Datasets are represented by name of the first author **(A)**, which are denoted numerically in **(B–D)**. **(A)** Oprm1 expression values were distributed below or along the median in the Zhang and Gosselin datasets (#1 and 2–9) but consistently below the median in all the Galatro datasets. **(B)** In the Myeloid gene clusters, the Microglia- related genes were the most highly expressed in all datasets analyzed, with expression values distributed above the 75th quantile, except in dataset #17 which had higher expression values for the Monocytes-Neutrophils-related genes. **(C)** Activation pattern gene clusters were highly expressed in purified human microglia, distributed within, and above the 75th quantile. The majority of the datasets reported the highest expression values for the LPS-related genes. Interferon-related genes had the highest expression values in the datasets #5, 10, 14, and 16 with causes of death associated with blood circulation and aneurysm. **(D)** Neuron-, GABAergic-, and Astrocyte-related genes had expression values along and below the median in dataset#1 and those provided by Gosselin (#2–9). Expression values were all below the median in the aged samples (#10–17).

gene sets (**Figure 1C**). The three neuronal (Neurons, Excitatory and GABAergic) gene sets were positioned below the median in nine of the datasets (**Figure 1D**). The Astrocyte cluster was below the median in eight of the datasets (**Figure 1D**).

Microglia From Murine Cortex, Hippocampus, and Striatum

In the cortex, *Oprm1* expression was expressed in seven out of the eight datasets generated by either MA (53–55) or RNAseq (40, 56, 57) and not detected in the dataset by Matcovitch-Natan et al. derived from Massively parallel Single-cell RNA-seq (Mars-seq) (58). Expression ranged from above the median in two datasets, between median, and first quartile in three datasets, below the first quartile in two datasets, to not being expressed in the Mars-seq dataset (**Figure 2A**). The dataset with very high *Oprm1* expression was generated using the Agilent MA technology (53) while Affymetrix arrays were used in the other MA studies. In the hippocampus, *Oprm1* was above the median in one dataset, ranged between the median, and first quartile in two datasets, but not expressed in the Mars-seq dataset. In the striatum dataset by Grabert et al., the *Oprm1* was above the first quartile. The Microglia gene cluster was also highly expressed and the highest in all datasets (**Figure 2B**), similarly to whole mouse brain datasets. Macrophage and Neutrophil-monocyte module expression ranged among datasets from over to under the median, with the Macrophage cluster higher than that of Neutrophil-monocyte (**Figure 2B**). The Proliferation cluster stood out as the most expressed among the Activation pattern gene sets, followed by the LPS or Interferon and then Neurodegeneration gene sets (**Figure 2C**), comparably whole mouse brain data. The neuronal (Neurons, Excitatory and GABAergic) as well as the Astrocyte gene set were positioned below the median in nine of the datasets (**Figure 2D**).

Rodent Spinal Cord

The expression of *Oprm1* was analyzed in four mouse and one rat spinal microglia datasets obtained by RNA-seq (58–62). *Oprm1* is expressed above the median in two mouse spinal cord datasets, below the first quartile in one data set, and not expressed in the Mars-seq dataset by Matcovitch-Natan et al. The rat spinal cord dataset showed *Oprm1* level above the first quartile (**Figure 3A**). The Microglia gene cluster was also highly expressed in the five datasets (**Figure 3B**). Macrophage and Neutrophil-monocyte cluster expression varied among datasets from over to below the median (**Figure 3B**). In the Activation pattern gene modules, the Proliferation gene sets were the most expressed in mouse but not rat, whereas Neurodegeneration gene sets showed the lowest level (**Figure 3C**), similarly to what was found in mouse brain. The three neuronal (Neurons, Excitatory and GABAergic) gene sets were positioned below or at the median level while Astrocyte cluster expression differed across the datasets (**Figure 3D**).

Human Cortex

OPRM1 level was analyzed in microglia RNA-seq datasets generated from temporal cortex gray matter (63), different cortical areas (64), and parietal cortex (65) (**Figure 4A**). *OPRM1* is expressed above the median in the dataset generated by Zhang et al. (63) for which microglia were purified from cortex gray matter collected on juvenile-adult individuals. In the study by Gosselin et al. (64) on cortical microglia enriched from young adults, *OPRM1* levels ranged between the median and first quartile. In the S037 dataset issued from microglia collected from a brain tumor, *OPRM1* was highly expressed. In the microglia datasets by Galatro et al. (65), collected from the parietal cortex of aged people (57–102 years), *OPRM1* was around the first quartile and not expressed in the S12067 sample (**Figure 4A**). The Microglia gene module was the most expressed in all sets except one and the Macrophage cluster had generally a higher level than the Neutrophil-monocyte cluster (**Figure 4B**). Among the activation pattern gene sets, the LPS and Interferon sets showed mostly the highest levels, followed by the Proliferation cluster. The Neurodegeneration gene sets showed the lowest level of expression (**Figure 4C**). The values for the Excitatory and GABAergic clusters were higher than those of the Neurons cluster, which showed the lowest expression levels (**Figure 4D**), differing from mouse datasets. The Astrocyte cluster was below the median on most datasets (**Figure 4D**).

Single Cell RNAseq Data Sets

We investigated *Oprm1* expression in microglia datasets from published single cell RNA-seq studies. Transcripts for *Oprm1* were not found in the datasets which contained about 1,300, 1,985, 6,000, 1,179, 1,900–3,300, and 1,169 and 800 genes expressed per cell, respectively (27, 67–72). Microglia datasets generated from microdissected basal ganglia nuclei led to the identification of 2,647 expressed genes per cell that did not include *Oprm1* (73).

Relationships Between Oprm1 Expression and Gene Clusters

Myeloid Cells

We investigated the relationships between Oprm1 expression and the expression of myeloid molecular signatures (modules) that are genes found to be co-expressed in the study by Friedman et al. (40). Microglia, in comparison with Macrophages cluster, had inverse trends regarding their correlations with *Oprm1* in the rodent analyses (**Figures 5–9**). The Microglia module was positively correlated with *Oprm1* (mouse cortex, mouse hippocampus, and rodent spinal cord), while the Macrophage cluster was negatively correlated with *Oprm1*.

In whole brain analysis, *Oprm1* expression had a negative correlation with the Macrophage and Monocyte-neutrophil clusters, which was statistically significant with the Macrophage gene cluster only ($p = 0.030$). In the mouse cortex, there was a strong positive correlation between *Oprm1* and the Microglia gene set which was statistically significant only for this cluster ($r = 0.955$; $p = 0.0032$) but not for the other myeloid sets (**Figure 6**). No significant correlation was found between the Monocyte-neutrophil gene cluster and any dataset (**Figures 5–8**).

Activation Patterns

We investigated the relationships between the expression of *Oprm1* and of the myeloid activation modules as described (40). There was a negative correlation between *Oprm1* and the LPS

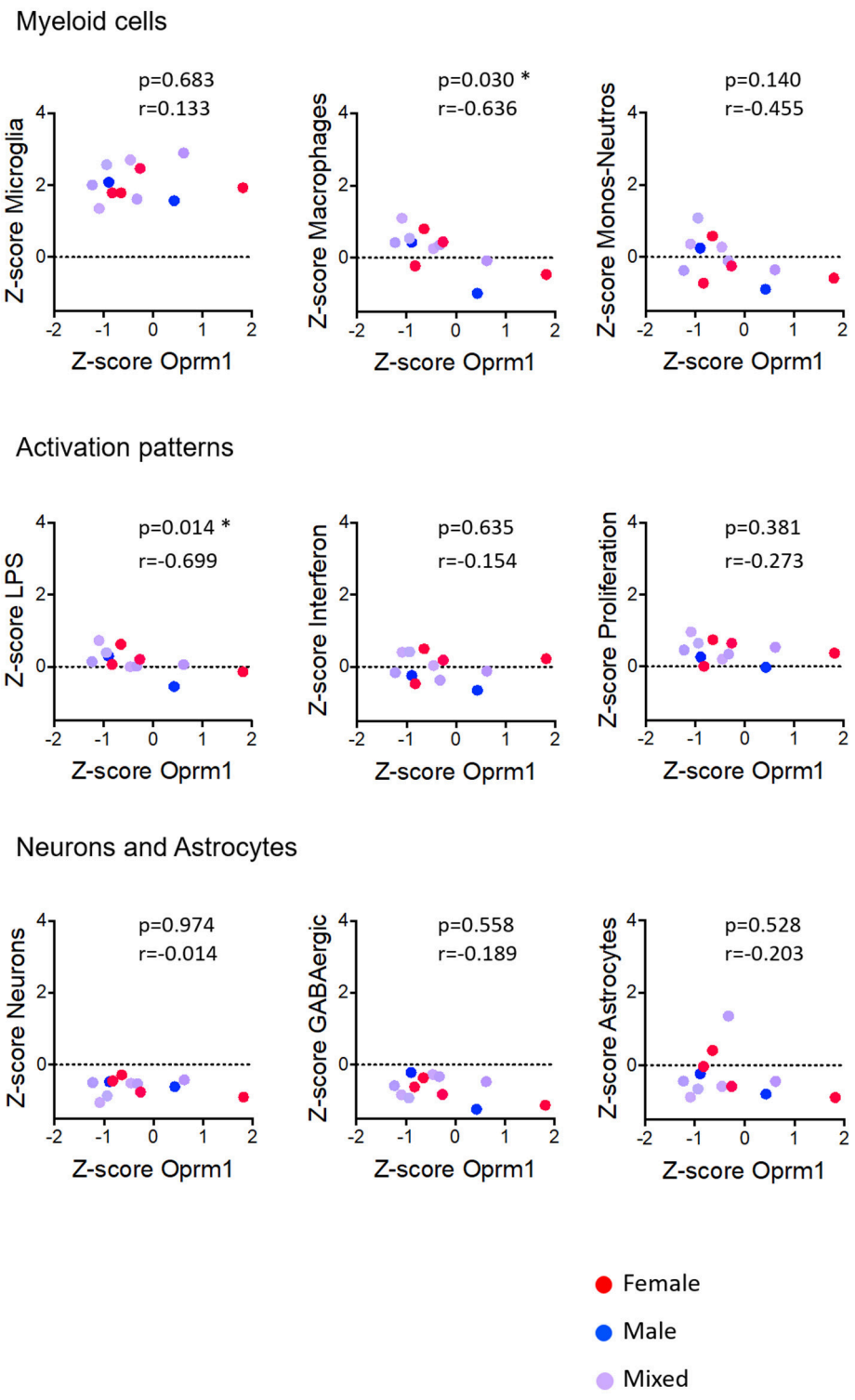

FIGURE 5 | Correlation of Oprm1 gene expression within the Myeloid cells, Activation patterns, and Neurons and Astrocytes gene clusters in mouse whole brain datasets. Oprm1 expression does not correlate with the microglia-related and the Monocyte-Neutrophil-related genes ($p > 0.05$, ns) but is significantly negatively correlated with the Macrophages-related genes ($r = 0.636$; $^*p = 0.03$). Oprm1 expression has a negative correlation ($r < 0$) with all the Activation patterns-, Neurons, and Astrocytes gene clusters, which is only statistically significant for the LPS-related gene module ($^*p = 0.014$). All correlations were determined with the Spearman's correlation test.

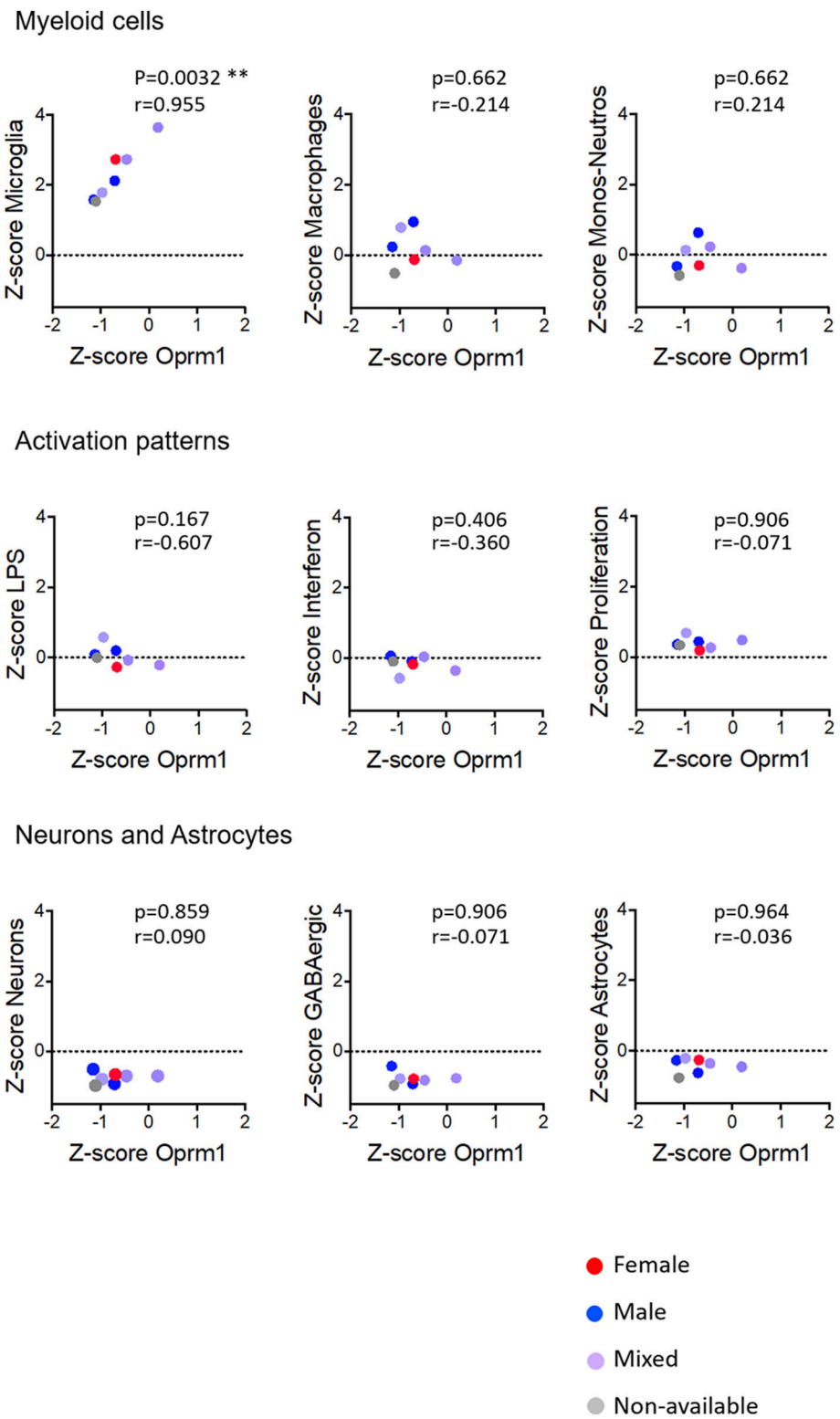

FIGURE 6 | Correlation of Oprm1 gene expression within the Myeloid cells, Activation patterns, and Neurons and Astrocytes gene clusters in mouse cortex datasets. Oprm1 expression is positively and significantly correlated with the microglia-related genes ($r = 0.955$; $^{**}p = 0.0032$) but does not correlate with the Macrophages- and Monocyte-Neutrophils-related genes ($r \leq 0.214$; $p > 0.05$, ns). Oprm1 expression does not correlate with any of the Activation patterns- ($r < 0$; $p > 0.05$, ns), Neurons or Astrocytes gene clusters ($r \leq 0.09$; $p > 0.05$, ns). All correlations were determined with the Spearman's correlation test.

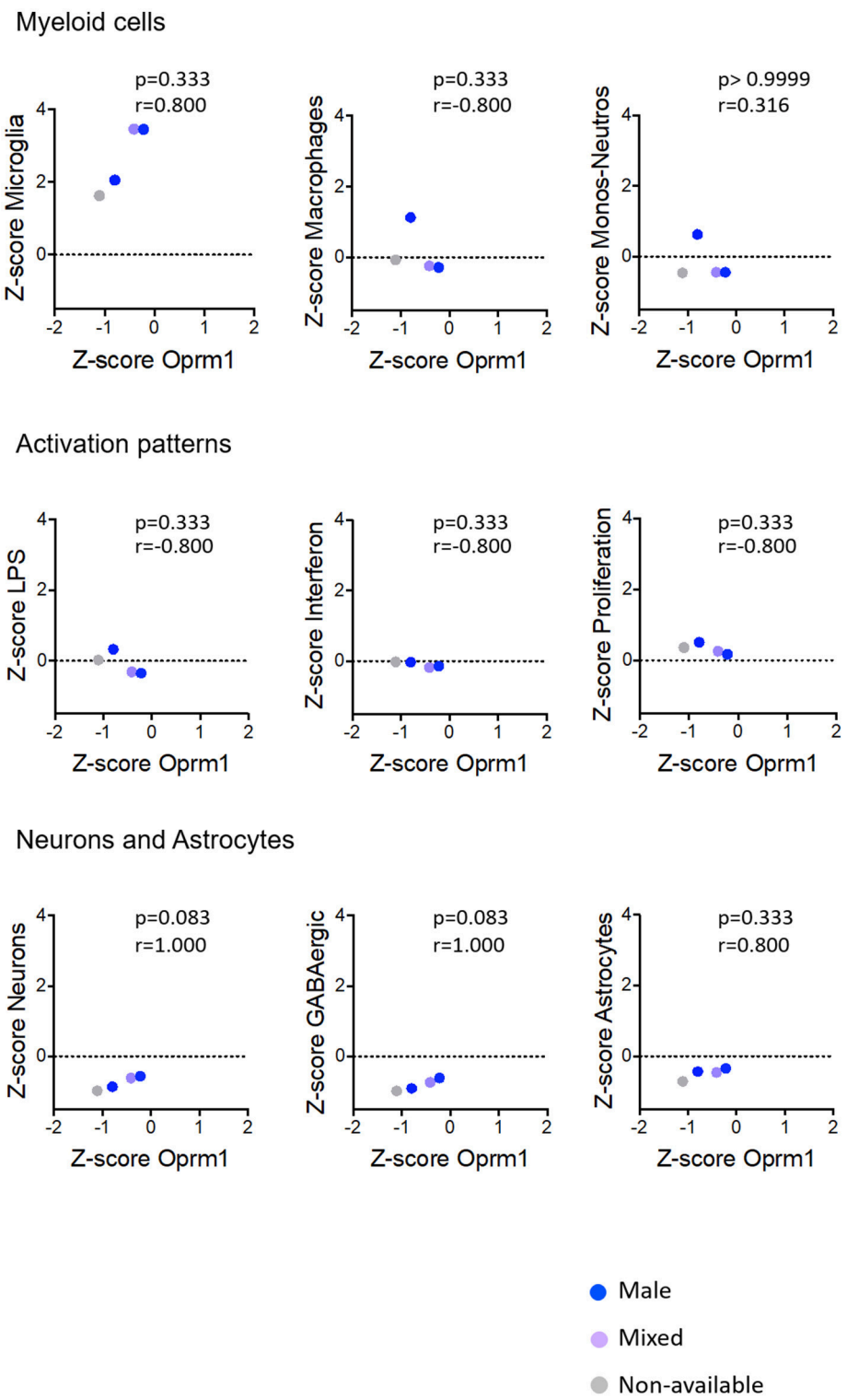

FIGURE 7 | Correlation of Oprm1 gene expression within the Myeloid cells, Activation patterns and Neurons and Astrocytes gene clusters in mouse hippocampus datasets. There is no correlation between Oprm1 expression and all the gene clusters investigated in the mouse hippocampus datasets ($p > 0.05$, ns). Although Oprm1 expression seems to have a strongly positive correlation with the Neurons and Astrocytes gene clusters ($r = 1$ and $r = 0.800$, respectively) this is not statistically significant ($p > 0.05$, ns). All correlations were determined with the Spearman's correlation test.

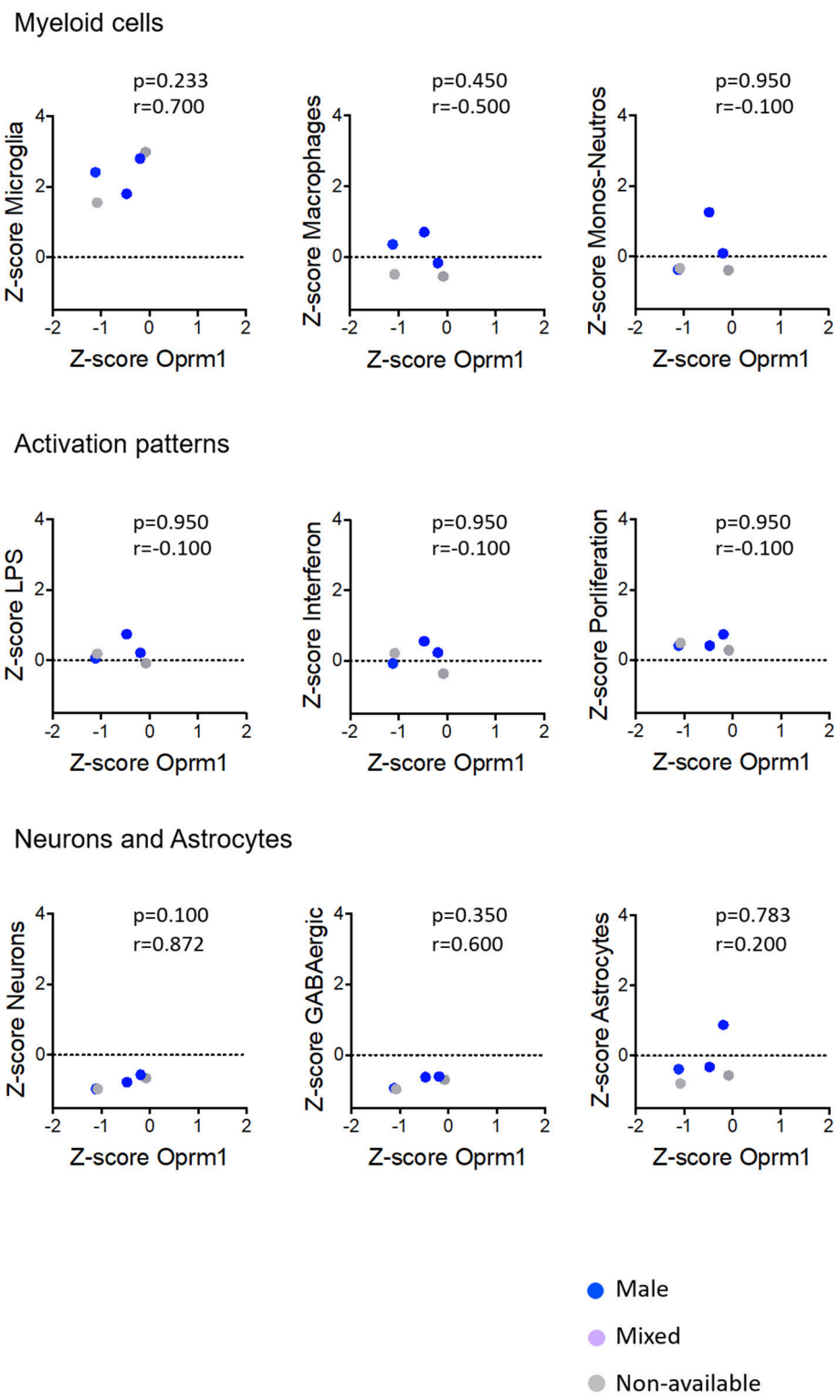

FIGURE 8 | Correlation of Oprm1 gene expression within the Myeloid cells, Activation patterns, and Neurons and Astrocytes gene clusters in rodent spinal cord datasets. Oprm1 expression has a positive correlation ($r = 0.700$) with the Microglia-related genes but which is not statistically significant ($p > 0.05$, ns). Oprm1 expression is negatively correlated ($r < 0$) with Macrophages, Monocytes-Neutrophils- related genes, and Activation patterns gene clusters, but this is not statistically significant ($r < 0$; $p > 0.05$, ns). Oprm1 expression has a positive correlation with the Neurons ($r > 0.5$) which is not statistically significant ($p > 0.05$, ns). There is no correlation between Oprm1 expression and Astrocytes gene clusters ($r = 0.2$; $p = 0.783$, ns). All correlations were determined with the Spearman's correlation test.

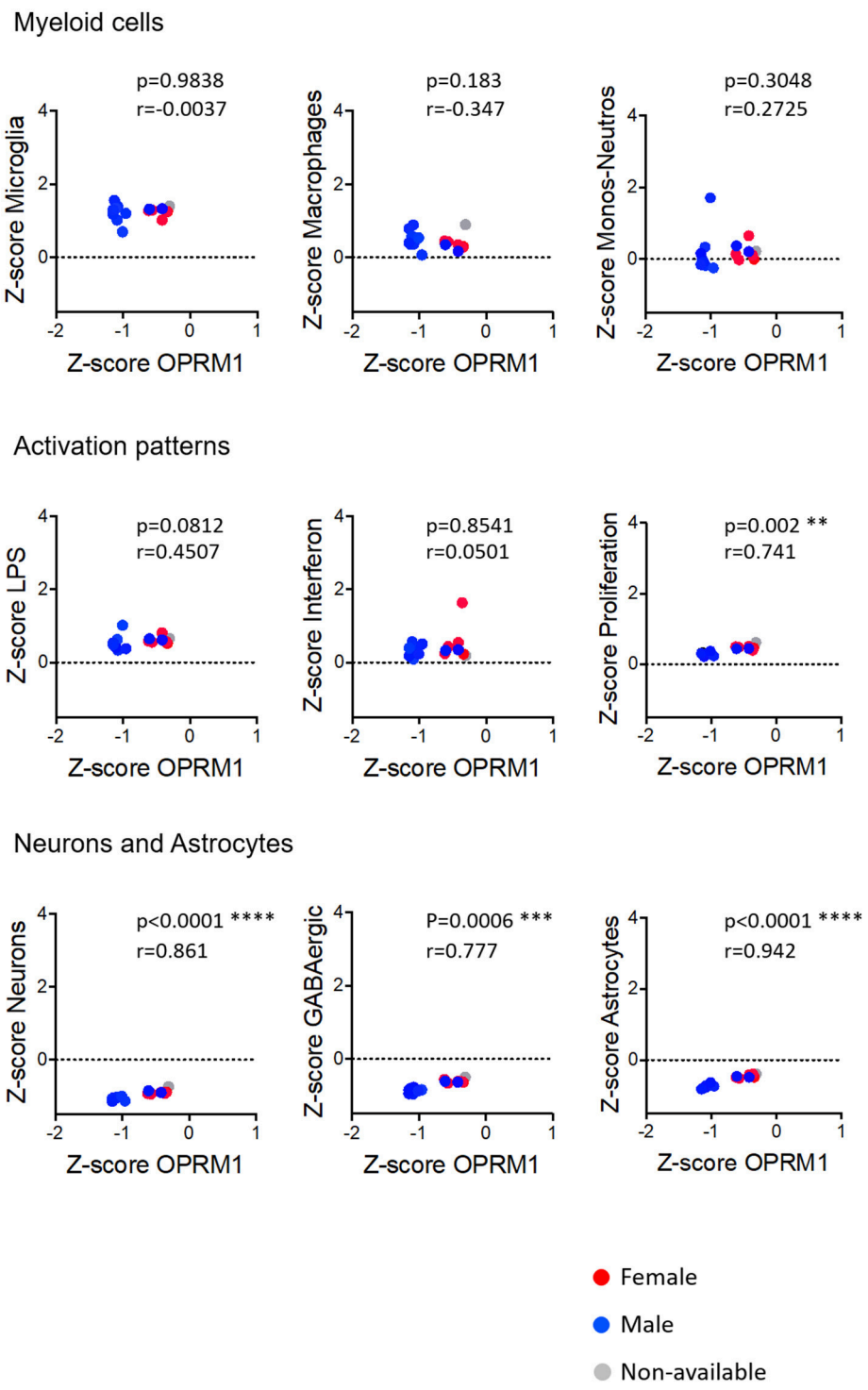

FIGURE 9 | Correlation of Oprm1 gene expression within the Myeloid cells, Activation patterns and Neurons and Astrocytes gene clusters in human cortex datasets. Oprm1 expression had a negative correlation with Microglia- and Macrophage-related genes ($r < 0$) and a positive correlation with the Monocytes-Neutrophils-related genes which were not statistically significant ($p > 0.05$; ns). Within the Activation patterns-related genes, Oprm1 expression has a weak positive correlation ($r < 0.5$) which is not statistically significant for the LPS-related genes ($p > 0.5$). There is a statistically significant positive correlation between Oprm1 expression and the Proliferation-related genes ($r = 0.741$; $^{**}p = 0.002$). There is a statistically significant positive correlation between Oprm1 expression and the GABAergic- ($r > 0.5$; $^{***}p = 0.0006$), Neurons-, and Astrocytes-related genes ($r > 0.5$; $^{****}p < 0.0001$).

gene cluster in the mouse whole brain datasets (**Figure 5**) and a positive correlation between *OPRM1* and the Proliferation-related gene level for the human cortex datatsets (**Figure 9**). No correlation was found between *Oprm1* level and the Interferon cluster in any of the dataset studied (**Figures 5–9**).

Neurons and Astrocytes

The analysis for relationships between *Oprm1* expression and expression of the Neuron and GABAergic neuronal signatures indicated no correlation between *Oprm1* and these modules for whole mouse brain, cortex, hippocampus, and rodent spinal cord datasets (**Figures 5–8**). The *p* and r values obtained from the analysis of hippocampus and spinal cord data suggest that more datasets are required to increase the power of the correlation analysis. For the human cortex datasets, a positive correlation was found between *Oprm1* expression and expression of the Neuronal and Astrocyte gene modules (**Figure 9**).

MOR Expression in CX3cr1-eGFP Microglia

MOR Expression in Brain and Spinal Cord

The **Figures 10**, **11** show that Cx3cr1-eGFP-positive cells display the typical microglia morphology with no neuronal, astrocytic or lymphocytic shapes, confirming previous findings on colocalization of CX3CR1-eGFP with the CD11b or Iba-1 microglia and macrophage markers (37, 74, 75) but not neuronal, astrocytic, or oligodendrocytic markers (76, 77) in the brain. MOR is expressed in microglia of different brain regions in female and male Cx3cr1-eGFP-MOR-mCherry mice, in the Frontal Cortex (FCx), Nucleus Accumbens (NAcc), Central Amygdala (CeA), Ventral Tegmental Area (VTA), and Periaqueductal Gray (PAG) (**Figure 10**). The percent of microglia expressing MOR-mCherry protein ranges from 35.4 ± 4.1 to 51.6 ± 3.5 (**Supplementary Table 5**). The percentage of MOR-positive microglia was calculated in the FCx, NAcc, CeA, and PAG of female and male brains. The VTA showed a lower percentage of microglia containing MOR in females than in males which was statistically significant ($p = 0.0037$) (**Supplementary Table 5**).

In spinal cord dorsal horn, MOR is expressed in microglia of both female and male Cx3cr1-eGFP-MOR-mCherry mice (**Figures 11A,B**, **Supplementary Video 1**, **Supplementary Table 5**). The percentage of microglia expressing MOR ranged from 37.1 ± 2.7 to 44.5 42.4 ± 2.6 in cervical, thoracic and lumbar segments, with no statistical difference between the segments (**Supplementary Table 5**). There were no statistically significant differences in the percentages of MOR-expressing microglia between males and females with 36.9 ± 2.3% and 36 ± 2.4%, 38.8 ± 2.1% and 42.4 ± 2.5%, and 37.1 ± 2.7% and 39.5 ± 2.3% in cervical, thoracic, and lumbar spinal segments, respectively (**Supplementary Table 5**).

MOR-Golgi-lysosome Localization

To specifically localize MOR within the intracellular compartments of spinal microglia, we labeled Cis-Golgi, trans-Golgi, and lysosomes with GM130, TGN38, and Lamp1 antibodies, respectively. Microglial MOR co-localized with GM-130 (**Figure 11C**) and TGN38 (**Figure 11D**) but not with Lamp1 (**Figure 11E**). Together, this indicates that microglial MOR is localized in cis and trans-Golgi rather than lysosomes.

DISCUSSION

Altogether the transcriptomics and imaging data show MOR expression in microglia of all analyzed mammalian brain areas and the spinal cord. The expression of Oprm1 transcripts and MOR protein in microglia was shown previously by using RT-qPCR or antibody based techniques, respectively (9–14, 16). Although two papers found no expression of MOR in microglia by using the same above-mentioned techniques (21, 22), a link between microglial activation and MOR expression and function was demonstrated, mostly in *in vitro* studies on murine primary microglia cultures. Low concentrations of morphine and DAMGO (a selective MOR agonist) activated rat microglia and this activation was blocked by the MOR-selective antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP) (15). Morphine increased Toll-like Receptors (TLRs) expression which was attenuated by the MOR-selective antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP). Additionally, this morphine effect was abolished in cultured microglia of MOR knockout (KO) mice (78). In murine microglia, morphine increased the expression of cytokines and activated the PKCe-Akt-ERK1/2 kinase pathway and these effects were inhibited by *Oprm1* RNA silencing as well as by CTAP (14, 16). A low dose of morphine enhanced NF-kB activity via MOR that was blocked by CTAP while a morphine high dose triggered a TLR4-mediated non-opioid response (18). One study has shown that, *in vivo,* the morphine-induced increase in microglial P2X4R ATP-receptor was blocked by (-)-naloxone but not by its (+)-naloxone enantiomer, implicating microglial MOR rather than TLR4 in OIH (17). MOR implication in OIH has been confirmed at the genetic level with the use of global MOR KO mice (21, 79). Regarding the implication of TLR4 in OIH, the study of TLR4 KO mice has led to controversial results as two papers showed that OIH was abolished in TLR4 KO mice while two others showed that it was maintained (6, 7). Moreover, TLR4-independent activation of NF-kB by morphine was shown together with increased Tumor Necrosis Factor alpha (TNFα), suggesting TNFα signaling as a novel pathway for morphine action on microglia (80). However, whether this effect of morphine on microglia activation occurs directly through the microglial MOR or indirectly via a neuronal MOR still remains to be determined and could be addressed in upcoming studies such as with conditional MOR-KO targeted at microglia or other cell types.

The detection of *Oprm1/OPRM1* in 27 out of 30 rodent datasets and 16 out of 17 human whole genome transcriptomics datasets issued from microglia *ex vivo* validates the presence of MOR transcripts in microglia. From this analysis, we could conclude that in the mammalian central nervous system, microglia of the whole brain, cerebral cortex, hippocampus, and spinal cord all contain *Oprm1/OPRM1* messenger. Single-cell RNA-seq currently allows detection of only a few thousand

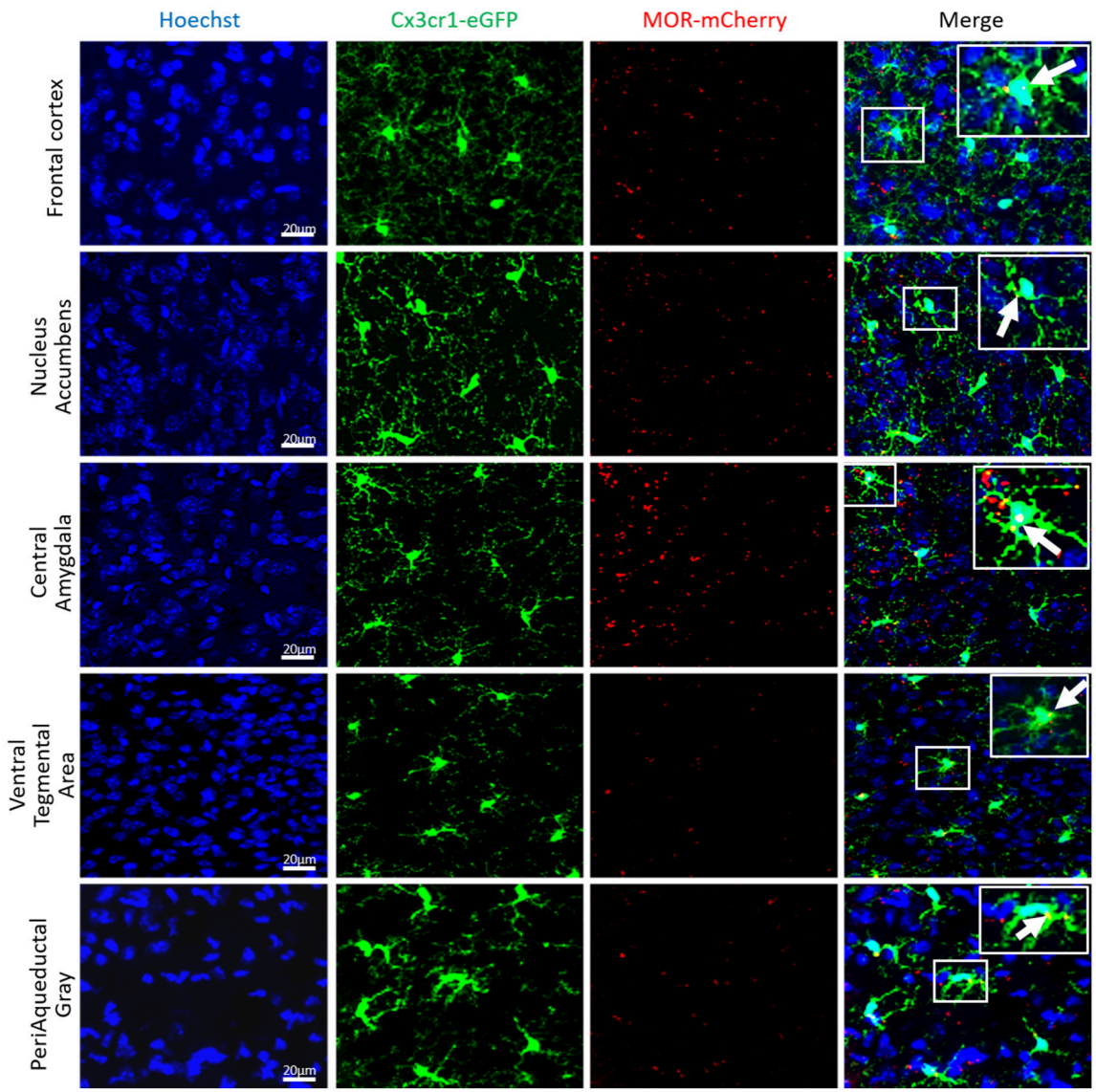

FIGURE 10 | MOR is expressed in mouse brain microglia. Photomicrographs from coronal sections of Frontal cortex (FCx), Nucleus Accumbens (NAcc), Central Amygdala (CeA), Ventral Tegmental Area (VTA), and Periaqueductal Gray (PAG) of Cx3Cr1-eGFP-MOR-mCherry mice show the colocalization (orange) of CX3CR1-eGFP (green), and MOR-mCherry (red) denoted by white arrows. Scale bar = 20 µm.

of the most highly expressed transcripts within a specimen. Therefore, this approach is used for determining differential gene expression among the highly expressed genes and cannot analyze complete transcriptomes (81). As *Oprm1* is overall expressed around or below the median within the reported transcriptomes, the transcripts would not be contained among the few thousands of highly expressed genes detected by single-cell RNA-seq studies. This lack of expression is found also in the Mars-seq datasets (58).

The analysis of the relationships between expression of *Oprm1* and of the different gene clusters led to interesting insights. First, **Figures 5–9** show that the z-scores for Microglia-related gene expression are globally above 1 while z-scores for neurons or astrocytes were below 0, indicating a significant enrichment in microglia compared to other cell types. The positive correlation observed between *Oprm1* and the Microglia module in mouse cortex further supports the presence of *Oprm1* expression in microglia. Such a correlation was not found for the mouse whole brain or spinal cord datasets, possibly reflecting the diverse cellular heterogeneity in these tissues compared to a defined region like the cortex. Indeed, the whole brain comprises many distinct regions, and microglia from other brain regions implicated in opioid response or pain, such as VTA, NAcc, or PAG, should be further profiled at the molecular level in order to characterize the global expression of microglial *Oprm1*. Neurons, GABAergic neurons and Astrocytes gene clusters correlate with *OPRM1* in the human cortex datasets but not in datasets

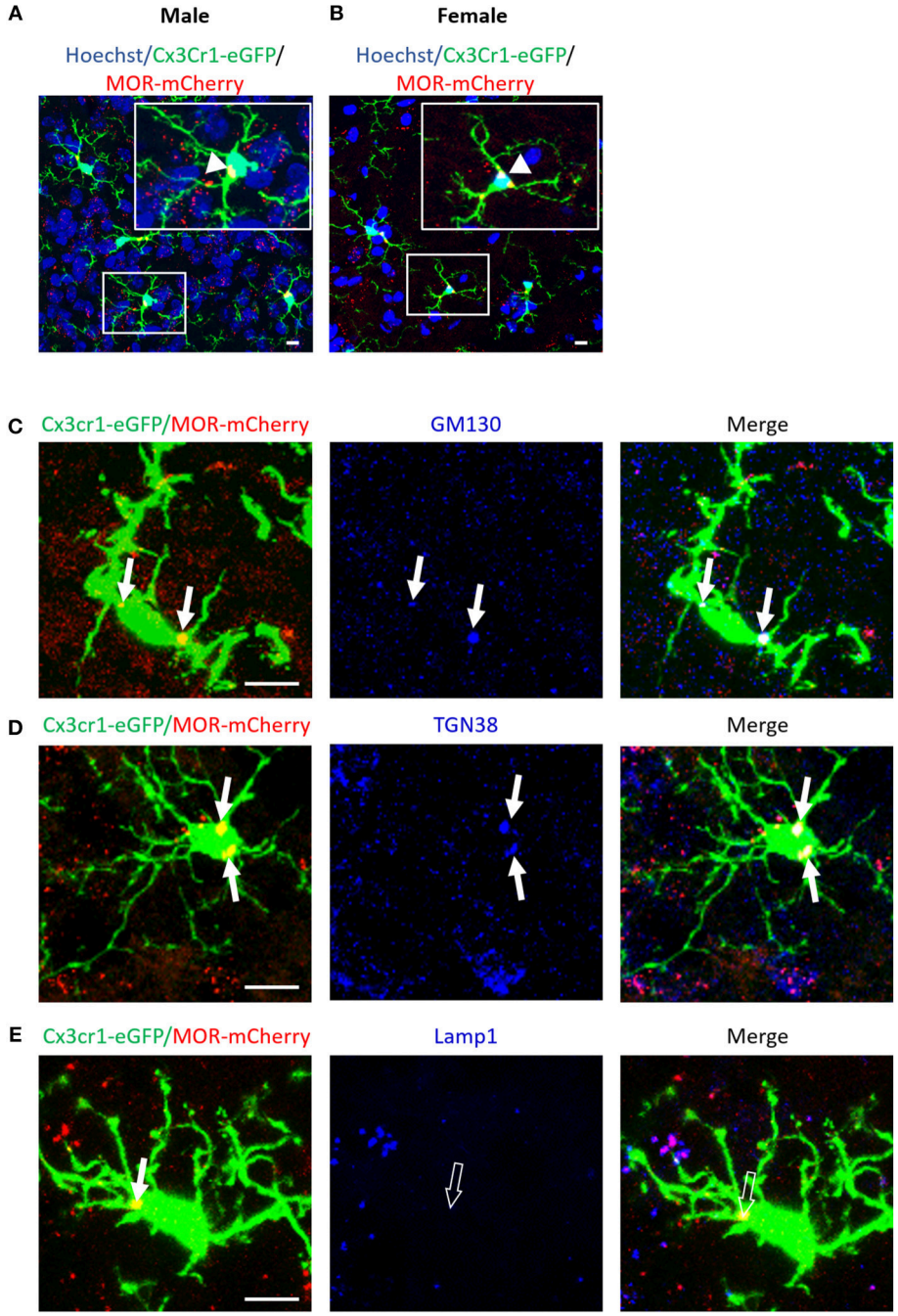

FIGURE 11 | MOR is expressed in microglia of the mouse spinal cord. **(A,B)** Photomicrographs from transverse sections of the lumbar spinal cord, at the level of the dorsal horn, demonstrate a colocalization (orange) of MOR-mCherry (red) with CX3CR1-eGFP-positive microglia (green) in adult male **(A)** and female **(B)** Cx3Cr1-eGFP-MOR-mCherry mice, indicated by white arrow heads. Scale bar = 10 μm. **(C–E)** High magnification photomicrographs of CX3CR1-eGFP positive microglia (green) that colocalize (orange) with MOR-mCherry (red) are indicated with white arrows. Scale bar = 10 μm. **(C)** Anti-GM130 labeling (blue) colocalizes (pink-white) with MOR-mCherry in the cis-Golgi of CX3CR1-eGFP positive microglia, indicated with white arrows. **(D)** Anti-TGN38 labeling (blue) colocalizes (pink-white) with MOR-mCherry in the trans-Golgi of CX3CR1-eGFP positive microglia, indicated with white arrows. **(E)** Anti-Lamp1 labeling (blue) of lysosomes does not colocalize with MOR-mCherry in CX3CR1-eGFP positive microglia, indicated with open arrows. The white (closed) arrow indicate colocalization of CX3CR1-eGFP with MOR-mCherry (orange) alone.

from rodents. A significantly higher content of neuronal and astrocyte gene transcripts in FACS-isolated microglia compared to cultured microglia has already been reported, and was suggested to result from synapse-related mRNAs that were phagocytosed by the microglia (19). The same phenomenon may contribute to the positive correlation found between *OPRM1*

and Neurons and Astrocytes modules in the human datasets, despite their very low expression levels in the datasets. In addition to the transcriptomic analysis, imaging of the Cx3cr1-eGFP-MOR-mCherry reporter line confirmed MOR expression by microglia of the FCx, NAcc, CeA, VTA, PAG, and spinal cord. Furthermore, co-localization of microglial MOR with cis- and trans-Golgi compartments but not lysosomes strongly supports the protein synthesis and post-translational processing of MOR within microglial cells.

Importantly, as discussed by Richard M Ransohoff, microglia polarization into M1 and M2 subtypes appears too simplistic and does not reflect the diversity of microglia phenotypes or profiles induced by internal and external environmental factors that include central nervous system region, sex, age, and health state revealed by high-throughput analyses (82). Some reports on microglia transcriptomics have defined co-regulated gene subsets or modules to allow for classification of microglia into subtypes expressing high levels of specific gene modules. We analyzed the 47 microglia datasets and established three main activation module profiles related to Interferon, LPS and Proliferation, in relation to *Oprm1* expression. We found a lack of, or a poor correlation between *Orpm1* and these modules. There is a negative correlation between *Oprm1* and the LPS-related module in whole mouse brain datasets, and a positive correlation between *OPRM1* and the Proliferation-related module in human cortex datasets. These low correlations may be explained by the use of datasets from naive, non-stimulated rodents microglia in which the LPS-related, Interferon-related, and Proliferation related genes are expressed at the basal low level.

Several lines of evidence support a functional impact of MOR on microglia activity. Morphine increased spinal microglial p38 and extracellular receptor kinase (ERK) phosphorylation, leading to an augmented microglial production of proinflammatory cytokines, and other pronociceptive molecules as well as their receptors and hence to increased pain (15). Morphine also enhances the activity of microglial ATP-gated P2X7R and P2X4R receptors in the spinal cord, leading to increased Brain-derived neurotrophic factor (BDNF) release from these cells. This in turn downregulates K-Cl co-transporter KCC2 expression in GABAergic neurons enhancing their excitability and thus increased pain (8, 17, 20, 83). Other spinal microglial-mediated responses for morphine analgesic tolerance and hyperalgesia involve large conductance Ca2+-activated K+ channels (84) and genes of the DPA12/TREM2 and potassium intermediate/small conductance calcium activated channels KCNN4 pathways (62). Furthermore, bidirectional cross-talks between chemokine receptors and opioid receptors mediate opioid analgesic tolerance (85). Also, microglial pannexin-1 channels attenuate morphine withdrawal in rodents but are not involved in opioid analgesic tolerance, thus identifying pannexin-1 as a novel mediator of specific morphine actions in microglia (86, 87). Peripheral nerve injury leads to microglial activation in several brain structures involved in pain sensation or emotion including the VTA and amygdala (88). Within the VTA, chronic pain, chronic opiate treatment, and opioid withdrawal induce a dysregulation of BDNF in microglia that impacts on GABAergic neurons and disrupts the dopaminergic pathway leading to defects in reward (89, 90). Therefore, specific regulations in microglia appear to mediate opioid reward and link chronic pain and opioid dependence (91, 92) that may be mediated by MOR activity within different subsets of microglia.

With the use of the Cx3cr1-eGFP-MOR-mCherry reporter mouse, we have shown that, overall, 35–51% microglia express MOR, suggesting that the receptor may be expressed by a particular microglial subset and that only MOR-expressing microglia will respond to opioid treatment. Future studies are necessary to address how MOR expression within different microglial subsets may impact on their functionality, under physiological, and pathological conditions. The specific ablation of the Oprm1 gene in the different subtypes of resting or activated microglia by targeted Cre/Lox technology would require the identification of unique and very selective gene markers for each of these subtypes and the generation of the corresponding Cre mouse lines. In the same line, optogenetic approaches allow the evaluation of opioid signaling and behavior elicited by specific spatiotemporal patterns of MOR activation in neurons (93) that could be applied to microglia. Another aspect of MOR activation is ligand-biased signaling where different ligands stimulate differential cell responses (94, 95). We report an intracellular localization of MOR within the Golgi apparatus of microglia that suggests a local production of MOR in these cells. MOR function in microglia could be investigated by novel biosensors that have been assayed on transfected cell lines and primary neuronal cultures, revealing a cell localization bias for opioid receptor activation by endogenous, and exogenous opioids (96).

As sex differences are an important factor for microglial contribution to pain (35, 36), we investigated MOR expression in microglia from adult females and males. The transcriptomic datasets did not contain enough numbers per sex for a full determination of sex effect, see **Figures 5–9**. The analysis of brain sections from the Cx3cr1-eGFP-MOR-mCherry mice indicated a comparable ratio of microglia containing MOR in female and male animals in different brain and spinal regions, except in the VTA where significantly lower percentages of microglia expressed MOR in females than males. It would therefore be interesting to determine whether microglial MOR would influence the regulatory mechanisms in the dopaminergic system in females as has been previously described for males in the VTA (89, 90). Also, sex differences in microglia activity were found in the PAG where a significantly higher microglial activation profile was found in females compared to males (97). In the spinal cord, the microglial P2X4R-mediated hyperalgesia showed sexual dimorphism (98). Altogether this suggests that transcriptomic profiles of microglia in chronic pain models or following chronic drug administration may help to identify *Oprm1*-linked mechanisms associated with alterations in microglial functions. However, sex differences may exert a weaker influence on morphine-induced analgesic tolerance and hyperalgesia than on other forms of chronic pain like neuropathic pain. Indeed, these morphine adverse effects occurred similarly in male and female mice (79) and no clear impact was shown on OIH (6).

The question of the factors influencing the opioid effects on microglia physiology should be further explored in the future by taking into account the genetic diversity, sex differences, and

pathological states (26). The importance of genetic background on opioid tolerance, hyperalgesia (6), and misuse (99) has been well-established in both clinical and preclinical settings. The rodent datasets analyzed in the present study were from mouse of C57BL/6 genetic background except four datasets issued from other strains, genetic background, or species. In the future, the study of *Oprm1* expression may be broadened to a larger number of rodent strains to get a more comprehensive view. In addition, all human microglia datasets were collected from cortical areas and there is a need for studies on microglia from spinal cord and other brain regions involved in pain and addiction. The correlations between rodent and human transcriptomes may be studied as well. Galatro et al. have shown an overlap between the two species, with similar microglia core genes expressed by both human and mouse microglia including the main markers CX3CR1, P2YR12, and Itgam. There were also dissimilarities due to differences in environment and medical condition as mouse microglia were isolated from healthy mice while human donors suffered from pathologies that led to their death, potentially influencing microglial genes regulation, and expression (65).

Neuroinflammatory mechanisms underlie opioid-induced pain sensitization (6, 7) comprising of reciprocal signaling between neurons and neuroinflammatory cells including microglia (100). OIH, together with abuse liability, contribute to the opioid crisis that is epidemic in North America and becoming prevalent in Europe as well (101). Therefore, the elucidation of mechanisms involved in opioid deleterious effects would help to develop new strategies for safer analgesic clinical intervention. Altogether, this work on microglial *Oprm1* expression and previous studies open the way to the exploration of the role of microglial MOR in response to opiates, in particular to analgesic tolerance, OIH, and physical dependence elicited by their chronic use.

DATA AVAILABILITY STATEMENT

Data are available on request.

AUTHOR CONTRIBUTIONS

TM analyzed data and contributed to the writing of the manuscript. EA, DD, NM, and CG-R designed experiments, analyzed data and contributed to the writing of the manuscript. DR performed experiments. DM provided the MOR-mCherry mouse line and contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2018.00726/full#supplementary-material

REFERENCES

1. Gaveriaux-Ruff C. Opiate-induced analgesia: contributions from mu, delta and kappa opioid receptors mouse mutants. *Curr Pharm Des.* (2013) 19:7373–81. doi: 10.2174/13816128194214010516372 7
2. Maldonado R, Banos JE, Cabanero D. Usefulness of knockout mice to clarify the role of the opioid system in chronic pain. *Br J Pharmacol.* (2018) 175:2791–808. doi: 10.1111/bph.14088
3. Siuda ER, Carr R, III, Rominger DH, Violin JD. Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. *Curr Opin Pharmacol.* (2017) 32:77–84. doi: 10.1016/j.coph.2016.11.007
4. Gunther T, Dasgupta P, Mann A, Miess E, Kliewer A, Fritzwanker S, et al. Targeting multiple opioid receptors - improved analgesics with reduced side effects? *Br J Pharmacol.* (2018) 175:2857–68. doi: 10.1111/bph.13809
5. Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. *Science* (2016) 354:572–7. doi: 10.1126/science.aaf8924
6. Roeckel LA, Le Coz GM, Gaveriaux-Ruff C, Simonin F. Opioid-induced hyperalgesia: Cellular and molecular mechanisms. *Neuroscience* (2016) 338:160–82. doi: 10.1016/j.neuroscience.2016.06.029
7. Rivat C, Ballantyne J. The dark side of opioids in pain management: basic science explains clinical observation. *Pain Rep.* (2016) 1:e570. doi: 10.1097/PR9.0000000000000570
8. Leduc-Pessah H, Weilinger NL, Fan CY, Burma NE, Thompson RJ, Trang T. Site-specific regulation of P2X7 receptor function in microglia gates morphine analgesic tolerance. *J Neurosci.* (2017) 37:10154–72. doi: 10.1523/JNEUROSCI.0852-17.2017
9. Chao CC, Hu S, Shark KB, Sheng WS, Gekker G, Peterson PK. Activation of mu opioid receptors inhibits microglial cell chemotaxis. *J Pharmacol Exp Ther.* (1997) 281:998–1004.
10. Calvo CF, Cesselin F, Gelman M, Glowinski J. Identification of an opioid peptide secreted by rat embryonic mixed brain cells as a promoter of macrophage migration. *Eur J Neurosci.* (2000) 12:2676–84. doi: 10.1046/j.1460-9568.2000.00145.x
11. Turchan-Cholewo J, Dimayuga FO, Ding Q, Keller JN, Hauser KF, Knapp PE, et al. Cell-specific actions of HIV-Tat and morphine on opioid receptor expression in glia. *J Neurosci Res.* (2008) 86:2100–10. doi: 10.1002/jnr.21653
12. Mika J, Popiolek-Barczyk K, Rojewska E, Makuch W, Starowicz K, Przewlocka B. Delta-opioid receptor analgesia is independent of microglial

activation in a rat model of neuropathic pain. *PLoS ONE* (2014) 9:e104420. doi: 10.1371/journal.pone.0104420
13. Popiolek-Barczyk K, Piotrowska A, Makuch W, Mika J. Biphalin, a dimeric enkephalin, alleviates LPS-induced activation in rat primary microglial cultures in opioid receptor-dependent and receptor-independent manners. *Neural Plast.* (2017) 2017:3829472. doi: 10.1155/2017/3829472
14. Merighi S, Gessi S, Varani K, Fazzi D, Stefanelli A, Borea PA. Morphine mediates a proinflammatory phenotype via mu-opioid receptor-PKCvarepsilon-Akt-ERK1/2 signaling pathway in activated microglial cells. *Biochem Pharmacol.* (2013) 86:487–96. doi: 10.1016/j.bcp.2013.05.027
15. Horvath RJ, DeLeo JA. Morphine enhances microglial migration through modulation of P2X4 receptor signaling. *J Neurosci.* (2009) 29:998–1005. doi: 10.1523/JNEUROSCI.4595-08.2009
16. Merighi S, Gessi S, Varani K, Fazzi D, Mirandola P, Borea PA. Cannabinoid CB(2) receptor attenuates morphine-induced inflammatory responses in activated microglial cells. *Br J Pharmacol.* (2012) 166:2371–85. doi: 10.1111/j.1476-5381.2012.01948.x
17. Ferrini F, Trang T, Mattioli TA, Laffray S, Del'Guidice T, Lorenzo LE, et al. Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. *Nat Neurosci.* (2013) 16:183–92. doi: 10.1038/nn.3295
18. Gessi S, Borea PA, Bencivenni S, Fazzi D, Varani K, Merighi S. The activation of mu-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia. *FEBS Lett.* (2016) 590:2813–26. doi: 10.1002/1873-3468.12313
19. Beutner C, Linnartz-Gerlach B, Schmidt SV, Beyer M, Mallmann MR, Staratschek-Jox A, et al. Unique transcriptome signature of mouse microglia. *Glia* (2013) 61:1429–42. doi: 10.1002/glia.22524
20. Horvath RJ, Romero-Sandoval EA, De Leo JA. Inhibition of microglial P2X4 receptors attenuates morphine tolerance, Iba1, GFAP and mu opioid receptor protein expression while enhancing perivascular microglial ED2. *Pain* (2010) 150:401–13. doi: 10.1016/j.pain.2010.02.042
21. Corder G, Tawfik VL, Wang D, Sypek EI, Low SA, Dickinson JR, et al. Loss of mu opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nat Med.* (2017) 23:164–73. doi: 10.1038/nm.4262
22. Kao SC, Zhao X, Lee CY, Atianjoh FE, Gauda EB, Yaster M, et al. Absence of mu opioid receptor mRNA expression in astrocytes and microglia of rat spinal cord. *Neuroreport* (2012) 23:378–84. doi: 10.1097/WNR.0b013e3283522e1b
23. Denk F. Don't let useful data go to waste. *Nature* (2017) 543:7. doi: 10.1038/543007a
24. Sousa C, Biber K, Michelucci A. Cellular and molecular characterization of microglia: a unique immune cell population. *Front Immunol.* (2017) 8:198. doi: 10.3389/fimmu.2017.00198
25. Crotti A, Ransohoff RM. Microglial physiology and pathophysiology: insights from genome-wide transcriptional profiling. *Immunity* (2016) 44:505–15. doi: 10.1016/j.immuni.2016.02.013
26. Hirbec H, Noristani HN, Perrin FE. Microglia responses in acute and chronic neurological diseases: what microglia-specific transcriptomic studies taught (and did Not Teach) us. *Front Aging Neurosci.* (2017) 9:227. doi: 10.3389/fnagi.2017.00227
27. Avey D, Sankararaman S, Yim AKY, Barve R, Milbrandt J, RDM. Single-Cell RNA-Seq uncovers a robust transcriptional response to morphine by glia. *Cell Rep*. (2018) 24:3619–29. doi: 10.1016/j.celrep.2018.08.080
28. Prinz M, Erny D, Hagemeyer N. Ontogeny and homeostasis of CNS myeloid cells. *Nat Immunol.* (2017) 18:385–92. doi: 10.1038/ni.3703
29. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* (2013) 38:79–91. doi: 10.1016/j.immuni.2012.12.001
30. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol.* (2000) 20:4106–14. doi: 10.1128/MCB.20.11.4106-4114.2000
31. Erbs E, Faget L, Scherrer G, Matifas A, Filliol D, Vonesch JL, et al. A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct Funct.* (2015) 220:677–702. doi: 10.1007/s00429-014-0717-9
32. Mogil JS. Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nat Rev Neurosci.* (2012) 13:859–66. doi: 10.1038/nrn3360
33. Sorge RE, Mapplebeck JC, Rosen S, Beggs S, Taves S, Alexander JK, et al. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci.* (2015) 18:1081–3. doi: 10.1038/nn.4053
34. Taves S, Berta T, Liu DL, Gan S, Chen G, Kim YH, et al. Spinal inhibition of p38 MAP kinase reduces inflammatory and neuropathic pain in male but not female mice: Sex-dependent microglial signaling in the spinal cord. *Brain Behav Immun.* (2016) 55:70–81. doi: 10.1016/j.bbi.2015.10.006
35. Sorge RE, Totsch SK. Sex differences in pain. *J Neurosci Res.* (2017) 95:1271–81. doi: 10.1002/jnr.23841
36. Mapplebeck JC, Beggs S, Salter MW. Molecules in pain and sex: a developing story. *Mol Brain* (2017) 10:9. doi: 10.1186/s13041-017-0289-8
37. Kim I, Mlsna LM, Yoon S, Le B, Yu S, Xu D, et al. A postnatal peak in microglial development in the mouse hippocampus is correlated with heightened sensitivity to seizure triggers. *Brain Behav.* (2015) 5:e00403. doi: 10.1002/brb3.403
38. Voronova A, Yuzwa SA, Wang BS, Zahr S, Syal C, Wang J, et al. Migrating interneurons secrete fractalkine to promote oligodendrocyte formation in the developing mammalian brain. *Neuron* (2017) 94:500–16.e9.
39. Tay TL, Savage JC, Hui CW, Bisht K, Tremblay ME. Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. *J Physiol.* (2017) 595:1929–45. doi: 10.1113/JP272134
40. Friedman BA, Srinivasan K, Ayalon G, Meilandt WJ, Lin H, Huntley MA, et al. Diverse brain myeloid expression profiles reveal distinct microglial activation states and aspects of alzheimer's disease not evident in mouse models. *Cell Rep.* (2018) 22:832–47. doi: 10.1016/j.celrep.2017.12.066
41. Wang Y, Cella M, Mallinson K, Ulrich JD, Young KL, Robinette ML, et al. TREM2 lipid sensing sustains the microglial response in an Alzheimer's disease model. *Cell* (2015) 160:1061–71. doi: 10.1016/j.cell.2015.01.049
42. Verheijden S, Beckers L, Casazza A, Butovsky O, Mazzone M, Baes M. Identification of a chronic non-neurodegenerative microglia activation state in a mouse model of peroxisomal beta-oxidation deficiency. *Glia* (2015) 63:1606–20. doi: 10.1002/glia.22831
43. Poliani PL, Wang Y, Fontana E, Robinette ML, Yamanishi Y, Gilfillan S, et al. TREM2 sustains microglial expansion during aging and response to demyelination. *J Clin Invest.* (2015) 125:2161–70. doi: 10.1172/JCI77983
44. Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci.* (2015) 18:965–77. doi: 10.1038/nn.4030
45. Szulzewsky F, Pelz A, Feng X, Synowitz M, Markovic D, Langmann T, et al. Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express Gpnmb and Spp1. *PLoS ONE* (2015) 10:e0116644. doi: 10.1371/journal.pone.0116644
46. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med.* (2013) 19:1264–72. doi: 10.1038/nm.3337
47. Bruttger J, Karram K, Wortge S, Regen T, Marini F, Hoppmann N, et al. Genetic cell ablation reveals clusters of local self-renewing microglia in the mammalian central nervous system. *Immunity* (2015) 43:92–106. doi: 10.1016/j.immuni.2015.06.012
48. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. *Cell* (2014) 159:1312–26. doi: 10.1016/j.cell.2014.11.018
49. Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB, et al. New tools for studying microglia in the mouse and human CNS. *Proc Natl Acad Sci USA* (2016) 113:E1738–46. doi: 10.1073/pnas.1525528113
50. Gosselin D, Link VM, Romanoski CE, Fonseca GJ, Eichenfield DZ, Spann NJ, et al. Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. *Cell* (2014) 159:1327–40. doi: 10.1016/j.cell.2014.11.023
51. Krasemann S, Madore C, Cialic R, Baufeld C, Calcagno N, El Fatimy R, et al. The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases. *Immunity* (2017) 47:566–81.e9. doi: 10.1016/j.immuni.2017.0

52. Zhao D, Mokhtari R, Pedrosa E, Birnbaum R, Zheng D, Lachman HM. Transcriptome analysis of microglia in a mouse model of Rett syndrome: differential expression of genes associated with microglia/macrophage activation and cellular stress. *Mol Autism.* (2017) 8:17. doi: 10.1186/s13229-017-0134-z
53. Orre M, Kamphuis W, Osborn LM, Melief J, Kooijman L, Huitinga I, et al. Acute isolation and transcriptome characterization of cortical astrocytes and microglia from young and aged mice. *Neurobiol Aging.* (2014) 35:1–14. doi: 10.1016/j.neurobiolaging.2013.07.008
54. Arumugam TV, Manzanero S, Furtado M, Biggins PJ, Hsieh YH, Gelderblom M, et al. An atypical role for the myeloid receptor Mincle in central nervous system injury. *J Cereb Blood Flow Metab.* (2017) 37:2098–111. doi: 10.1177/0271678X16661201
55. Grabert K, Michoel T, Karavolos MH, Clohisey S, Baillie JK, Stevens MP, et al. Microglial brain region-dependent diversity and selective regional sensitivities to aging. *Nat Neurosci.* (2016) 19:504–16. doi: 10.1038/nn.4222
56. Srinivasan K, Friedman BA, Larson JL, Lauffer BE, Goldstein LD, Appling LL, et al. Untangling the brain's neuroinflammatory and neurodegenerative transcriptional responses. *Nat Commun.* (2016) 7:11295. doi: 10.1038/ncomms11295
57. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci.* (2014) 34:11929–47. doi: 10.1523/JNEUROSCI.1860-14.2014
58. Matcovitch-Natan O, Winter DR, Giladi A, Vargas Aguilar S, Spinrad A, Sarrazin S, et al. Microglia development follows a stepwise program to regulate brain homeostasis. *Science* (2016) 353:aad8670. doi: 10.1126/science.aad8670
59. Chiu IM, Morimoto ET, Goodarzi H, Liao JT, O'Keeffe S, Phatnani HP, et al. A neurodegeneration-specific gene-expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model. *Cell Rep.* (2013) 4:385–401. doi: 10.1016/j.celrep.2013.06.018
60. Denk F, Crow M, Didangelos A, Lopes DM, McMahon SB. Persistent alterations in microglial enhancers in a model of chronic pain. *Cell Rep.* (2016) 15:1771–81. doi: 10.1016/j.celrep.2016.04.063
61. Noristani HN, Gerber YN, Sabourin JC, Le Corre M, Lonjon N, Mestre-Frances N, et al. RNA-Seq analysis of microglia reveals time-dependent activation of specific genetic programs following spinal cord injury. *Front Mol Neurosci.* (2017) 10:90. doi: 10.3389/fnmol.2017.00090
62. Jokinen V, Sidorova Y, Viisanen H, Suleymanova I, Tiilikainen H, Li Z, et al. Differential Spinal and supraspinal activation of glia in a rat model of morphine tolerance. *Neuroscience* (2018) 375:10–24. doi: 10.1016/j.neuroscience.2018.01.048
63. Zhang Y, Sloan SA, Clarke LE, Caneda C, Plaza CA, Blumenthal PD, et al. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron* (2016) 89:37–53. doi: 10.1016/j.neuron.2015.11.013
64. Gosselin D, Skola D, Coufal NG, Holtman IR, Schlachetzki JCM, Sajti E, et al. An environment-dependent transcriptional network specifies human microglia identity. *Science* (2017) 356:eaal3222. doi: 10.1126/science.aal3222
65. Galatro TF, Holtman IR, Lerario AM, Vainchtein ID, Brouwer N, Sola PR, et al. Transcriptomic analysis of purified human cortical microglia reveals age-associated changes. *Nat Neurosci.* (2017) 20:1162–71. doi: 10.1038/nn.4597
66. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* (2012) 9:676–82. doi: 10.1038/nmeth.2019
67. Darmanis S, Sloan SA, Zhang Y, Enge M, Caneda C, Shuer LM, et al. A survey of human brain transcriptome diversity at the single cell level. *Proc Natl Acad Sci USA* (2015) 112:7285–90. doi: 10.1073/pnas.1507125112
68. Zeisel A, Munoz-Manchado AB, Codeluppi S, Lonnerberg P, La Manno G, Jureus A, et al. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science* (2015) 347:1138–42. doi: 10.1126/science.aaa1934
69. Tasic B, Menon V, Nguyen TN, Kim TK, Jarsky T, Yao Z, et al. Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat Neurosci.* (2016) 19:335–46. doi: 10.1038/nn.4216
70. Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, Ulland TK, et al. A unique microglia type associated with restricting development of alzheimer's disease. *Cell* (2017) 169:1276–90.e17. doi: 10.1016/j.cell.2017.05.018
71. Mathys H, Adaikkan C, Gao F, Young JZ, Manet E, Hemberg M, et al. Temporal tracking of microglia activation in neurodegeneration at single-cell resolution. *Cell Rep.* (2017) 21:366–80. doi: 10.1016/j.celrep.2017.09.039
72. Saunders AME, Wysoker A, Goldman M, Krienen F, Bien E, Baum M, et al. Molecular diversity and specializations among the cells of the adult mouse brain. *Cell* (2018) 174:1015–30. doi: 10.1016/j.cell.2018.07.028
73. De Biase LM, Schuebel KE, Fusfeld ZH, Jair K, Hawes IA, Cimbro R, et al. Local cues establish and maintain region-specific phenotypes of basal ganglia microglia. *Neuron* (2017) 95:341–56.e6. doi: 10.1016/j.neuron.2017.06.020
74. Squarzoni P, Oller G, Hoeffel G, Pont-Lezica L, Rostaing P, Low D, et al. Microglia modulate wiring of the embryonic forebrain. *Cell Rep.* (2014) 8:1271–9. doi: 10.1016/j.celrep.2014.07.042
75. Kälin S, Miller KR, Kälin RE, Jendrach M, Witzel C, FL H. CNS myeloid cells critically regulate heat hyperalgesia. *J Clin Invest.* (2018) 128:2774–86. doi: 10.1172/JCI95305
76. Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, et al. Control of microglial neurotoxicity by the fractalkine receptor. *Nat Neurosci.* (2006) 9:917–24. doi: 10.1038/nn1715
77. Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR III, Lafaille JJ, et al. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell* (2013) 155:1596–609. doi: 10.1016/j.cell.2013.11.030
78. Dutta R, Krishnan A, Meng J, Das S, Ma J, Banerjee S, et al. Morphine modulation of toll-like receptors in microglial cells potentiates neuropathogenesis in a HIV-1 model of coinfection with pneumococcal pneumoniae. *J Neurosci.* (2012) 32:9917–30. doi: 10.1523/JNEUROSCI.0870-12.2012
79. Roeckel LA, Utard V, Reiss D, Mouheiche J, Maurin H, Robe A, et al. Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Sci Rep.* (2017) 7:10406. doi: 10.1038/s41598-017-11120-4
80. Xie N, Gomes FP, Deora V, Gregory K, Vithanage T, Nassar ZD, et al. Activation of mu-opioid receptor and Toll-like receptor 4 by plasma from morphine-treated mice. *Brain Behav Immun.* (2017) 61:244–58. doi: 10.1016/j.bbi.2016.12.002
81. AlJanahi AA, Danielsen M, Dunbar CE. An Introduction to the analysis of single-cell RNA-sequencing data. *Mol Ther Methods Clin Dev.* (2018) 10:189–96. doi: 10.1016/j.omtm.2018.07.003
82. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci.* (2016) 19:987–91. doi: 10.1038/nn.4338
83. Liu S, Yao JL, Wan XX, Song ZJ, Miao S, Zhao Y, et al. Sonic hedgehog signaling in spinal cord contributes to morphine-induced hyperalgesia and tolerance through upregulating brain-derived neurotrophic factor expression. *J Pain Res.* (2018) 11:649–59. doi: 10.2147/JPR.S153544
84. Hayashi Y, Morinaga S, Zhang J, Satoh Y, Meredith AL, Nakata T, et al. BK channels in microglia are required for morphine-induced hyperalgesia. *Nat Commun.* (2016) 7:11697. doi: 10.1038/ncomms11697
85. Melik Parsadaniantz S, Rivat C, Rostene W, Reaux-Le Goazigo A. Opioid and chemokine receptor crosstalk: a promising target for pain therapy? *Nat Rev Neurosci.* (2015) 16:69–78. doi: 10.1038/nrn3858
86. Burma NE, Bonin RP, Leduc-Pessah H, Baimel C, Cairncross ZF, Mousseau M, et al. Blocking microglial pannexin-1 channels alleviates morphine withdrawal in rodents. *Nat Med.* (2017) 23:355–60. doi: 10.1038/nm.4281
87. Burma NE, Leduc-Pessah H, Trang T. Genetic deletion of microglial Panx1 attenuates morphine withdrawal, but not analgesic tolerance or hyperalgesia in mice. *Channels* (2017) 11:487–94. doi: 10.1080/19336950.2017.1359361
88. Taylor AM, Mehrabani S, Liu S, Taylor AJ, CM. C. Topography of microglial activation in sensory- and affect-related brain regions in chronic pain. *J Neurosci Res.* (2017) 95:1330–5. doi: 10.1002/jnr.23883
89. Taylor AM, Castonguay A, Taylor AJ, Murphy NP, Ghogha A, Cook C, et al. Microglia disrupt mesolimbic reward circuitry in chronic pain. *J Neurosci.* (2015) 35:8442–50. doi: 10.1523/JNEUROSCI.4036-14.2015

90. Taylor AM, Castonguay A, Ghogha A, Vayssiere P, Pradhan AA, Xue L, et al. Neuroimmune regulation of GABAergic neurons within the ventral tegmental area during withdrawal from chronic morphine. *Neuropsychopharmacology* (2016) 41:949–59. doi: 10.1038/npp.2015.221
91. Cahill CM, Taylor AM. Neuroinflammation-a co-occurring phenomenon linking chronic pain and opioid dependence. *Curr Opin Behav Sci.* (2017) 13:171–7. doi: 10.1016/j.cobeha.2016.12.003
92. Cahill CM, Walwyn W, Taylor AMW, Pradhan AAA, Evans CJ. Allostatic mechanisms of opioid tolerance beyond desensitization and downregulation. *Trends Pharmacol Sci.* (2016) 37:963–76. doi: 10.1016/j.tips.2016.08.002
93. Siuda ER, Copits BA, Schmidt MJ, Baird MA, Al-Hasani R, Planer WJ, et al. Spatiotemporal control of opioid signaling and behavior. *Neuron* (2015) 86:923–35. doi: 10.1016/j.neuron.2015.03.066
94. Madariaga-Mazon A, Marmolejo-Valencia AF, Li Y, Toll L, Houghten RA, Martinez-Mayorga K. Mu-Opioid receptor biased ligands: A safer and painless discovery of analgesics? *Drug Discov Today* (2017) 22:1719–29. doi: 10.1016/j.drudis.2017.07.002
95. Valentino RJ, Volkow ND. Untangling the complexity of opioid receptor function. Neuropsychopharmacology (2018) 43:2514–20. doi: 10.1038/s41386-018-0225-3
96. Stoeber M, Jullie D, Lobingier BT, Laeremans T, Steyaert J, Schiller PW, et al. A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* (2018) 98:963–76.e5. doi: 10.1016/j.neuron.2018.04.021
97. Doyle HH, Eidson LN, Sinkiewicz DM, Murphy AZ. Sex differences in microglia activity within the periaqueductal gray of the rat: a potential mechanism driving the dimorphic effects of morphine. *J Neurosci.* (2017) 37:3202–14. doi: 10.1523/JNEUROSCI.2906-16.2017
98. Mapplebeck JCS, Dalgarno R, Tu Y, Moriarty O, Beggs S, Kwok CHT, et al. Microglial P2X4R-evoked pain hypersensitivity is sexually dimorphic in rats. *Pain* (2018) 159:1752–63. doi: 10.1097/j.pain.0000000000001265
99. Berrettini W. A brief review of the genetics and pharmacogenetics of opioid use disorders. *Dialogues Clin Neurosci.* (2017) 19:229–36.
100. Grace PM, Hutchinson MR, Maier SF, Watkins LR. Pathological pain and the neuroimmune interface. *Nat Rev Immunol.* (2014) 14:217–31. doi: 10.1038/nri3621
101. Helmerhorst GT, Teunis T, Janssen SJ, Ring D. An epidemic of the use, misuse and overdose of opioids and deaths due to overdose, in the United States and Canada: is Europe next? *Bone Joint J.* (2017) 99-B:856–64. doi: 10.1302/0301-620X.99B7.BJJ-2016-1350.R1

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Mu-Opioid Receptor Agonist Induces Kir3 Currents in Mouse Peripheral Sensory Neurons – Effects of Nerve Injury

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Neuropathic pain often arises from damage to peripheral nerves and is difficult to treat. Activation of opioid receptors in peripheral sensory neurons is devoid of respiratory depression, sedation, nausea, and addiction mediated in the brain, and ameliorates neuropathic pain in animal models. Mechanisms of peripheral opioid analgesia have therefore gained interest, but the role of G protein-coupled inwardly rectifying potassium (Kir3) channels, important regulators of neuronal excitability, remains unclear. Whereas functional Kir3 channels have been detected in dorsal root ganglion (DRG) neurons in rats, some studies question their contribution to opioid analgesia in inflammatory pain models in mice. However, neuropathic pain can be diminished by activation of peripheral opioid receptors in mouse models. Therefore, here we investigated effects of the selective μ-opioid receptor (MOR) agonist [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) on potassium conductance in DRG neurons upon a chronic constriction injury (CCI) of the sciatic nerve in mice. For verification, we also tested human embryonic kidney (HEK) 293 cells transfected with MOR and Kir3.2. Using patch clamp, we recorded currents at −80 mV and applied voltage ramps in high extracellular potassium concentrations, which are a highly sensitive measures of Kir3 channel activity. We found a significantly higher rate of HEK cells responding with potassium channel blocker barium-sensitive inward current (233 ± 51 pA) to DAMGO application in transfected than in untransfected group, which confirms successful recordings of inward currents through Kir3.2 channels. Interestingly, DAMGO induced similar inward currents (178 ± 36–207 ± 56 pA) in 15–20% of recorded DRG neurons from naïve mice and in 4–27% of DRG neurons from mice exposed to CCI, measured in voltage clamp or voltage ramp modes. DAMGO-induced currents in naïve and CCI groups were reversed by barium and a more selective Kir3 channel blocker tertiapin-Q. These data indicate the coupling of Kir3 channels with MOR in mouse peripheral sensory neuron cell bodies, which was unchanged after CCI. A comparative analysis of opioid-induced potassium conductance at the axonal injury site and peripheral terminals of DRG neurons could clarify the role of Kir3 channel–MOR interactions in peripheral nerve injury and opioid analgesia.

Keywords: neuropathy, DRG neurons, DAMGO, peripheral opioid receptors, potassium channels, GIRK channels, patch clamp

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INTRODUCTION

In European countries, 12–30% of adults suffer from chronic pain and many of them experience severe limitations in managing daily life activities (Breivik et al., 2006). Besides the impairment of patients' quality of life, chronic pain is a major economic challenge for social and health care systems. Neuropathic pain is defined as pain caused by a lesion or disease of the somatosensory nervous system (IASP, 2017). Patients experience reduced thresholds to painful mechanical or thermal stimuli, or pain sensations elicited by normally innocuous stimuli such as touch, warm or cool temperatures (Baron et al., 2010). Common causes of this chronic condition are diabetes, herniated vertebral discs, cancer, chemotherapy, human immunodeficiency virus or varicella zoster virus, and injuries to nerves due to accidents or medical procedures (Jensen and Finnerup, 2014).

The treatment of neuropathic pain is challenging and the side effects restrict the use of many medications. Among these are opioids such as morphine and fentanyl, which are the most powerful analgesics available. However, they also induce constipation which predominantly results from activation of intestinal opioid receptors (Imam et al., 2018), as well as respiratory depression, sedation, dizziness, and nausea mediated in the central nervous system (CNS) (Li and van den Pol, 2008; Imam et al., 2018). Furthermore, their prolonged use leads to the development of addiction, which has resulted in a worldwide opioid epidemic (Volkow et al., 2018). Importantly, the activation of opioid receptors in peripheral sensory neurons can provide analgesia without CNS side effects (Stein, 1995; Kalso et al., 2002). In fact, peripheral opioid receptors mediate a large proportion of the analgesic effects produced by systemically administered opioids (Stein and Machelska, 2011; Gaveriaux-Ruff, 2013; Jagla et al., 2014; Spahn et al., 2017). Numerous preclinical studies have also shown peripheral opioid analgesia in models of neuropathic pain in rats (Truong et al., 2003; Kabli and Cahill, 2007; Obara et al., 2007, 2009; Spahn et al., 2018) and mice (Kolesnikov et al., 2007; Cayla et al., 2012; Hervera et al., 2012; Labuz and Machelska, 2013; Labuz et al., 2016). Furthermore, a clinical trial reported attenuation of neuropathic pain after peripherally applied morphine in patients (Azad et al., 2000). Activation of peripheral opioid receptors leads to the inhibition of voltage-gated calcium and sodium channels, acid-sensing ion channels, transient receptor potential cation channel subfamily V member 1 (TRPV1), and subfamily M member 3 (TRPM3) (Gold and Levine, 1996; Endres-Becker et al., 2007; Cai et al., 2014; Dembla et al., 2017). A particularly prominent mechanism underlying peripheral opioid analgesia is the activation of potassium channels in peripheral sensory neurons (Ocana et al., 2004). Hence, interactions between opioid receptors and potassium channels pose a promising basis for the development of novel therapies with an improved side effect profile.

Potassium channels play a pivotal role in the regulation of neuronal excitability and their dysregulation contributes to neuropathic pain (Prescott et al., 2014; Waxman and Zamponi, 2014). Inwardly rectifying potassium (Kir) channels have gained particular interest due to their crucial role in maintaining the resting membrane potential of neurons. Of these channels the G protein-coupled inwardly rectifying potassium (GIRK or Kir3) channels form membrane bound signaling complexes with opioid receptors (Luscher and Slesinger, 2010; Nagi and Pineyro, 2014). Four Kir3 channel subunits have been identified, Kir3.1–3.4. To form a functional channel, the subunits assemble in heterotetramers (Kir3.1, 3.3, 3.4) or both hetero- and homo-tetramers (Kir3.2). The Kir3.1–3.3 subunits are common in the CNS (Hibino et al., 2010; Luscher and Slesinger, 2010; Nagi and Pineyro, 2014). In the peripheral neuron cell bodies in dorsal root ganglia (DRG), the Kir3 mRNA and protein expression, and function have been shown in rats and humans (Khodorova et al., 2003; Gao et al., 2007; Nockemann et al., 2013; Chung et al., 2014; Gorham et al., 2014; Lyu et al., 2015). In contrast, the data on Kir3 in the mouse DRG are conflicting. Whereas some studies detected Kir3 mRNA in DRG (Manteniotis et al., 2013; Saloman et al., 2016), others did not find Kir3 mRNA or protein in DRG or cutaneous nerves in wild-type mice (Mitrovic et al., 2003; Nockemann et al., 2013). The latter study suggested that the absence of Kir3 in DRG neurons underlie the weak or absent peripheral mu-opioid receptor (MOR)-mediated analgesia in an inflammatory pain model in wild-type mice; this analgesia was established by transgenic expression of Kir3.2 in DRG neurons (Nockemann et al., 2013). However, in neuropathic pain models, opioids effectively alleviate mechanical and heat hypersensitivity via activation of peripheral MOR in wild-type mice (Kolesnikov et al., 2007; Cayla et al., 2012; Hervera et al., 2012; Labuz and Machelska, 2013; Labuz et al., 2016). These findings raise the question whether Kir3 channels functionally couple to MOR in peripheral sensory neurons in mice following neuropathy.

Therefore, our goal in this study was to investigate the effects of MOR agonist [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) on potassium conductance in DRG neurons upon peripheral nerve injury in mice. We hypothesized that the nerve lesion results in enhanced MOR-induced potassium conductance in the corresponding DRG sensory neuron cell bodies. As a model of neuropathic pain we used a chronic constriction injury (CCI) of the sciatic nerve, which resembles human peripheral neuropathy (e.g., nerve entrapment or compression) (Bennett and Xie, 1988). To this end, we examined the effects of DAMGO on potassium conductance using patch clamp in cultured DRG neurons from naïve mice and mice exposed to CCI. As a reference, equivalent experiments were performed in human embryonic kidney (HEK) 293 cells transfected with MOR and Kir3.2.

MATERIALS AND METHODS

HEK 293 Cell Culture and Transfection

Human embryonic kidney 293 cells transfected with rat MOR and mouse Kir3.2 and untransfected HEK 293 cells (control) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were maintained in DMEM (Sigma-Aldrich, Steinheim, Germany) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum

(Biochrom, Berlin, Germany) in 5% CO_2 at 37°C, and were passaged every 2–3 days. The cells were seeded onto plastic culture dishes (35 mm) a day prior to transfection. The transfection mixture consisted of 1 μg pcDNA3.1-MOR (kindly provided by Prof. Christian Zöllner), 1 μg pFLAG-Kir3.2 (kindly provided by Dr. Dinah Nockemann), 6 μl XtremeGene added to 88 μl DMEM without supplements per culture dish. Untransfected cells were cultured accordingly.

Animals

Animal experiments were approved by the State animal care committee (Landesamt für Gesundheit und Soziales, Berlin, Germany) and followed the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the ARRIVE guidelines (Kilkenny et al., 2010). Wild-type male C57BL/6J mice (18–35 g, 6–8 weeks old; Janvier Laboratories, France) were housed in groups of 2–4 per cage lined with ground corncob bedding, with free access to standard laboratory rodent chow and tap water, on a 12 h/12 h (8 am/8 pm) light/dark cycle. Room temperature was 22 ± 0.5°C and humidity was 60–65%.

Chronic Constriction Injury

The CCI was induced in deeply isoflurane-anesthetized mice by exposing the sciatic nerve at the level of the right mid-thigh, and placing three loose silk ligatures (4-0) around the nerve with about 1-mm spacing. The ligatures were tied until they elicited a brief twitch in the respective hind limb. The wound was closed with silk sutures (Labuz et al., 2009; Labuz and Machelska, 2013).

DRG Tissue Preparation and Neuron Culture

Dorsal root ganglia were isolated from naïve mice and mice 2 days after CCI. Briefly, mice were killed by an overdose of isoflurane, the vertebral column was removed, washed in PBS, placed in ice-cold PBS, and the lumbar (three to five) DRG innervating sciatic nerve ipsilateral to CCI or from the right side of naïve mice were dissected. The DRG were collected in ice-cold serum-free working medium (DMEM/HAM's F12 supplemented with 1% penicillin/streptomycin). DRG from one animal were used for one culture. Further handling of the tissue was performed under a laminar air flow hood in sterile conditions. The collected DRG tissue was incubated in 1.25% collagenase (Sigma-Aldrich) for 50 min at 37°C in a thermoshaker, washed with PBS and incubated in 2.5% trypsin (Sigma-Aldrich) for 5 min at 37°C in a thermoshaker. After digestion, the tissue was triturated using plastic pipette tips and subsequently filtered through a 40-μm cell strainer. The filtrate was centrifuged, the supernatant discarded and the cell pellet resuspended in 300–1000 μl culture medium (DMEM/HAM's F12 supplemented with 1% penicillin/streptomycin and 10% horse serum), depending on the required cell density. The cell suspensions (30–100 μl) were then seeded onto poly-L-lysine coated plastic culture dishes (35 mm) and placed in an incubator (5% CO_2 at 37°C). An hour later (to allow the cells to settle down), the cell cultures were topped up to a total of 2 ml of culture medium and cultured until patch clamp recordings, as previously described (Nockemann et al., 2013).

Patch Clamp Experiments

Human embryonic kidney 293 cells were recorded 40–50 h after plating (untransfected cells) or transfection with MOR and Kir3.2. DRG neurons (medium diameter of 20–35 μm) (Stucky and Lewin, 1999) were used 20–30 h after cultivation. Cell viability was evaluated before first experiment by Trypan Blue exclusion assay. Recordings were carried out in whole-cell voltage clamp mode. After washing with PBS, cells were bathed in low potassium extracellular buffer (5.6 mM KCl, 140 mM NaCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, 2.6 mM glucose; adjusted to pH 7.4 using NaOH; all from Sigma-Aldrich) and visualized using a Zeiss Axiovert 200 inverse microscope. Patch pipettes (resistance 3.5–8 MΩ) were fabricated from Borosilicate glass capillaries using a Sutter P-97 puller (Sutter Instrument, Novato, CA, United States) and filled with intracellular buffer (122 mM KCl, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 11 mM EGTA, 4 mM MgATP, 0.25 mM NaGTP; adjusted to pH 7.4 using KOH; all from Sigma-Aldrich). Currents were amplified and recorded using an EPC-10 patch amplifier and Pulse software (HEKA, Lambrecht, Germany), and were sampled at a frequency of 100 Hz. Cells were superfused by steady flow of extracellular buffer at a flow rate of 800–1000 μl/min using a pressurized application system (Perfusion Pressure Kit VPP-6; Warner Instruments, Hamden, CT, United States) and a suction pump. Test compounds, DAMGO (10 μM), $BaCl_2$ (3 mM; both from Sigma-Aldrich), and tertiapin-Q (100 nM; Alomone Labs, Jerusalem, Israel) were applied using a perfusion valve control systems (VC-6; Warner Instruments) to switch between vehicle buffer and buffers containing the test compounds. All recordings were performed at room temperature. Fast capacitive currents (i.e., pipette potential) were canceled before seal formation. After reaching "giga-seal," the membrane patch was ruptured to achieve whole-cell configuration. In DRG neurons, the resting membrane potential was estimated in current-clamp mode shortly after gaining whole-cell access and action potentials were recorded in current-clamp mode using stepwise increasing current injections of 100 ms from 100 to 600 pA. Only cells showing proper action potential overshoot were included for further experiments. Cell capacitance, series and input resistance were monitored by applying test pulses of 10 mV for 10 ms before each recording. The currents were recorded in voltage-clamp mode at a constant holding potential of −80 mV in high potassium buffer (140 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES; adjusted to pH 7.4 using KOH) for 120 s in the absence or presence of DAMGO without or with $BaCl_2$ (Nockemann et al., 2013). Hyperpolarized state and high concentration of potassium in extracellular buffer were used to increase the electro-chemical gradient for potassium to drive it into the cell when Kir3 channels are opened. To reduce "stress" to the cells, high potassium buffer was carefully washed in over a period of 2 min and cells were allowed to stabilize for at least 2 min before recording. For tertiapin-Q experiments we applied voltage ramps from a holding potential of −40 mV and measured the induced

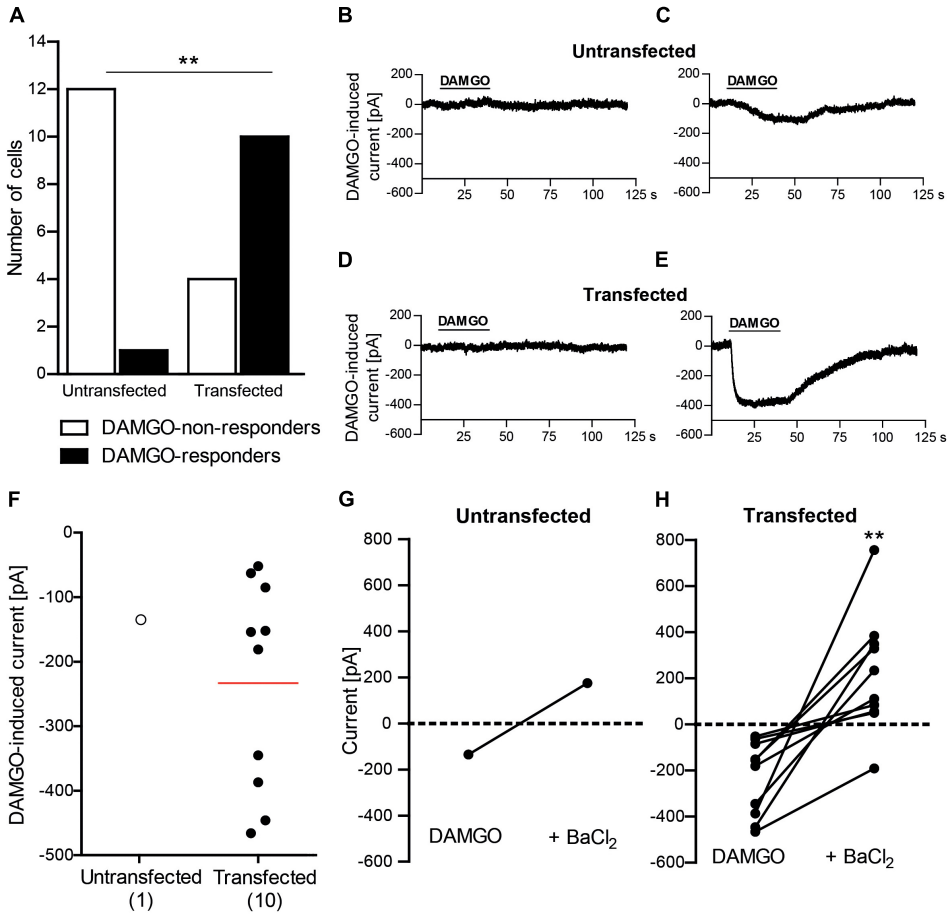

FIGURE 1 | DAMGO (10 μM) induces potassium currents in HEK 293 cells transfected with rat MOR and mouse Kir3.2. **(A)** Number of DAMGO-responders and non-responders in untransfected and transfected cells. $^{**}P = 0.0013$ (Fisher's exact test) indicates higher proportion of DAMGO-responders to DAMGO-non-responders in transfected vs. untransfected cells. The cells were sampled from three untransfected and four transfected cell cultures. **(B–E)** Exemplary traces of DAMGO-non-responder **(B)** and DAMGO-responder **(C)** in untransfected cells, and of DAMGO-non-responder **(D)** and DAMGO-responder **(E)** in transfected cells. **(F)** Single cell currents in DAMGO-responders. The data points represent single cell values, and the red horizontal line indicates the mean. Numbers in brackets indicate the number of cells. **(G,H)** $BaCl_2$ (3 mM)-mediated reversibility of DAMGO-induced currents in untransfected cells ($n = 1$) **(G)** and transfected cells ($n = 10$; $^{**}P = 0.002$, paired t-test) **(H)**. Only DAMGO-responders are shown. Data points represent DAMGO-induced currents of the same cell before and after application of $BaCl_2$. Dotted lines represent zero current. In all experiments, the currents were recorded in voltage clamp mode at –80 mV in high potassium extracellular buffer (140 mM). Cells were defined as responding to DAMGO if the resulting current was larger than three times the noise range.

current at −80 mV, based on previously published protocols (Gao et al., 2007; Gorham et al., 2014). These experiments were performed in a 45 mM high potassium extracellular buffer (45 mM KCl, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose; adjusted to pH 7.4 using KOH) and an intracellular solution consisting of 120 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1 mM EGTA, 3 mM NaATP, 0.3 mM NaGTP (adjusted to pH 7.4 using KOH) (Gorham et al., 2014). The ramps were applied every 10 s for 200 s in the absence or presence of DAMGO without or with tertiapin-Q. The analysis of patch clamp recordings was performed using Nest-o-Patch v1.2 and Prism v6 software (GraphPad Software, Inc., La Jolla, CA, United States). Effects of DAMGO were measured as departure from holding current (in voltage clamp mode experiments) or baseline currents (in voltage ramp experiments) while running vehicle buffer. Cells were considered responding to DAMGO application (DAMGO-responders) if the resulting current was larger than three times the noise range from the holding and baseline currents, respectively. Effects of $BaCl_2$ were measured as departure from holding current while running DAMGO buffer. Effects of tertiapin-Q were measured as departure from the mean baseline current while running DAMGO buffer. Drift of baseline was corrected manually or using the Nest-o-Patch baseline correction tool when necessary.

Statistical Analyses

Data are shown in raw values as bars representing cell numbers, representative currents, individual data points representing single cell currents, or means ± SEM. The number of cells per group was 13–41; the exact numbers are given in Figures. Statistical analyses were performed using GraphPad Prism software (Version 5.02 for Windows; GraphPad Software, Inc.).

The data were normally distributed as evaluated by Kolmogorov–Smirnov test. The comparison of DAMGO-induced currents between HEK 293 cells and DRG neurons was done by one-way analysis of variance (ANOVA). The comparison of DAMGO-induced currents between DRG neurons from naïve and CCI mice was analyzed by unpaired *t*-test. The reversibility of DAMGO-induced currents by $BaCl_2$ or tertiapin-Q was assessed by paired *t*-test. To compare ratios of DAMGO-responders to DAMGO-non-responders between naïve and CCI mice as well as between untransfected and transfected HEK 293 cells, the Fisher's exact test was used. The differences were defined as statistically significant if $P < 0.05$. The statistical tests and the degree of significance are specified in the Section "Results" or figure captions.

RESULTS

DAMGO Induces Potassium Currents in HEK 293 Cells Transfected With MOR and Kir3.2

To establish the protocol for patch clamp recordings of inward potassium currents, we first used HEK 293 cells transfected with MOR and Kir3.2 and untransfected (control) HEK 293 cells. Effects of DAMGO (10 μM) were recorded in the whole-cell voltage clamp mode at constant holding potential of −80 mV and high potassium extracellular buffer (140 mM). Analysis of all recorded HEK 293 cells revealed a significantly higher rate of cells responding with inward current to DAMGO application (DAMGO-responders) in transfected than in untransfected group (**Figure 1A**). In the untransfected group, vast majority of cells did not respond to DAMGO (92%; 12 of total 13 recorded cells) (**Figures 1A,B**), and only one cell showed very small, questionable response to DAMGO (**Figures 1A,C**) (see also section "Discussion"). In contrast, most of the cells in the transfected group showed prompt and reversible (by washout) inward currents upon DAMGO application (71%; 10 of total 14 recoded cells), whereas four cells (29%) were classified as DAMGO-non-responders (**Figures 1A,D,E**). The currents were similar in all DAMGO-responders, although statistical analysis could not be performed as there was only one responder among untransfected cells (**Figure 1F**). Application of the potassium channel blocker barium (3 mM $BaCl_2$) reversed DAMGO-mediated currents in one untransfected cell responding to DAMGO (**Figure 1G**) and in all DAMGO-responding transfected cells (**Figure 1H**), indicating that inward currents were mediated by potassium channels. These results clearly show functional coupling of MOR and Kir3.2 in transfected HEK 293 cells.

DAMGO Induces Potassium Currents in Mouse DRG Neurons

Conferring the recording conditions established for HEK 293 cells, in the next set of experiments we investigated the effects of nerve injury on DAMGO (10 μM)-induced potassium conductance in mouse DRG neurons. In addition, we used voltage ramp experiments in 45 mM potassium extracellular buffer to reduce the ionic stress in tertiapin-Q experiments, since the viability of the neurons dramatically decreased during tertiapin-Q application in voltage clamp mode in 140 mM potassium buffer. We analyzed neurons from DRG of naïve mice and from DRG ipsilateral to the CCI (2 days after CCI). In previous studies, we have shown that mechanical and heat hypersensitivities are fully established and can be attenuated by peripherally applied DAMGO at this time point following CCI in mice *in vivo* (Cayla et al., 2012; Labuz and Machelska, 2013; Labuz et al., 2016). Here we found that the rate of DAMGO-responding neurons between naïve and CCI mice was not significantly different (**Figures 2A**, **3A**). Thus, in the voltage clamp mode experiments (**Figure 2A**), we recorded 15% DAMGO-responders (5 of total 33 recorded neurons) from naïve mice and 4% DAMGO-responders (1 of total 26 recorded neurons) from mice exposed to CCI. In voltage ramp experiments (**Figure 3A**), we recorded 20% DAMGO-responders (8 of total 41 recorded neurons) from naïve mice and 27% DAMGO-responders (9 of total 33 recorded neurons) from CCI mice. DAMGO-induced inward currents were comparable between neurons from naïve and CCI mice; due to low number of DAMGO-responders in the voltage clamp mode (**Figure 2B**), the statistical analysis could only be performed for the voltage ramp experiments (**Figure 3B**) ($P = 0.6781$, unpaired *t*-test). Nevertheless, in both experimental conditions the DAMGO-responding neurons showed prompt inward currents, which could be reversed by barium (3 mM $BaCl_2$) (**Figures 2C–H**) and attenuated by a more selective Kir3 channel blocker tertiapin-Q (100 nM) (**Figures 3C–H**). Additionally, the currents of DAMGO-responding neurons from naïve mice (**Figures 2B**, **3B**) and CCI mice (**Figure 3B**) were comparable to currents of DAMGO-responders in MOR- and Kir3.2-transfected HEK 293 cells (**Figure 1F**) ($P = 0.8866$, one-way ANOVA).

DISCUSSION

In this study, we found that the MOR-selective agonist DAMGO induces potassium currents in DRG neurons of both naïve mice and mice with CCI of the sciatic nerve, which were diminished by barium and tertiapin-Q indicating the involvement of Kir3 channels. The rate of DAMGO-responding neurons and the DAMGO-induced inward currents did not change following CCI.

In initial experiments, we determined DAMGO-induced inward potassium currents using a whole-cell voltage clamp approach in hyperpolarized untransfected and transfected with MOR and Kir3.2 HEK 293 cells. Among the untransfected cells, one cell was classified as DAMGO-responder according to the criterion that the DAMGO-mediated current is three times larger than the noise range. However, based on the response kinetics (slow and not clearly corresponding to DAMGO application, very small current amplitude; **Figure 1C**), this cell did not appear to reliably respond to DAMGO. In contrast, the response of transfected cells was fast and had distinct and tightly correlated to DAMGO application onset (**Figure 1E**), similar to the literature (Kohno et al., 2005; Kobayashi et al., 2006; Nockemann et al., 2013; Gorham et al., 2014). Furthermore, substantially higher

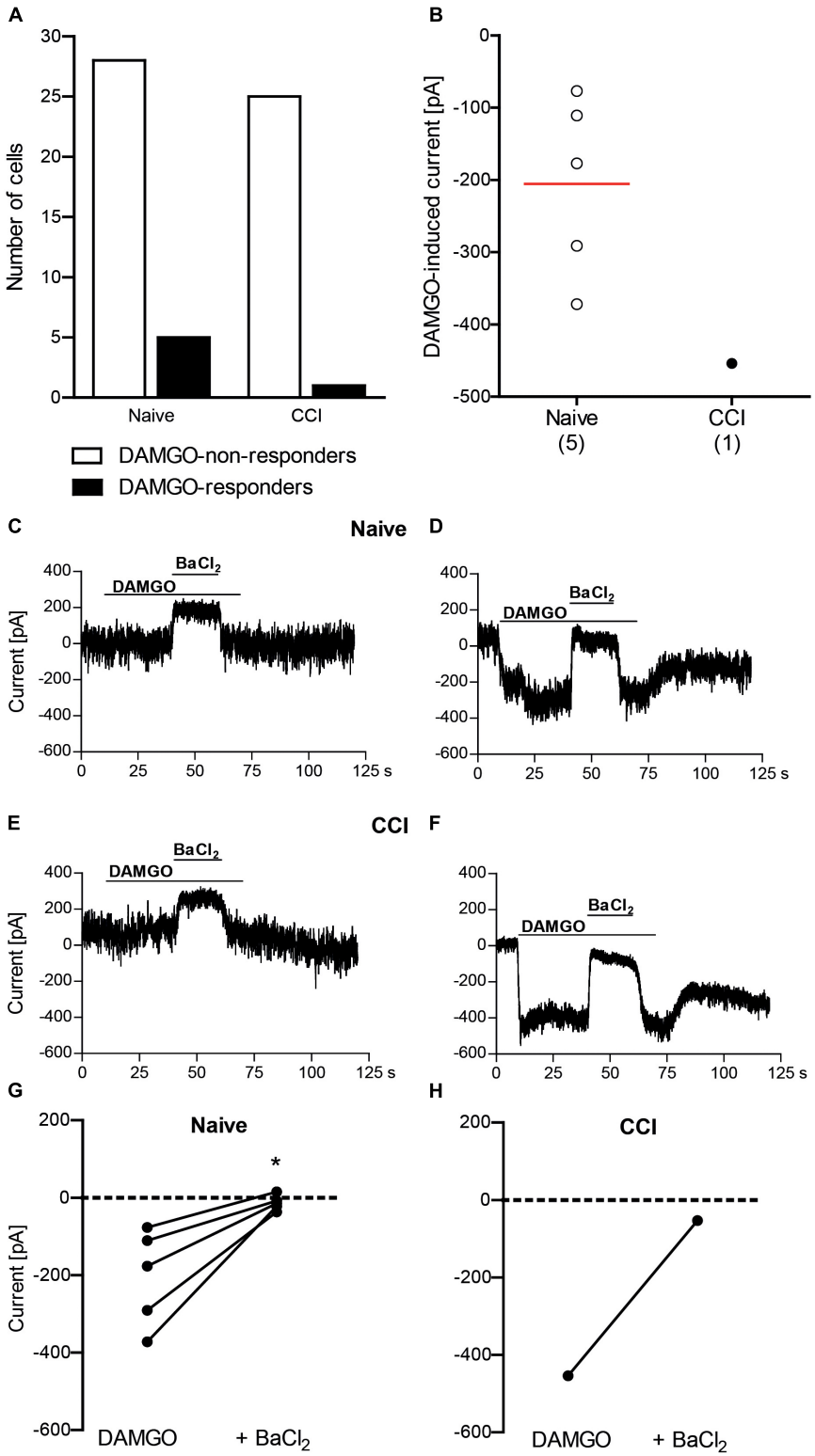

FIGURE 2 | DAMGO (10 μM) induces potassium currents in mouse DRG neurons, assessed in the voltage clamp mode. **(A)** Number of neurons responding and non-responding to DAMGO from naïve and CCI mice. The proportion of DAMGO-responding to DAMGO-non-responding neurons from naïve vs. CCI mice did not differ significantly ($P = 0.2148$; Fisher's exact t-test). The neurons were sampled from cultures obtained from DRG of nine naïve mice and eight CCI mice. **(B)** Single neuron currents in DAMGO-responders. The data points represent single neuron values, and the red horizontal line indicates the mean. Numbers in brackets indicate

(Continued)

FIGURE 2 | Continued
the number of neurons. **(C–F)** Exemplary traces of DRG neurons non-responding **(C)** and responding **(D)** to DAMGO from naïve mice, and DRG neurons non-responding **(E)** and responding **(F)** to DAMGO from mice on day 2 following CCI. The DAMGO effects are shown before and during $BaCl_2$ (3 mM) application. **(G,H)** $BaCl_2$ (3 mM)-mediated reversibility of DAMGO-induced currents in individual neurons from naïve mice (n = 5 neurons; $^*P$ = 0.017, paired t-test) **(G)** and CCI mice (n = 1 neuron) **(H)**. Only DAMGO-responding neurons are shown. Data points represent DAMGO-induced currents of the same neuron before and after application of $BaCl_2$. Dotted lines represent zero current. In all experiments, the currents were recorded in voltage clamp mode at –80 mV in high potassium extracellular buffer (140 mM). Neurons were defined as responding to DAMGO if the resulting current was larger than three times the noise range.

ratio of DAMGO-responders in MOR- and Kir3.2-transfected compared to untransfected HEK 293 cells, and the reversibility of DAMGO-induced currents by potassium channel blocker barium clearly demonstrate successful identification of DAMGO-induced inward currents and suggest they were mediated by Kir3.2 channels.

Our finding that DAMGO induced similar currents in DRG neurons of naïve wild-type mice is somewhat intriguing. Whereas functional Kir3 channels have been consistently identified in rat peripheral sensory neurons (Gao et al., 2007; Nockemann et al., 2013; Chung et al., 2014), only a few studies investigated Kir3 channels in these neurons in mice, and the data are inconsistent. Nockemann et al. (2013) showed very low amounts of Kir3.1 and Kir3.2 mRNA transcripts and no immunoreactivity of the corresponding proteins in mouse DRG. Using patch clamp recordings, the authors reported "negligible" inward currents upon DAMGO application and concluded on the absence of Kir3 from mouse DRG neurons. However, the size of DAMGO-induced currents they measured in naïve wild-type mouse DRG neurons are substantial (1.8 ± 0.4 nA) (Nockemann et al., 2013) and in fact, much higher compared to currents recorded under similar conditions and defined as opioid-mediated responses in rat DRG and spinal cord neurons, or in Xenopus oocytes transfected with Kir3 (40–800 pA) in other studies (Kohno et al., 2005; Kobayashi et al., 2006; Gao et al., 2007). The results in these latter publications are indeed similar to our recordings in MOR- and Kir3.2-transfected HEK 293 cells (233 ± 51 pA; **Figure 1F**), and DRG neurons from naïve mice (206 ± 55 pA, **Figure 2B**; 178 ± 36 pA, **Figure 3B**) and CCI mice (207 ± 56 pA, **Figure 3B**). Kanjhan et al. (2005) reported the absence of hyperpolarization-activated potassium currents characteristic for Kir3 in DRG neurons of newborn mice and argued that Kir3 expression might occur later in the development of the nervous system, but did not examine older animals. Mitrovic et al. (2003) stated a lack of Kir3.2-immunostaining in mouse DRG, but did not present the corresponding data. In a comprehensive RNA expression analysis of mouse sensory ganglia, Manteniotis et al. (2013) reported moderate levels of KCNJ3 mRNA coding for Kir3.1 in DRG and trigeminal ganglia. This, however, is insufficient to form a functional channel, since functional Kir3 channels are formed by Kir3.1/Kir3.2 heterotetramers and Kir3.2 homotetramers (Luscher and Slesinger, 2010). Interestingly, a recent study found mRNAs encoding Kir3.1 and Kir3.2 in mouse DRG, which would allow formation of functional Kir3 channels (Saloman et al., 2016). Taken together, the current literature suggests low to moderate Kir3 mRNA expression (Nockemann et al., 2013; Saloman et al., 2016), which may result in low protein level difficult to detect by immunostaining (Mitrovic et al., 2003; Nockemann et al., 2013) and functional analysis (Nockemann et al., 2013) in mouse DRG neurons. Yet, we found a moderate number of neurons (15–20%) reliably responding to DAMGO with inward currents in DRG of naïve wild-type mice. DAMGO-induced currents in our experiments were diminished by both a general potassium channel blocker barium, and by tertiapin-Q, currently considered the most selective Kir3 channel blocker (Jin and Lu, 1998; Kitamura et al., 2000). Furthermore, considering the patch clamp conditions in our experiments such as high potassium concentration in the extracellular buffer and hyperpolarizing holding potential or voltage ramp mode, which are highly sensitive measures of Kir3 channel activity (Kobayashi et al., 2006; Gao et al., 2007; Nockemann et al., 2013; Gorham et al., 2014), the DAMGO-mediated potassium currents in mouse DRG neurons in our experiments likely resulted from the activation of Kir3 channels. This is also supported by the finding that currents of DAMGO-responding neurons were comparable to Kir3 currents measured in our MOR- and Kir3.2-transfected HEK 293 cell experiments, and in rat neurons and Xenopus oocytes in other studies (Kohno et al., 2005; Kobayashi et al., 2006; Gao et al., 2007). Although we have not used MOR antagonist, DAMGO is the MOR-selective agonist (Labuz and Machelska, 2013), and its effects in the dose of 10 μM (we used here) were reversible by opioid receptor antagonist naloxone in patch clamp experiments (Nockemann et al., 2013), suggesting that DAMGO-induced potassium currents in our experiments are MOR-mediated.

We have also analyzed DRG neurons from mice following sciatic nerve CCI, and found one DAMGO-responding neuron (i.e., 4% of all recorded neurons) in the voltage clamp mode, and nine DAMGO-responding neurons (i.e., 27% of all recorded neurons) in the voltage ramp experiments. This was not significantly different compared to DAMGO-responding neurons from naïve mice (15% and 20%, respectively). Also the DAMGO-induced inward currents were comparable between neurons from naïve and CCI mice (see paragraph above and **Figure 3B**). As such functional analysis following CCI has not been performed previously, several studies examined expression of Kir3 channels or MOR. Following CCI of the sciatic nerve in transgenic Kir3.2 mice, the Kir3.2 mRNA in the DRG was not altered (Nockemann et al., 2013). The MOR mRNA or protein levels were either decreased (Obara et al., 2009), not altered (Briscini et al., 2002; Kolesnikov et al., 2007), or elevated (Truong et al., 2003) following CCI. Regardless of these effects in the DRG cell bodies, the activation of MOR on DRG neuron peripheral terminals consistently attenuated CCI-induced hypersensitivity *in vivo* and nociceptor excitability *ex vivo* in mice (Kolesnikov et al., 2007; Cayla et al., 2012; Hervera et al., 2012;

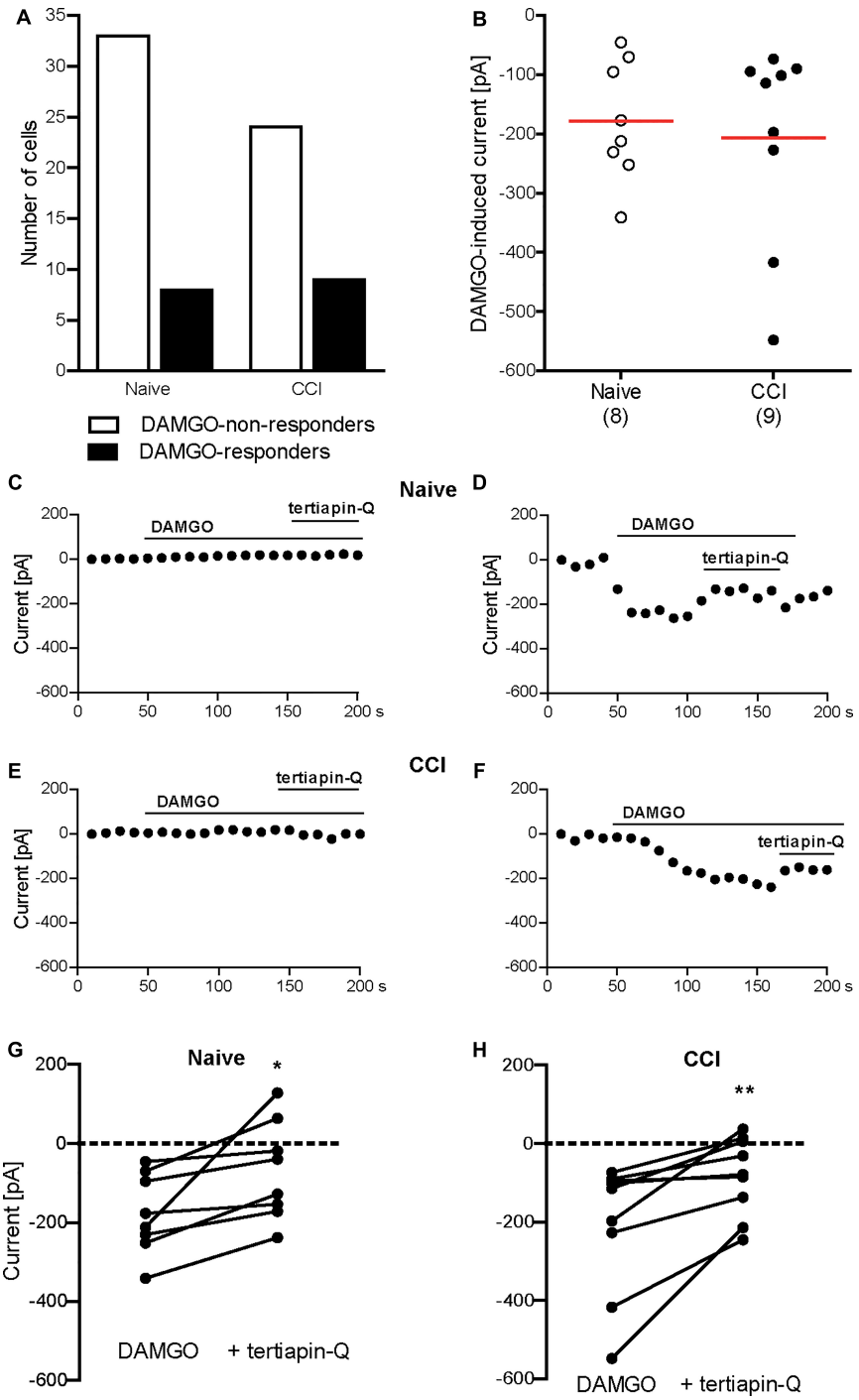

FIGURE 3 | DAMGO (10 μM)-induced potassium currents in mouse DRG neurons obtained in the voltage ramp mode. **(A)** Number of neurons responding and non-responding to DAMGO from naïve and CCI mice. The proportion of DAMGO-responding to DAMGO-non-responding neurons from naïve vs. CCI mice did not differ significantly ($P = 0.596$; Fisher's exact t-test). The neurons were sampled from cultures obtained from DRG of seven naïve and eight CCI mice. **(B)** Single neuron currents in DAMGO-responders. The data points represent single neuron values, and the red horizontal lines indicate the means. Numbers in brackets indicate the number of neurons. **(C–F)** Exemplary currents of DRG neurons non-responding **(C)** and responding **(D)** to DAMGO from naïve mice, and DRG neurons non-responding **(E)** and responding **(F)** to DAMGO from mice on day 2 following CCI. The DAMGO effects are shown before and during tertiapin-Q (100 nM) application. **(G,H)** Tertiapin-Q (100 nM)-mediated attenuation of DAMGO-induced currents in individual neurons from naïve mice ($n = 8$ neurons; $^*P = 0.0204$, paired t-test) **(G)** and CCI mice ($n = 9$ neurons; $^{**}P = 0.0073$, paired t-test) **(H)**. Only DAMGO-responding neurons are shown. Data points represent DAMGO-induced currents of the same neuron before and after application of tertiapin-Q. Dotted lines represent zero current. In all experiments, the currents were obtained by voltage ramps from a holding potential of –40 mV and measured at –80 mV in high potassium extracellular buffer (45 mM). Neurons were defined as responding to DAMGO if the resulting current was larger than three times the noise range.

Schmidt et al., 2012; Labuz and Machelska, 2013; Labuz et al., 2016). This is in line with the accumulation of Kir3.1, Kir3.2, and MOR proteins at the site of nerve ligation (Schmidt et al., 2013; Lyu et al., 2015) and the alleviation of CCI-induced hypersensitivity by DAMGO applied at the CCI site (Cayla et al., 2012; Labuz and Machelska, 2013; Labuz et al., 2016). Therefore, the corresponding electrophysiological recordings from the injury site, for example using *in vitro* skin-nerve preparations, appear appealing, but are technically very challenging.

CONCLUSION AND RELEVANCE

Using electrophysiology, we addressed here for the first time the effect of MOR agonist DAMGO on potassium currents in mouse peripheral neurons following CCI of the sciatic nerve. Our data indicate a coupling of MOR and Kir3 in DRG neurons in naïve mice and following CCI. The number of responding neurons and the size of DAMGO-induced potassium currents were comparable between both groups. Hence, the MOR–Kir3 interactions in peripheral sensory neurons in attenuation of neuropathic pain presents a worthwhile target for further investigations. Particularly, site-specific analysis of opioid-mediated Kir3 conductance along the peripheral pain pathway, including injury site at the axon and peripheral terminals, could elucidate the role of Kir3 and MOR in peripheral neuropathies and their alleviation.

AUTHOR CONTRIBUTIONS

PS designed and performed the experiments, analyzed and interpreted the results, and wrote the manuscript. VS designed and performed the experiments, analyzed and interpreted the results, and wrote the manuscript. MÖC and DL performed animal surgeries. HM conceptualized the project, designed experiments, interpreted the results, and wrote the manuscript. All authors accepted the final version of the manuscript.

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The data presented in this study constitute a part of the MD thesis by PS (Stötzner, 2018) available at https://refubium.fu-berlin.de/handle/fub188/7834. This is the only medium the data have appeared in and is in line with the policy of the Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health. We thank Dr. Paul A. Heppenstall for advice on performance and interpretation of patch clamp recordings.

REFERENCES

Azad, S. C., Beyer, A., Romer, A. W., Galle-Rod, A., Peter, K., and Schops, P. (2000). Continuous axillary brachial plexus analgesia with low dose morphine in patients with complex regional pain syndromes. *Eur. J. Anaesthesiol.* 17, 185–188. doi: 10.1097/00003643-200003000-00010

Baron, R., Binder, A., and Wasner, G. (2010). Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. *Lancet Neurol.* 9, 807–819. doi: 10.1016/S1474-4422(10)70143-5

Bennett, G. J., and Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33, 87–107. doi: 10.1016/0304-3959(88)90209-6

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., and Gallacher, D. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *Eur. J. Pain* 10, 287–333. doi: 10.1016/j.ejpain.2005.06.009

Briscini, L., Corradini, L., Ongini, E., and Bertorelli, R. (2002). Up-regulation of ORL-1 receptors in spinal tissue of allodynic rats after sciatic nerve injury. *Eur. J. Pharmacol.* 447, 59–65. doi: 10.1016/S0014-2999(02)01833-2

Cai, Q., Qiu, C. Y., Qiu, F., Liu, T. T., Qu, Z. W., Liu, Y. M., et al. (2014). Morphine inhibits acid-sensing ion channel currents in rat dorsal root ganglion neurons. *Brain Res.* 1554, 12–20. doi: 10.1016/j.brainres.2014.01.042

Cayla, C., Labuz, D., Machelska, H., Bader, M., Schafer, M., and Stein, C. (2012). Impaired nociception and peripheral opioid antinociception in mice lacking both kinin B1 and B2 receptors. *Anesthesiology* 116, 448–457. doi: 10.1097/ALN.0b013e318242b2ea

Chung, M. K., Cho, Y. S., Bae, Y. C., Lee, J., Zhang, X., and Ro, J. Y. (2014). Peripheral G protein-coupled inwardly rectifying potassium channels are involved in delta-opioid receptor-mediated anti-hyperalgesia in rat masseter muscle. *Eur. J. Pain* 18, 29–38. doi: 10.1002/j.1532-2149.2013.00343.x

Dembla, S., Behrendt, M., Mohr, F., Goecke, C., Sondermann, J., Schneider, F. M., et al. (2017). Anti-nociceptive action of peripheral mu-opioid receptors by G-beta-gamma protein-mediated inhibition of TRPM3 channels. *eLife* 6:e26280. doi: 10.7554/eLife.26280

Endres-Becker, J., Heppenstall, P. A., Mousa, S. A., Labuz, D., Oksche, A., Schafer, M., et al. (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol. Pharmacol.* 71, 12–18. doi: 10.1124/mol.106.026740

Gao, X. F., Zhang, H. L., You, Z. D., Lu, C. L., and He, C. (2007). G protein-coupled inwardly rectifying potassium channels in dorsal root ganglion neurons. *Acta Pharmacol. Sin.* 28, 185–190. doi: 10.1111/j.1745-7254.2007.00478.x

Gaveriaux-Ruff, C. (2013). Opiate-induced analgesia: contributions from mu, delta and kappa opioid receptors mouse mutants. *Curr. Pharm. Des.* 19, 7373–7381. doi: 10.2174/13816128194214010516372 7

Gold, M. S., and Levine, J. D. (1996). DAMGO inhibits prostaglandin E2-induced potentiation of a TTX-resistant Na+ current in rat sensory neurons in vitro. *Neurosci. Lett.* 212, 83–86. doi: 10.1016/0304-3940(96)12791-9

Gorham, L., Just, S., and Doods, H. (2014). Somatostatin 4 receptor activation modulates G-protein coupled inward rectifying potassium channels and voltage stimulated calcium signals in dorsal root ganglion neurons. *Eur. J. Pharmacol.* 736, 101–106. doi: 10.1016/j.ejphar.2014.04.016

Hervera, A., Leanez, S., and Pol, O. (2012). The inhibition of the nitric oxide-cGMP-PKG-JNK signaling pathway avoids the development of tolerance to the local antiallodynic effects produced by morphine during neuropathic pain. *Eur. J. Pharmacol.* 685, 42–51. doi: 10.1016/j.ejphar.2012.04.009

Hibino, H., Inanobe, A., Furutani, K., Murakami, S., Findlay, I., and Kurachi, Y. (2010). Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiol. Rev.* 90, 291–366. doi: 10.1152/physrev.00021.2009

IASP (2017). *IASP Terminology: Pain Terms, A Current List With Definitions and Notes on Usage*. Available: http://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698 [accessed July 02, 2018].

Imam, M. Z., Kuo, A., Ghassabian, S., and Smith, M. T. (2018). Progress in understanding mechanisms of opioid-induced gastrointestinal adverse effects and respiratory depression. *Neuropharmacology* 131, 238–255. doi: 10.1016/j.neuropharm.2017.12.032

Jagla, C., Martus, P., and Stein, C. (2014). Peripheral opioid receptor blockade increases postoperative morphine demands–a randomized, double-blind, placebo-controlled trial. *Pain* 155, 2056–2062. doi: 10.1016/j.pain.2014.07.011

Jensen, T. S., and Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *Lancet Neurol.* 13, 924–935. doi: 10.1016/S1474-4422(14)70102-4

Jin, W., and Lu, Z. (1998). A novel high-affinity inhibitor for inward-rectifier K+ channels. *Biochemistry* 37, 13291–13299. doi: 10.1021/bi981178p

Kabli, N., and Cahill, C. M. (2007). Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain. *Pain* 127, 84–93. doi: 10.1016/j.pain.2006.08.003

Kalso, E., Smith, L., Mcquay, H. J., and Andrew Moore, R. (2002). No pain, no gain: clinical excellence and scientific rigour–lessons learned from IA morphine. *Pain* 98, 269–275. doi: 10.1016/S0304-3959(02)00019-2

Kanjhan, R., Coulson, E. J., Adams, D. J., and Bellingham, M. C. (2005). Tertiapin-Q blocks recombinant and native large conductance K+ channels in a use-dependent manner. *J. Pharmacol. Exp. Ther.* 314, 1353–1361. doi: 10.1124/jpet.105.085928

Khodorova, A., Navarro, B., Jouaville, L. S., Murphy, J. E., Rice, F. L., Mazurkiewicz, J. E., et al. (2003). Endothelin-B receptor activation triggers an endogenous analgesic cascade at sites of peripheral injury. *Nat. Med.* 9, 1055–1061. doi: 10.1038/nm885

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., and Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *J. Pharmacol. Pharmacother.* 1, 94–99. doi: 10.4103/0976-500X.72351

Kitamura, H., Yokoyama, M., Akita, H., Matsushita, K., Kurachi, Y., and Yamada, M. (2000). Tertiapin potently and selectively blocks muscarinic K(+) channels in rabbit cardiac myocytes. *J. Pharmacol. Exp. Ther.* 293, 196–205.

Kobayashi, T., Washiyama, K., and Ikeda, K. (2006). Inhibition of G protein-activated inwardly rectifying K+ channels by ifenprodil. *Neuropsychopharmacology* 31, 516–524. doi: 10.1038/sj.npp.1300844

Kohno, T., Ji, R. R., Ito, N., Allchorne, A. J., Befort, K., Karchewski, L. A., et al. (2005). Peripheral axonal injury results in reduced mu opioid receptor pre- and post-synaptic action in the spinal cord. *Pain* 117, 77–87. doi: 10.1016/j.pain.2005.05.035

Kolesnikov, Y., El-Maarouf, A., Rutishauser, U., and Pasternak, G. (2007). Reorganization of dorsal root ganglion neurons following chronic sciatic nerve constriction injury: correlation with morphine and lidocaine analgesia. *Eur. J. Pharmacol.* 568, 124–133. doi: 10.1016/j.ejphar.2007.04.045

Labuz, D., and Machelska, H. (2013). Stronger antinociceptive efficacy of opioids at the injured nerve trunk than at its peripheral terminals in neuropathic pain. *J. Pharmacol. Exp. Ther.* 346, 535–544. doi: 10.1124/jpet.113.205344

Labuz, D., Schmidt, Y., Schreiter, A., Rittner, H. L., Mousa, S. A., and Machelska, H. (2009). Immune cell-derived opioids protect against neuropathic pain in mice. *J. Clin. Invest.* 119, 278–286. doi: 10.1172/JCI36246

Labuz, D., Spahn, V., Celik, M. O., and Machelska, H. (2016). Opioids and TRPV1 in the peripheral control of neuropathic pain–Defining a target site in the injured nerve. *Neuropharmacology* 101, 330–340. doi: 10.1016/j.neuropharm.2015.10.003

Li, Y., and van den Pol, A. N. (2008). Mu-opioid receptor-mediated depression of the hypothalamic hypocretin/orexin arousal system. *J. Neurosci.* 28, 2814–2819. doi: 10.1523/JNEUROSCI.5447-07.2008

Luscher, C., and Slesinger, P. A. (2010). Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nat. Rev. Neurosci.* 11, 301–315. doi: 10.1038/nrn2834

Lyu, C., Mulder, J., Barde, S., Sahlholm, K., Zeberg, H., Nilsson, J., et al. (2015). G protein-gated inwardly rectifying potassium channel subunits 1 and 2 are down-regulated in rat dorsal root ganglion neurons and spinal cord after peripheral axotomy. *Mol. Pain* 11:44. doi: 10.1186/s12990-015-0044-z

Manteniotis, S., Lehmann, R., Flegel, C., Vogel, F., Hofreuter, A., Schreiner, B. S., et al. (2013). Comprehensive RNA-Seq expression analysis of sensory ganglia with a focus on ion channels and GPCRs in Trigeminal ganglia. *PLoS One* 8:e79523. doi: 10.1371/journal.pone.0079523

Mitrovic, I., Margeta-Mitrovic, M., Bader, S., Stoffel, M., Jan, L. Y., and Basbaum, A. I. (2003). Contribution of GIRK2-mediated postsynaptic signaling to opiate and alpha 2-adrenergic analgesia and analgesic sex differences. *Proc. Natl. Acad. Sci. U.S.A.* 100, 271–276. doi: 10.1073/pnas.0136822100

Nagi, K., and Pineyro, G. (2014). Kir3 channel signaling complexes: focus on opioid receptor signaling. *Front. Cell Neurosci.* 8:186. doi: 10.3389/fncel.2014.00186

Nockemann, D., Rouault, M., Labuz, D., Hublitz, P., Mcknelly, K., Reis, F. C., et al. (2013). The K(+) channel GIRK2 is both necessary and sufficient for peripheral opioid-mediated analgesia. *EMBO Mol. Med.* 5, 1263–1277. doi: 10.1002/emmm.201201980

Obara, I., Makuch, W., Spetea, M., Schütz, J., Schmidhammer, H., Przewlocki, R., et al. (2007). Local peripheral antinociceptive effects of 14-O-methyloxymorphone derivatives in inflammatory and neuropathic pain in the rat. *Eur. J. Pharmacol.* 558, 60–67. doi: 10.1016/j.ejphar.2006.11.037

Obara, I., Parkitna, J. R., Korostynski, M., Makuch, W., Kaminska, D., Przewlocka, B., et al. (2009). Local peripheral opioid effects and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. *Pain* 141, 283–291. doi: 10.1016/j.pain.2008.12.006

Ocana, M., Cendan, C. M., Cobos, E. J., Entrena, J. M., and Baeyens, J. M. (2004). Potassium channels and pain: present realities and future opportunities. *Eur. J. Pharmacol.* 500, 203–219. doi: 10.1016/j.ejphar.2004.07.026

Prescott, S. A., Ma, Q., and De Koninck, Y. (2014). Normal and abnormal coding of somatosensory stimuli causing pain. *Nat. Neurosci.* 17, 183–191. doi: 10.1038/nn.3629

Saloman, J. L., Scheff, N. N., Snyder, L. M., Ross, S. E., Davis, B. M., and Gold, M. S. (2016). Gi-DREADD expression in peripheral nerves produces ligand-dependent analgesia, as well as ligand-independent functional changes in sensory neurons. *J. Neurosci.* 36, 10769–10781.

Schmidt, Y., Gaveriaux-Ruff, C., and Machelska, H. (2013). mu-Opioid receptor antibody reveals tissue-dependent specific staining and increased neuronal mu-receptor immunoreactivity at the injured nerve trunk in mice. *PLoS One* 8:e79099. doi: 10.1371/journal.pone.0079099

Schmidt, Y., Labuz, D., Heppenstall, P. A., and Machelska, H. (2012). Cutaneous nociceptors lack sensitisation, but reveal mu-opioid receptor-mediated reduction in excitability to mechanical stimulation in neuropathy. *Mol. Pain* 8:81. doi: 10.1186/1744-8069-8-81

Spahn, V., Del Vecchio, G., Labuz, D., Rodriguez-Gaztelumendi, A., Massaly, N., Temp, J., et al. (2017). A nontoxic pain killer designed by modeling of pathological receptor conformations. *Science* 355, 966–969. doi: 10.1126/science.aai8636

Spahn, V., Del Vecchio, G., Rodriguez-Gaztelumendi, A., Temp, J., Labuz, D., Kloner, M., et al. (2018). Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist. *Sci. Rep.* 8:8965. doi: 10.1038/s41598-018-27313-4

Stein, C. (1995). The control of pain in peripheral tissue by opioids. *N. Engl. J. Med.* 332, 1685–1690. doi: 10.1056/NEJM199506223322506

Stein, C., and Machelska, H. (2011). Modulation of peripheral sensory neurons by the immune system: implications for pain therapy. *Pharmacol. Rev.* 63, 860–881. doi: 10.1124/pr.110.003145

Stötzner, P. (2018). *Opioids in Neuropathic pain – The Role of Potassium Channels in Peripheral Sensory Neurons*. M.D. thesis, Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin.

Stucky, C. L., and Lewin, G. R. (1999). Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J. Neurosci.* 19, 6497–6505. doi: 10.1523/JNEUROSCI.19-15-06497.1999

Truong, W., Cheng, C., Xu, Q. G., Li, X. Q., and Zochodne, D. W. (2003). Mu opioid receptors and analgesia at the site of a peripheral nerve injury. *Ann. Neurol.* 53, 366–375. doi: 10.1002/ana.10465

Volkow, N., Benveniste, H., and Mclellan, A. T. (2018). Use and misuse of opioids in chronic pain. *Annu. Rev. Med.* 69, 451–465. doi: 10.1146/annurev-med-011817-044739

Waxman, S. G., and Zamponi, G. W. (2014). Regulating excitability of peripheral afferents: emerging ion channel targets. *Nat. Neurosci.* 17, 153–163. doi: 10.1038/nn.3602

Zimmermann, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16, 109–110. doi: 10.1016/0304-3959(83)90201-4

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Deformation-based Morphometry MRI Reveals Brain Structural Modifications in Living Mu Opioid Receptor Knockout Mice

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Mu opioid receptor (MOR) activation facilitates reward processing and reduces pain, and brain networks underlying these effects are under intense investigation. Mice lacking the MOR gene (MOR KO mice) show lower drug and social reward, enhanced pain sensitivity and altered emotional responses. Our previous neuroimaging analysis using Resting-state (Rs) functional Magnetic Resonance Imaging (fMRI) showed significant alterations of functional connectivity (FC) within reward/aversion networks in these mice, in agreement with their behavioral deficits. Here we further used a structural MRI approach to determine whether volumetric alterations also occur in MOR KO mice. We acquired anatomical images using a 7-Tesla MRI scanner and measured deformation-based morphometry (DBM) for each voxel in subjects from MOR KO and control groups. Our analysis shows marked anatomical differences in mutant animals. We observed both local volumetric contraction (striatum, nucleus accumbens, bed nucleus of the stria terminalis, hippocampus, hypothalamus and periacqueducal gray) and expansion (prefrontal cortex, amygdala, habenula, and periacqueducal gray) at voxel level. Volumetric modifications occurred mainly in MOR-enriched regions and across reward/aversion centers, consistent with our prior FC findings. Specifically, several regions with volume differences corresponded to components showing highest FC changes in our previous Rs-fMRI study, suggesting a possible function-structure relationship in MOR KO-related brain differences. In conclusion, both Rs-fMRI and volumetric MRI in live MOR KO mice concur to disclose functional and structural whole-brain level mechanisms that likely drive MOR-controlled behaviors in animals, and may translate to MOR-associated endophenotypes or disease in humans.

Keywords: MRI, *in-vivo*, mice, structural change, mu opioid receptor, anatomical

INTRODUCTION

The muopioid receptor (MOR) is an inhibitory G protein-coupled receptor (GPCR) belonging to the opioid receptor family (1, 2). MOR is activated by endogenous opioid peptides and by exogeneous opiates like morphine (3). MOR activation alleviates aversive states such as physical or social pain (4–7) and drives natural and drug reward processes (2, 8). Misuse and abuse of MOR agonists may cause addiction and overdose, a main cause for the rising opioid epidemics in North America (9).

Mice lacking MOR display several behavioral alterations such as increased pain perception (7) and reduced drug reward (10). Furthermore, mice with a deletion of MOR show altered sensitivity to natural rewards, as shown by their lower motivation to eat (11) and reduced maternal attachment (12). We further demonstrated that adult MOR knockout (MOR KO) animals recapitulate core and multiple comorbid behavioral symptoms of autism, including deficient social abilities, aggressiveness and stereotyped behaviors, high anxiety, impaired motor coordination and increased sensitivity to seizures, and these behavioral symptoms are associated with anatomical alterations (13). Additionally, the gene MOR deletion in mice reshapes functional connectivity in live animals (14). In the latter study, we combined blood oxygenation level–dependent (BOLD) Resting-state (Rs) functional Magnetic Resonance Imaging (fMRI) and diffusion tractography (DTI), and found pronounced modifications of whole-brain functional connectivity (FC) with only minor changes in structural connectivity (14). Strongest perturbations occurred in connectional patterns across the reward/aversion circuitry, with predominant alterations of pain/aversion-related networks (14).

Because these modifications reveal circuit mechanisms underlying behavioral alterations in these mice (increased pain sensitivity and reward deficits), we sought to further explore structural abnormalities in MOR KO mice using most state-of-the-art anatomical MRI. We used high-resolution structural image acquisition in living MOR KO mice and their controls (CTLs), and identified anatomical differences caused by the deletion of MOR using a voxelwise deformation-based morphometry (DBM) analysis (**Figure 1A**). Our data extend a previous study (15) and reveal structural changes that parallel FC alterations that we observed in our previous study using Rs-fMRI approach in MOR KO and controls (14).

MATERIALS AND METHODS

Mice

All experiments were performed following the guidelines on animal experimentation established by the Canadian Council of Animal Care and animal protocol was approved by the Animal Care Committees of McGill University/Douglas Mental Health University Institute, Montreal, Canada (#2014-2018/7466).18 MOR KO and 18 CTLs mice were produced as described in Matthes et al. (3) and were bred at the Douglas Research Center, Montreal, Canada. Mice were kept under standard conditions at $22 \pm 1^{\circ}$C, 60% relative humidity, and 12-h light-dark cycle with food and water available *ad libitum*. All *in-vivo* MRI experiments were performed in homozygote MOR CTLs and KO of 10–12 weeks old male mice.

MRI Anatomical Acquisition

Mouse brain MRI data acquisition was performed with a 7T small animal scanner (BioSpec 70/30USR, Bruker) using 23 mm volumetric coil (Bruker). Animals were anesthetized by inhalation of isoflurane during the image acquisition. Five minutes before the start of the acquisition, mice were placed in an anesthesia chamber receiving 5% isoflurane. Then, the animals were placed into a specific animal bed system (Bruker) receiving 1.5–2% isoflurane during the whole procedure. Respiration was monitored and maintained between 45 and 70 breaths per minute using a 1025-IBP-50 Small Animal Monitoring Gating System (SA instruments). Body temperature was maintained constant at 37°C inside the magnetic bore by blowing warm air on the animal and eye lubricant was applied. MRI data were collected using 3D True-fast imaging with steady state precession (3D-true FISP) with the following parameters: matrix $128 \times 128 \times 64$, image resolution $140 \times 140 \times 140$ μm^3, echo time (TE)/repetition time (TR) = 2.6 ms/800 ms. Eight radio-frequency (RF) angles (180, 0, 90, 270, 45, 225, 135, and 315 degree) were used to remove banding artifacts and the time of the whole sequence lasted for 40 min for each subject (16). Finally, root mean square (RMS) of the 8 angle acquisitions were calculated for each subject.

Registration and Analysis

Voxelwise deformation-based morphometry (DBM) was used to analyze the anatomical differences all over the brain (17, 18) following the procedure detailed in Lerch et al. (19). Briefly, mice brains were registered together through a series of linear (6 parameter followed by a 12 parameter) and nonlinear registration steps (20) to create a group-wise average. The deformation fields map the minimum deformation required at a voxel-level to map each subject to the average neuroanatomy of the group. Then, the Jacobian determinants of the deformation fields were used to measure local anatomical differences (19). These alterations could either be expansions or contractions and are dependent of the magnitude of the deformation at each voxel (19). The deformations were then mapped voxelwise by using standard tools including minc-toolkit (http://www.bic.mni.mcgill.ca/ServicesSoftware/ServicesSoftwareMincToolKit, RMINC (https://wiki.mouseimaging.ca/display/MICePub/RMINC, (rstudio (https://www.rstudio.com/, anaconda (https://repo.continuum.io/archive/and pydpiper (https://github.com/Mouse-Imaging-Center/pydpiper. For statistical analysis, we used the general linear model, with the log of the local deformation being modeled with 18 covariates and group. Group differences were measured via the t-statistic from the linear model and corrected for multiple comparisons using FDR at 20% (RMINC, https://wiki.mouseimaging.ca/display/MICePub/RMINCRMINC was also used for single voxel boxcar plotting from Jacobians of individual subjects from each group.

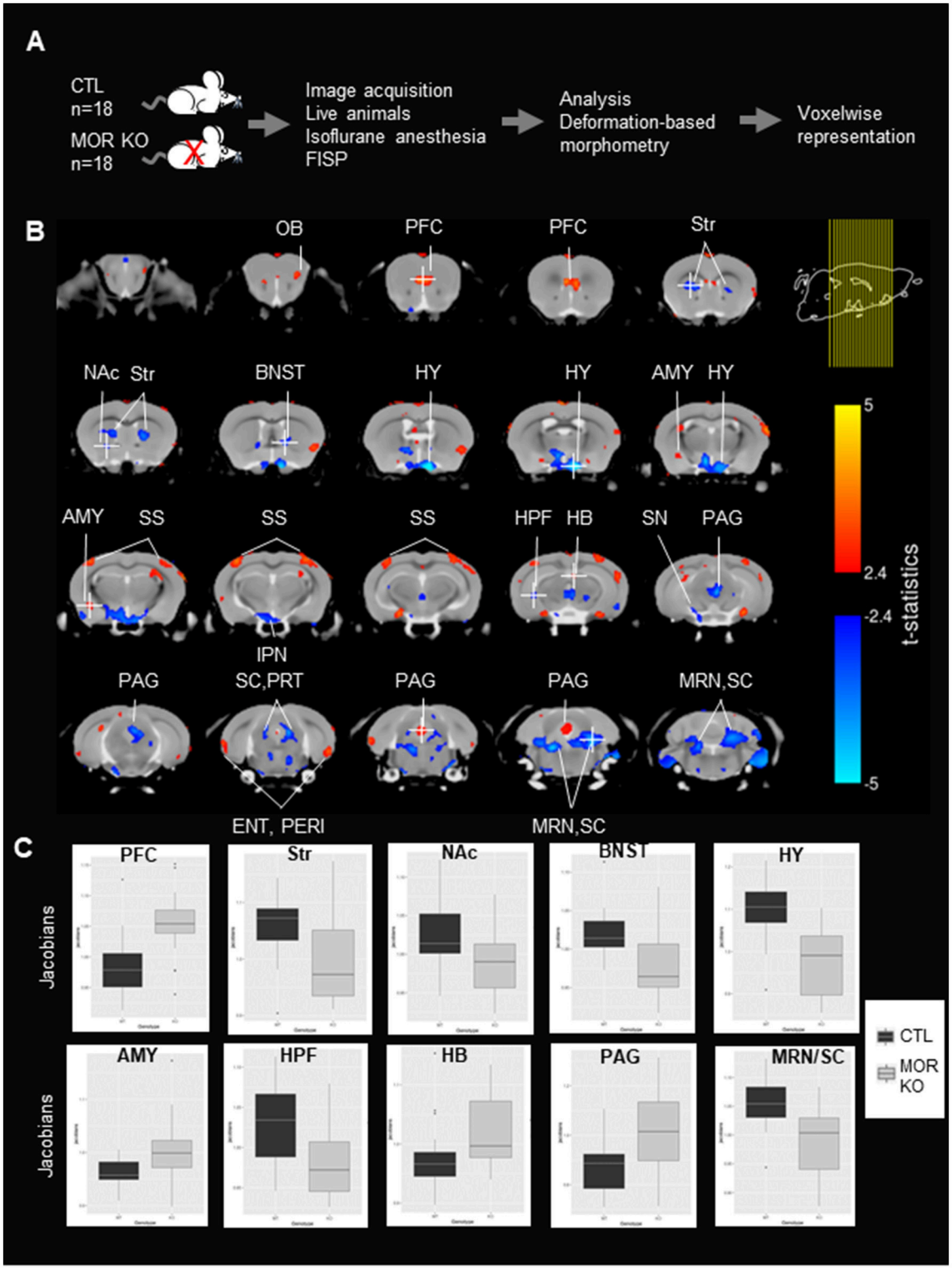

FIGURE 1 | Local volumes significantly differ between controls (CTLs) and MOR Knockout (KO) mice. **(A)** Experimental timeline. Experiment was performed in CTLs and MOR KO groups ($n = 18$). MRI images were scanned following true-FISP sequences in live animals under isoflurane anesthesia. Anatomical differences in MOR KO animals were calculated using the deformation-based morphometry (DBM) and are represented voxelwise. **(B)** Significant anatomical differences (*t-statistic*, $p <$ 0.05 and FDR correction) between CTLs and MOR KO represented on a coronal. Coronal slices are represented from anterior to posterior. Right top corner shows the

(Continued)

FIGURE 1 | location of brain mouse slices (yellow lines). Red and blue colors represent statistically significant increase and decrease of anatomical volume of MOR KO in comparison to CTLs mice. The t-statistics scale for the significant expansion are in yellow to red and for the significant contraction are in light blue to blue. The brain regions were identified using Allen brain atlas. Most regions with significant modifications are annotated. Voxels of the Str, NAc, BNST, HY, IPN, HPF, SN SC/PRT, MRN/SC are contracted, whereas voxels of the PFC, AMY, SS, HB, and PAG have expanded in mutant mice. **(C)**. Boxcar plot of relative Jacobians from both CTL and KO groups on 10 selected voxels indicated in **(B)**, and the voxel is identified with a white cross. OB, Olfactory Bulb; PFC, prefrontal cortex; NAc, Nucleus Accumbens; BNST, Bed nucleus of the stria terminalis; AMY, Amygdala; HY, hypothalamus; IPN, Interpeduncular nucleus; SS, Somatosensory cortex; HB, Habenula; TH, Thalamus; SN, Substantia Nigra; Str, striatum; HPF, hippocampal formation; SC, Superior Colliculus; PRT, Pretectal Region; PAG, Periaqueductal Gray; MRN, Midbrain Reticular Nucleus.

RESULTS

Volume Changes Are Observed in the Brain of Live MOR KO Mice

The experimental flow is shown in **Figure 1A**. MOR KO mice and their CTLs (18 mice/group) were slightly sedated with isoflurane to avoid motion, and True-FISP sequences were used to acquire high-resolution anatomical images (16). Images were registered together through a series of linear and nonlinear fits, and deformation-based morphometry was calculated on relative Jacobians and used to compare brain local volumes (total number of voxels in the field of view = 702 720 voxels) between the two groups (see section Materials and Methods). Statistical differences between anatomical volumes from MOR KO and CTL groups were determined using parametric *t-statistic* ($p < 0.05$) and FDR correction (see section Materials and Methods), and significant anatomical differences are represented in coronal (**Figure 1B**) views. Local volume changes were detected in several brain areas, which we identified based on the Allen Brain Atlas. Groups of voxels showing an expansion of volume in MOR KO brains were located in the Olfactory Bulb (OB), Prefrontal Cortex (PFC), amygdala (AMY), somatosensory cortex (SS), habenula (HB) and periaqueductal gray (PAG) (**Figure 1B**). Sets of voxels with a local contraction were in the striatum (Str), Nucleus Accumbens (NAc), the bed nucleus of the stria terminalis (BNST), hypothalamus (HY), hippocampal formation (HPF), Interpeduncular nucleus (IPN), substantia nigra (SN), Superior Colliculus (SC) and midbrain reticular nucleus (MRN) (**Figure 1B**). Several groups of voxels showed a bilateral alteration, and these include the dorsal striatum, SS, MRN, HY and SC (**Figure 1B**). Furthermore, quantification of relative Jacobians in regional center-voxel confirmed the reduction (Str, NAc, BNST, HY, HPF, and MRN/SC) or increase (PFC, AMY, and HAB) volumes of altered regions, which were identified using DBM (**Figure 1C**). All these areas are known to express the receptor (21), with some of them particularly MOR-enriched (PAG, HB, AMY, Str, NAc, HY, SC), and all belong to reward/aversion centers (see summary in **Figure 2A**) networks (14).

DISCUSSION

Previous high-resolution MRI studies mainly used post-mortem brains to characterize anatomical modifications in knockout mouse lines (17, 18) or drug effects (22), to take advantage of extended scanning time without motion artifacts**,** and increased tissue contrast, however the brain fixation process could induce some deformation artifacts (23). We therefore conducted this study in live animals and used a reasonably long scanning time to maximize resolution while minimizing motion issues. We achieved an isotropic resolution of 140 μm with our live imaging acquisitions of 40 min, which reached about a third of what is achieved with post-mortem imaging of 702 min (isotropic resolution of 56 (18)). Our volumetric analysis of live MOR KO brains demonstrates significant modifications of local brain volumes in brains areas with known implication for reward and/or aversion processing.

MOR is distributed throughout the brain with enriched expression in the striatum, the medial habenula and moderate density in NAc and SN (21). These brain areas correspond to regions with reduced (Str, NAc, and SN) or increased (HB) brain volume in MOR KO mice, suggesting that MOR activity locally influences brain microstructure. Notably also the MOR deletion had significant effects in areas with poor receptor density (PFC and HPF), suggesting long distance influence of MOR activity on brain structure.

An earlier study investigated the brain of live MOR KO mice using voxel-based morphometry (15). The authors used distinct acquisition parameters that led to a resolution (125 × 125 × 300 μm) and their DBM analysis revealed significant volume increase for OB, HY, PAG, and cerebellum. Our study (140 × 140 × 140 μm) corroborates some of the previous findings as we also observed expanded PAG and OB local volumes, among others (**Figures 1B,C**). In addition, we also observed contraction of voxel volumes in several brain areas, including HPF, Str, NAc, HY, BST, SN, SC, and MRN, further supported by the observation of bilateral volume reduction for Str and HY. Our study therefore expands previous knowledge about MOR effects on brain structure. Mechanisms underlying changes in volume after the MOR deletion will require further investigations. Sasaki and colleagues showed a higher number of glial and neuronal cells in the PAG of MOR KO mice (24), which may explain some variations in brain volumes in their report and also our own study. Furthermore, it will be important in the future to determine whether volume changes arise from a developmental role of endogenous opioids, or from the lack of MOR activity at the adult age. Further imaging studies using pharmacological MOR blockade will appraise the developmental contribution of MOR activity in brain volumes.

Overall, volume changes occur throughout centers of reward and aversion processing (summarized in **Figure 2A**). Voxel contraction is consistently observed across reward centers, whereas both expansion and contraction are observed in aversion

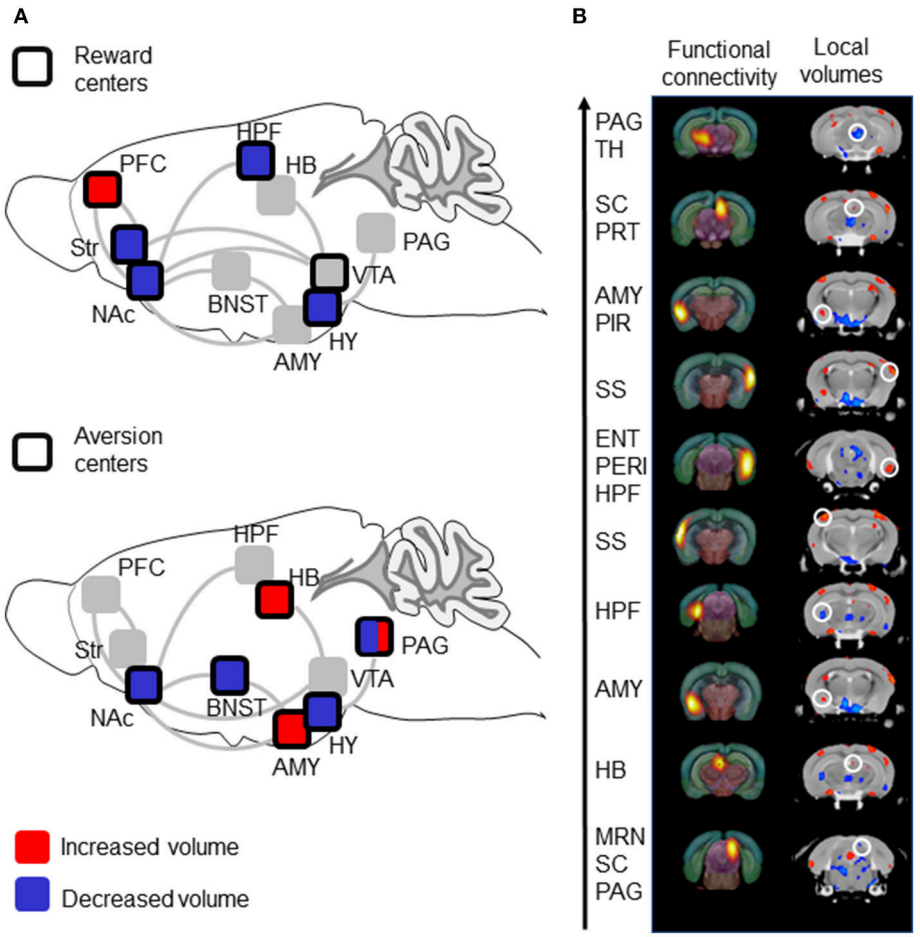

FIGURE 2 | Volumetric data show decreased volumes in reward centers with contrasted effects in aversion centers, and show correspondence with previous Rs-fMRI data. **(A)** Summary of volumetric analysis. Reward centers show reduced volumes while aversion center show both reduced and increased volumes. The scheme is adapted from Darcq and Kieffer (2) and shows main brain structures involved in reward/aversion, as well as addiction. Top: Areas belonging to reward circuitry are highlighted (dark square) and volume alterations in MOR KO mice are color-coded. Bottom: Areas belonging to aversion circuitry are highlighted (dark square) and volume alterations in MOR KO mice are color-coded. PFC, Prefrontal cortex; PAG, Periaqueductal Gray; TH, Thalamus; AMY, Amygdala; SC, Superior Colliculus; PRT, Pretectal Region; PIR, Piriform area; SS, Somatosensory cortex; ENT, Entorhinal area; PERI, Perirhinal area; HPF, hippocampal; MRN, Midbrain Reticular Nucleus; HY, hypothalamus; VTA, ventral tegmental Area; BNST, Bed nucleus of the stria terminalis. **(B)** Correspondence of functional nodes (Rs-fMRI) and regions (DBM) showing significant modifications in MOR KO from our previous study (14) and in this study, respectively. The 10 nodes with highest number of statistically significant FC changes from the Rs-fMRI study are represented in the left column, and ranked (arrow) from higher (top) to lower (bottom) modifications (14). Volumetric differences between MOR KO and CTLs in this study are represented in the right column. Comparable coronal sections are displayed, and a white circle highlights local volume changes corresponding to the 10-top nodes.

centers. Although this particular observation should not be overinterpreted, it is fair to propose that anatomical sites of volume changes are consistent with behavioral phenotypes of MOR KO mice. The genetic MOR deletion reduces both natural and drug rewards (2, 8), and we found reduced volumes for Str and NAc that are critical for reward processing (25). The MOR deletion also increases intracranial electrical stimulation in lateral hypothalamus (26), and bilateral reduction of the entire HY volume observed here may reflect a reduced function for this other key area of motivated behavior and reward (27). The MOR deletion increases pain perception (4, 7) and we found increased volume of PAG, critical in pain signal processing (28). Emotional behaviors are altered in MOR KO mice (29) paralleling volume changes in BNST and AMY known to regulate anxiety and stress responses (30–32).

An important aspect of the study is the observation that volumetric modifications parallel FC alterations that we previously reported using Rs-fMRI ((14) and see **Figure 2B**). In this previous Rs-FC study, we used data-driven spatial independent component analysis (100-ICASSO) of Rs-fMRI datasets, and identified 87 functional components (clusters of voxel showing correlated and/or anticorrelated activities), which we used as nodes to establish whole brain FC matrixes for each MOR KO and CTL group and compare the two groups. A MOR-dependent FC signature emerged and, to identify most

prominent alterations in MOR KO mice, we ranked these nodes based on the number of statistically significant FC changes (14). Here we considered the top-10 nodes from the Rs-fMRI study, and found that these nodes visually correspond with groups of voxels, which also show significant changes in local volumes (**Figure 2B**). Specifically, PAG/Thalamus (Th), SC/PRT, AMY/Piriform (PIR), right and left SS, entorhinal area (ENT), AMY, HB components from the Mechling study match regions with enhanced volumes in this study, while HPF and MRN/SC/PAG nodes match with reduced volumes (**Figure 2B**). Although volumetric images in this study were not co-registered with the same atlas than in Mechling et al. (14), the visual inspection therefore strongly suggest that the top-10 components with highest number of FC alterations overlap with regions showing volume modifications in MOR KO mice (**Figure 2B**). Concomitant structural and functional alterations for these 10 nodes, therefore, strengthen the notion that activity of these brain centers is regulated by MOR. Of note however, there is no obvious correspondence between modifications of volume size (expanded or contracted) and FC strength/diversity (enhanced or reduced), and further analysis will be necessary to determine whether variations of brain volume reflect FC plasticity upon the MOR genetic deletion.

In conclusion, progress in human anatomical MRI has greatly advanced our understanding of brain structure–function relations (33), and animal MRI is developing to allow translatable insights into drug effects (34) or vulnerability to disease (17). Future similar studies using humanized mice (35), and/or mouse exposure to chronic opiates, may pave the way to understanding mechanisms underlying the link between MOR gene variability and vulnerability to disease, or differential activities of MOR opioid agonists with distinct therapeutic profile at whole-brain level (2).

AUTHOR CONTRIBUTIONS

MC, BK, and ED designed the study; AM, L-AH, and ED acquired the data, MN, GD, AM, L-AH, and ED performed the analysis; MN, BK, and ED wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REFERENCES

1. Stein C. Opioid receptors. *Annu Rev Med*. (2016) 67:433–51. doi: 10.1146/annurev-med-062613-093100
2. Darcq E, Kieffer BL. Opioid receptors: drivers to addiction? *Nat Rev Neurosci*. (2018) 19:499–514. doi: 10.1038/s41583-018-0028-x
3. Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, et al. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* (1996) 383:819–23. doi: 10.1038/383819a0
4. Leknes S, Tracey I. A common neurobiology for pain and pleasure. *Nat Rev Neurosci*. (2008) 9:314–20. doi: 10.1038/nrn2333
5. Eisenberger NI. The pain of social disconnection: examining the shared neural underpinnings of physical and social pain. *Nat Rev Neurosci*. (2012) 13:421–34. doi: 10.1038/nrn3231
6. Borsook D, Edwards R, Elman I, Becerra L, Levine J. Pain and analgesia: the value of salience circuits. *Prog Neurobiol*. (2013) 104:93–105. doi: 10.1016/j.pneurobio.2013.02.003
7. Corder G, Castro DC, Bruchas MR, Scherrer G. Endogenous and exogenous opioids in pain. (2018). *Annu Rev Neurosci*. 41:453–73. doi: 10.1146/annurev-neuro-080317-061522
8. Contet C, Kieffer BL, Befort K. Mu opioid receptor: a gateway to drug addiction. *Curr Opin Neurobiol*. (2004) 14:370–8. doi: 10.1016/j.conb.2004.05.005
9. Compton WM, Jones CM, Baldwin GT. Relationship between nonmedical prescription-opioid use and heroin use. *N Engl J Med*. (2016) 374:154–63. doi: 10.1056/NEJMra1508490
10. Charbogne P, Kieffer BL, Befort K. 15 years of genetic approaches *in vivo* for addiction research: opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology* (2014) 76(Pt B):204–17. doi: 10.1016/j.neuropharm.2013.08.028
11. Charbogne P, Gardon O, Martin-Garcia E, Keyworth HL, Matsui A, Mechling AE, et al. Mu opioid receptors in gamma-aminobutyric acidergic forebrain neurons moderate motivation for heroin and palatable food. *Biol Psychiatry* (2017) 81:778–88. doi: 10.1016/j.biopsych.2016.12.022
12. Moles A, Kieffer BL, D'amato FR. Deficit in attachment behavior in mice lacking the mu-opioid receptor gene. *Science* (2004) 304:1983–6. doi: 10.1126/science.1095943
13. Becker JA, Clesse D, Spiegelhalter C, Schwab Y, Le Merrer J, Kieffer BL. Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity. *Neuropsychopharmacology* (2014) 39:2049–60. doi: 10.1038/npp.2014.59
14. Mechling AE, Arefin T, Lee HL, Bienert T, Reisert M, Ben Hamida S, et al. Deletion of the mu opioid receptor gene in mice reshapes the reward-aversion connectome. *Proc Natl Acad Sci USA* (2016) 113:11603–8. doi: 10.1073/pnas.1601640113
15. Sasaki K, Sumiyoshi A, Nonaka H, Kasahara Y, Ikeda K, Hall FS, et al. Specific regions display altered grey matter volume in mu-opioid receptor knockout mice: MRI voxel-based morphometry. *Br J Pharmacol*. (2015) 172:654–67. doi: 10.1111/bph.12807
16. Guma E, Rocchetti J, Devenyi GA, Tanti A, Mathieu A, Lerch JP, et al. Regional brain volume changes following chronic antipsychotic administration are mediated by the dopamine D2 receptor. *Neuroimage* (2018) 176:226–38. doi: 10.1016/j.neuroimage.2018.04.054
17. Ellegood J, Anagnostou E, Babineau BA, Crawley JN, Lin L, Genestine M, et al. Clustering autism: using neuroanatomical differences in 26 mouse models to gain insight into the heterogeneity. *Mol Psychiatry* (2015) 20:118–25. doi: 10.1038/mp.2014.98
18. Ellegood J, Yee Y, Kerr TM, Muller CL, Blakely RD, Henkelman RM, et al. Analysis of neuroanatomical differences in mice with genetically modified serotonin transporters assessed by structural magnetic resonance imaging. *Mol Autism* (2018) 9:24. doi: 10.1186/s13229-018-0210-z

19. Lerch JP, Sled JG, Henkelman RM. MRI phenotyping of genetically altered mice. *Methods Mol Biol*. (2011) 711:349–61. doi: 10.1007/978-1-61737-992-5_17
20. Friedel M, Van Eede MC, Pipitone J, Chakravarty MM, Lerch JP. Pydpiper: a flexible toolkit for constructing novel registration pipelines. *Front Neuroinform*. (2014) 8:67. doi: 10.3389/fninf.2014.00067
21. Erbs E, Faget L, Scherrer G, Matifas A, Filliol D, Vonesch JL, et al. A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct Funct*. (2015) 220:677–702. doi: 10.1007/s00429-014-0717-9
22. Wheeler AL, Lerch JP, Chakravarty MM, Friedel M, Sled JG, Fletcher PJ, et al. Adolescent cocaine exposure causes enduring macroscale changes in mouse brain structure. *J Neurosci*. (2013) 33:1797–803a. doi: 10.1523/JNEUROSCI.3830-12.2013
23. Oguz I, Yaxley R, Budin F, Hoogstoel M, Lee J, Maltbie E, et al. Comparison of magnetic resonance imaging in live vs. post mortem rat brains. *PLoS ONE* (2013) 8:e71027. doi: 10.1371/journal.pone.0071027
24. Sasaki K, Hall FS, Uhl GR, Sora I. Larger numbers of glial and neuronal cells in the periaqueductal gray matter of mu-opioid receptor knockout mice. *Front Psychiatry* (2018) 9:441. doi: 10.3389/fpsyt.2018.00441
25. Schultz W. Multiple reward signals in the brain. *Nat Rev Neurosci*. (2000) 1:199–207. doi: 10.1038/35044563
26. Ide S, Takahashi T, Takamatsu Y, Uhl GR, Niki H, Sora I, et al. Distinct roles of opioid and dopamine systems in lateral hypothalamic intracranial self-stimulation. *Int J Neuropsychopharmacol*. (2017) 20:403–9. doi: 10.1093/ijnp/pyw113
27. Stuber GD, Wise RA. Lateral hypothalamic circuits for feeding and reward. *Nat Neurosci*. (2016) 19:198–205. doi: 10.1038/nn.4220
28. Behbehani MM. Functional characteristics of the midbrain periaqueductal gray. *Prog Neurobiol*. (1995) 46:575–605. doi: 10.1016/0301-0082(95)00009-K
29. Lutz PE, Kieffer BL. Opioid receptors: distinct roles in mood disorders. *Trends Neurosci*. (2013) 36:195–206. doi: 10.1016/j.tins.2012.11.002
30. Koob GF. Brain stress systems in the amygdala and addiction. *Brain Res*. (2009) 1293:61–75. doi: 10.1016/j.brainres.2009.03.038
31. Lebow MA, Chen A. Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol Psychiatry* (2016) 21:450–63. doi: 10.1038/mp.2016.1
32. Fox AS, Shackman AJ. The central extended amygdala in fear and anxiety: closing the gap between mechanistic and neuroimaging research. *Neurosci Lett*. (2017). doi: 10.1016/j.neulet.2017.11.056. [Epub ahead of print].
33. Lerch JP, Van Der Kouwe AJ, Raznahan A, Paus T, Johansen-Berg H, Miller KL, et al. Studying neuroanatomy using MRI. *Nat Neurosci*. (2017) 20:314–26. doi: 10.1038/nn.4501
34. Becerra L, Upadhyay J, Chang PC, Bishop J, Anderson J, Baumgartner R, et al. Parallel buprenorphine phMRI responses in conscious rodents and healthy human subjects. *J Pharmacol Exp Ther*. (2013) 345:41–51. doi: 10.1124/jpet.112.201145
35. Ramchandani VA, Umhau J, Pavon FJ, Ruiz-Velasco V, Margas W, Sun H, et al. A genetic determinant of the striatal dopamine response to alcohol in men. *Mol Psychiatry* (2011) 16:809–17. doi: 10.1038/mp.2010.56

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REVIEW
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Advances in Achieving Opioid Analgesia Without Side Effects

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Opioids are the most effective drugs for the treatment of severe pain, but they also cause addiction and overdose deaths, which have led to a worldwide opioid crisis. Therefore, the development of safer opioids is urgently needed. In this article, we provide a critical overview of emerging opioid-based strategies aimed at effective pain relief and improved side effect profiles. These approaches comprise biased agonism, the targeting of (i) opioid receptors in peripheral inflamed tissue (by reducing agonist access to the brain, the use of nanocarriers, or low pH-sensitive agonists); (ii) heteromers or multiple receptors (by monovalent, bivalent, and multifunctional ligands); (iii) receptor splice variants; and (iv) endogenous opioid peptides (by preventing their degradation or enhancing their production by gene transfer). Substantial advancements are underscored by pharmaceutical development of new opioids such as peripheral κ-receptor agonists, and by treatments augmenting the action of endogenous opioids, which have entered clinical trials. Additionally, there are several promising novel opioids comprehensively examined in preclinical studies, but also strategies such as biased agonism, which might require careful rethinking.

Keywords: opioid receptor signaling, opioid side effects, addiction, pain, peripheral opioid analgesia, biased agonists, heteromers, endogenous opioid peptides

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INTRODUCTION

Opioids relieve pain, but also produce numerous side effects. All actions of opioids are mediated by μ-, δ-, and κ-opioid receptors encoded by the three respective genes (Evans et al., 1992; Kieffer et al., 1992; Mestek et al., 1995; Simonin et al., 1995). Opioid receptors belong to the superfamily of guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) and their structures have been solved at high-resolution by X-ray crystallography (Granier et al., 2012; Huang et al., 2015; Che et al., 2018). Upon activation by an agonist, opioid receptors couple to pertussis toxin-sensitive heterotrimeric Gi/o proteins, which dissociate into Gαi/o and Gβγ subunits to interact with various intracellular effector systems (Law et al., 2000; Waldhoer et al., 2004; Stein, 2016). Gαi/o inhibits adenylyl cyclases (AC), cyclic adenosine monophosphate (cAMP) formation, and protein kinase A (PKA) activity, which leads to the blockade of a heat sensor transient receptor potential cation channel subfamily V member 1 (TRPV1) (Vetter et al., 2006; Endres-Becker et al., 2007). Gαi/o–cAMP pathway also suppresses hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, acid-sensing ion channels (ASIC), and voltage-gated Na^+ (Na_v) channels (Ingram and Williams, 1994; Gold and Levine, 1996; Cai et al., 2014). Gβγ blocks voltage-gated Ca^{2+} (Ca_v) channels and heat-sensing transient receptor potential cation channel subfamily M member 3 (TRPM3), and activates various K^+ channels such as G protein-coupled inwardly

rectifying K^+ (GIRK or $K_{ir}3$) channels and adenosine triphosphate-sensitive K^+ (K_{ATP}) channels (Law et al., 2000; Waldhoer et al., 2004; Cunha et al., 2010; Stein, 2016; Dembla et al., 2017). Ultimately, these opioid-mediated actions lead to the suppression of excitatory neurotransmitter release (e.g., substance P, calcitonin gene-related peptide, glutamate), hyperpolarization and an overall decrease in neuronal excitability, which culminates in analgesia (Yaksh, 1997; Ocaña et al., 2004; Stein, 2016; Yudin and Rohacs, 2018) (**Figure 1A**). Additionally, analgesia can be mediated by opioid receptors expressed in immune cells. Activation of leukocyte opioid receptors leads to the secretion of endogenous opioid peptides (β-endorphin, Met-enkephalin, and dynorphin A 1-17), which involves Gαi/o–Gβγ–phospholipase C (PLC)–inositol 1,4,5-trisphosphate receptor (IP_3R)– intracellular Ca^{2+} pathway. The released opioid peptides subsequently activate neuronal opioid receptors and alleviate pain (Celik et al., 2016) (**Figure 1B**).

Opioid receptors also mediate numerous adverse effects that limits opioid pain therapy. Activation of μ-receptors can lead to respiratory depression, sedation, constipation, nausea, vomiting, reward/euphoria, and dependence/withdrawal. Activation of δ-receptors can cause convulsions and may produce reward or contribute to rewarding effects of other drugs of abuse. Agonists of κ-receptors exert aversion/dysphoria, sedation, and diuresis (i.e., increased urine output). Each of these symptoms represents a complex phenomenon with multiplex cellular and molecular mechanisms (Kapusta, 1995; Li and van den Pol, 2008; Bruijnzeel, 2009; Koob and Volkow, 2010; Gendron et al., 2016; Dripps et al., 2018). Importantly, these side effects are brought about by G protein-mediated actions in response to opioid receptor activation (**Figure 2A**). Opioid-induced respiratory depression is mediated by Gβγ-dependent activation of GIRK channels, which results in inhibition of neurons in the brainstem respiratory center (Montandon et al., 2016). Sedation is a consequence of the suppression of neurons in the hypothalamic arousal system, which depends on Gβγ actions on GIRK and Ca_v channels (Li and van den Pol, 2008). Constipation results from Gβγ-mediated activation of GIRK channels and inhibition of Ca_v channels leading to the suppression of enteric neuronal activity, including acetylcholine and substance P secretion blockade in the gastrointestinal tract (Galligan and Akbarali, 2014). Indirect evidence suggests that nausea and vomiting may involve Gαi/o-mediated decrease of the cAMP–PKA pathway activity, blockade of Ca_v channels and thus, inhibition of neurons in the vestibular apparatus (Seseña et al., 2014; Imam et al., 2017). Opioid-induced diuresis results from the inhibition of arginine vasopressin secretion in the hypothalamus, suggestive of G protein involvement, although the exact signaling pathways have not been elucidated (Kapusta, 1995). Chronic opioid use leads to Gβγ–cAMP–PKA pathway activation resulting in enhanced activity of ion channels (e.g., Na_v channels) and receptors (e.g., dopamine and *N*-methyl-D-aspartic acid receptors) and thereby, in increased neuronal activity (Nestler and Aghajanian, 1997; Liu and Anand, 2001; Christie, 2008). Furthermore, prolonged activation of opioid receptors results in Gβγ-dependent activation of protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II, and extracellular signal-regulated kinases 1 and 2 of the mitogen-activated protein kinases (MAPKs). These kinases as well as PKA can phosphorylate opioid receptors, which results in their uncoupling from G protein-mediated effects (Liu and Anand, 2001; Christie, 2008; Al-Hasani and Bruchas, 2011). These events have been ascribed to alterations in opioid receptor signaling underlying analgesic tolerance, reward/euphoria, dependence/withdrawal, or aversion/dysphoria (Nestler and Aghajanian, 1997; Law et al., 2000; Liu and Anand, 2001; Waldhoer et al., 2004; Christie, 2008; Koob and Volkow, 2010; Al-Hasani and Bruchas, 2011; Gendron et al., 2016) (**Figure 2A**). Additionally, opioid receptors are phosphorylated by GPCR kinases (GRKs), which is followed by recruitment of β-arrestins (**Figure 2B**). This process occurs after even brief agonist exposure and it terminates G protein coupling and signaling to promote receptor desensitization and internalization. Dephosphorylated opioid receptors can be recycled to the plasma membrane, which reinstates signaling, or can be targeted to lysosomes and degraded (Waldhoer et al., 2004). β-arrestin-2 (also known as arrestin-3) might be involved in morphine-induced analgesic tolerance, respiratory depression, and constipation (Bohn et al., 2000; Raehal et al., 2005). It has also been proposed to mediate κ-receptor-induced aversion via activation of p38 MAPK (Bruchas et al., 2006; Land et al., 2009; Ehrich et al., 2015). Nevertheless, the exact β-arrestin-2-regulated signaling underlying opioid-induced side effects are yet unclear (**Figure 2B**). The cellular mechanisms of δ-receptor-mediated convulsions have not been identified, and do not seem to involve Gαo or β-arrestin-2 (Dripps et al., 2018).

Clearly, conventional opioids produce numerous side effects, yet they are the strongest painkillers. As all other, non-opioid pain medications also exert adverse actions, none of them produces as powerful pain relief as opioids (Stein and Kopf, 2009; Sondergaard and Gislason, 2017; Welsch et al., 2018). Therefore, opioids will remain the main therapy for moderate and severe pain, which makes efforts to improve their action profile highly desirable and relevant. In the following sections, we present several interesting strategies to achieve safer opioid analgesia, and discuss limitations associated with these new approaches.

TARGETING OPIOID RECEPTORS IN PAINFUL TISSUE

The Rationale

All three opioid receptors (μ, δ, and κ) are expressed in the central nervous system (CNS), including spinal cord and brain, as well as in peripheral sensory neurons (nociceptors). Peripheral opioid receptors are synthetized in nociceptor cell bodies in trigeminal and dorsal root ganglia (DRG), from where they are transported and accumulate in nociceptor peripheral terminals innervating peripheral tissue (skin, joints, viscera) (**Figures 1B**, **3A**). The concept of targeting peripheral opioid receptors comes from the fact that they mediate effective analgesia, but are not involved in fatal effects, in animal models and in humans (Kalso et al., 2002;

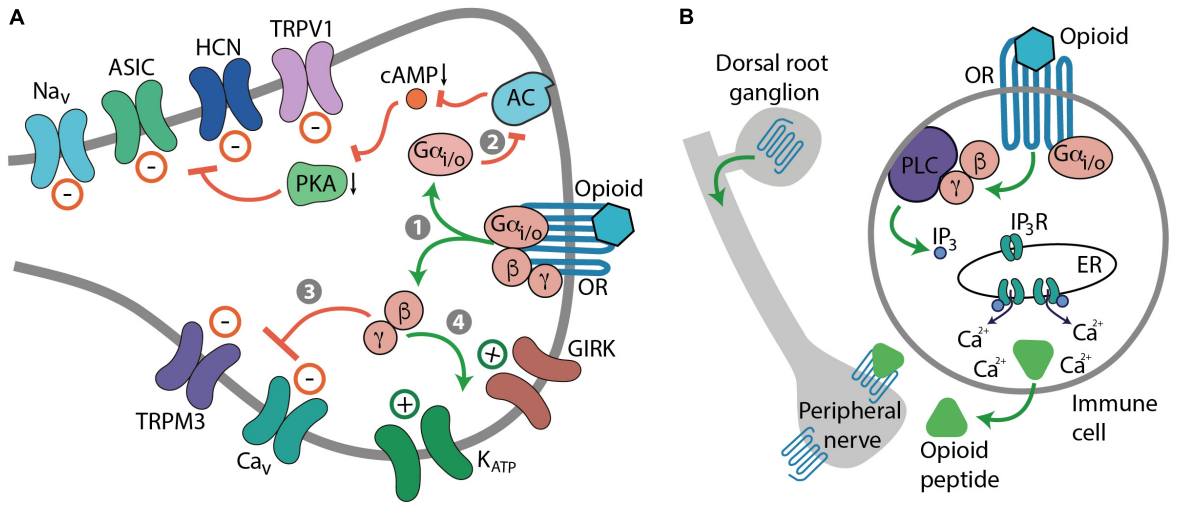

FIGURE 1 | Mechanisms of opioid-induced analgesia. **(A)** Cellular effects mediated by neuronal opioid receptors (OR). Activation of OR by an opioid leads to the dissociation of Gi/o proteins into Gαi/o and Gβγ subunits (step 1). Gαi/o inhibits AC, cAMP formation, and PKA activity, which blocks various ion channels, including TRPV1, HCN, ASIC, and Na_V channels (path 2). Gβγ blocks Ca_V and TRPM3 channels (path 3), and activates GIRK and K_{ATP} channels (path 4). Ultimately, these actions lead to the decrease in neuronal excitability, which culminates in analgesia. **(B)** Cellular effects mediated by OR in immune cells. Activation of leukocyte Gi/o-coupled OR leads to the Gβγ-mediated activation of PLC and production of IP_3, which activates IP_3R in endoplasmic reticulum (ER) to release intracellular Ca^{2+}, which results in the secretion of opioid peptides from immune cells. The released opioid peptides activate neuronal OR and decrease pain.

Stein et al., 2003; Stein and Machelska, 2011; Sawynok and Liu, 2014). Indeed, the serious side effects arise from opioid actions in the brain (**Figure 3B**). Respiratory depression results from activation of μ-receptors in the brainstem medulla (preBötzinger complex) and pons (Kölliker-Fuse nucleus, parabrachial nuclei, locus coeruleus), in cortical areas, thalamus, and amygdala, and to a lesser extent in the periphery in the carotid body (Pattinson, 2008; Imam et al., 2017). Reward and dependence/withdrawal mediated by μ-receptors, as well as aversion/dysphoria mediated by κ-receptors involve a widely distributed brain network, including the mesolimbic pathway (ventral tegmental area, nucleus accumbens), amygdala, cortex, hippocampus, and insula (Bruijnzeel, 2009; Koob and Volkow, 2010). Sedation is caused by μ- and κ-receptor activation in the hypothalamic and locus coeruleus neurons controlling arousal and sleep (Greco et al., 2008; Li and van den Pol, 2008; Chung et al., 2017). Convulsive actions of δ-receptor agonists involve hippocampus and thalamocortical circuits (Jutkiewicz et al., 2006). Constipation is mostly mediated by μ-receptors in peripheral sensory myenteric and submucosal neurons in the gastrointestinal tract, but spinal and supraspinal receptors may also be involved (Burks, 1990; Galligan and Akbarali, 2014; Imam et al., 2017). Nausea and vomiting are mostly mediated by μ-receptors in the medulla, cortex, and vestibular apparatus, and partially in the gastrointestinal tract, possibly secondary to constipation (Porreca and Ossipov, 2009; Imam et al., 2017). Diuresis results from activation of κ-receptors in the hypothalamus with some actions in adrenal glands (Kapusta, 1995). Thus, peripherally restricted opioids should be devoid of the CNS side effects, and produce fewer or less severe adverse actions having both CNS and peripheral components such as constipation, nausea, vomiting (μ-opioids), and diuresis (κ-opioids). Some authors suggested that peripheral μ-receptors mediate morphine-induced analgesic tolerance and paradoxical hyperalgesia, but not analgesia itself, using mice with μ-receptor deletion in TRPV1-expressing neurons (Corder et al., 2017). This is in contrast to studies in mice with μ-receptor deletion in $Na_V1.8$-expressing neurons, which showed that peripheral μ-receptors do not contribute to analgesic tolerance or hyperalgesia induced by morphine or its metabolite (Weibel et al., 2013; Roeckel et al., 2017). Further work will be required to find out whether these contradictory findings are related to different μ-receptor-expressing neuronal populations or unidentified knockout strategy-related alterations. Nevertheless, in agreement with the latter studies, experiments without genetic modifications showed that development of tolerance at peripheral μ-receptors is reduced in inflamed tissue in animals and humans, due to the continuous presence of immune cell-derived opioid peptides and enhanced μ-receptor recycling (Stein et al., 1996; Zöllner et al., 2008).

Additional advantage of peripheral opioid receptor targeting is the inhibition of pain at its source, since many painful syndromes originate in peripheral tissue and are usually associated with inflammation (including surgery, arthritis, neuropathy, cancer, and visceral disorders). Under such conditions, opioid receptor synthesis, transport, and G protein coupling in peripheral sensory neurons is increased, and disruption of the perineurial barrier facilitates the access of opioids to receptors (Hassan et al., 1993; Antonijevic et al., 1995; Zöllner et al., 2003; Hackel et al., 2012; Mousa et al., 2007, 2017). Moreover, damaged tissue is infiltrated by immune cells containing opioid peptides and expressing functional opioid receptors (Stein et al., 1990, 1993, 1996; Rittner et al., 2001; Labuz et al., 2009; Boué et al., 2014; Celik et al., 2016). All these events lead to enhanced analgesic efficacy of opioids at peripheral receptors. This has been shown

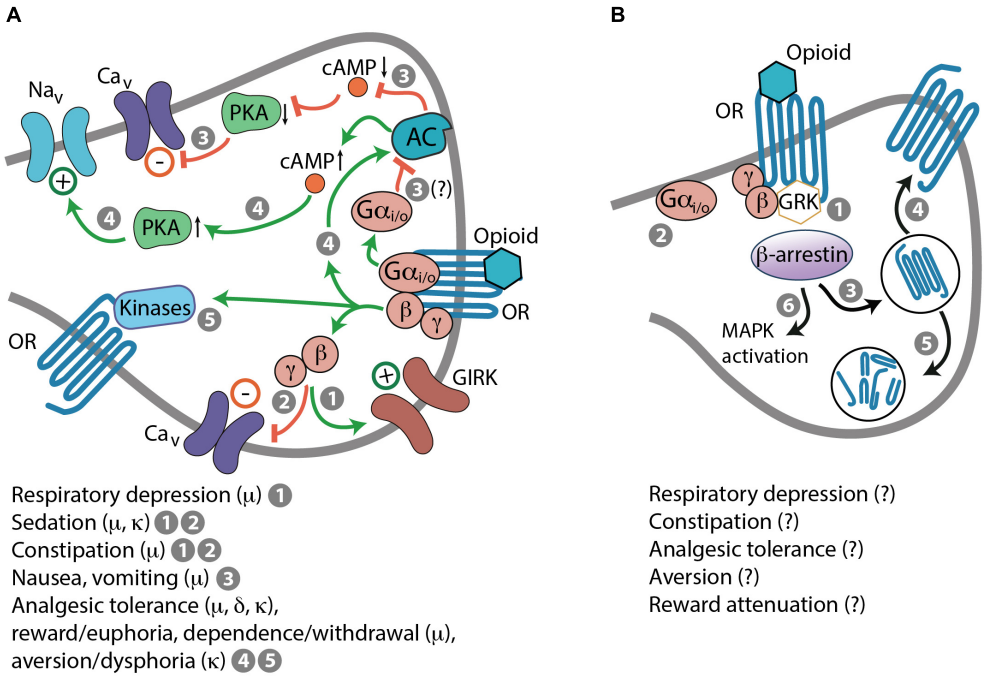

FIGURE 2 | Mechanisms of opioid-induced side effects. **(A)** G protein-mediated side effects in response to activation of opioid receptors (OR). (1) Respiratory depression: Gβγ-dependent activation of GIRK channels. (1 and 2) Sedation and constipation: Gβγ-dependent activation of GIRK channels (1) and inhibition of Ca_v channels (2). (3) Nausea and vomiting: Gαi/o-mediated inhibition of AC, decreased cAMP levels and PKA activity, and inhibition of Ca_v channels; this is based on indirect evidence (indicated by a question mark). (4 and 5) Analgesic tolerance, reward/euphoria, dependence/withdrawal, or aversion/dysphoria: Gβγ-mediated AC activation, elevated cAMP levels, enhanced PKA activity, and activation of Na_v channels (4). Phosphorylation of OR by various kinases (5), including PKA and activated by Gβγ PKC, CaMK II, and MAPK, which results in OR uncoupling form G protein-mediated effects. **(B)** β-arrestin-dependent actions. After even brief activation by an opioid, OR are phosphorylated by GRK recruited by Gβγ, followed by β-arrestin binding to phosphorylated OR (1), which terminates G protein coupling and signaling (2), and leads to OR internalization (3). Dephosphorylated OR can be recycled to the cell membrane (4) or directed to lysosomes and degraded (5). β-arrestin-2 might also promote morphine-induced respiratory depression, constipation, analgesic tolerance, and κ-receptor-mediated aversion, and dampen morphine-induced reward. Some of these effects may involve MAPK activation (6), but mechanisms are unknown (indicated by question marks).

following local application of small, systemically inactive doses of opioids in animal models and in humans (Kalso et al., 2002; Stein et al., 2003; Zeng et al., 2013; Stein, 2016). Importantly, pharmacologic, genetic, and clinical studies have demonstrated that peripheral opioid receptors mediate a large proportion of the analgesic effects produced by systemically administered opioids (Gavériaux-Ruff, 2013; Jagla et al., 2014; Stein and Jagla, 2014; Stein, 2016).

Reducing Opioid Access to the CNS

The above described findings stimulated the development of peripherally restricted opioid receptor agonists by limiting their ability to cross the blood-brain barrier (BBB) (**Figure 4A**). These strategies focused on κ-opioids, supported by a recent study (Snyder et al., 2018), and include agonist chemical modifications (e.g., incorporation of quaternary structures or amphiphilic molecules which contain hydrophilic and hydrophobic components), and synthesis of peptide-based compounds. However, these modifications often decreased agonist affinity to receptors, which required the use of relatively high doses and did not warrant complete BBB impermeability (Barber and Gottschlich, 1997; Rivière, 2004; Stein and Machelska, 2011). This also applies to peptides, as in contrast to previous beliefs, peptides can cross the BBB (Kastin and Pan, 2010). Nevertheless, two κ-receptor agonists gained pharmaceutical interests, asimadoline (initially termed EMD 61753) and CR845 (formerly FE 202845) (**Figure 4A** and **Table 1**). Asimadoline belongs to the amphiphilic molecules and possesses somewhat puzzling action profile. In animal models of hind paw inflammation or sciatic nerve injury, it alleviated pain (Barber et al., 1994) or produced bi-phasic effects, with analgesia at lower doses or shortly after injection, but paradoxically increased pain at higher doses or at later time points (Machelska et al., 1999; Walker et al., 1999). As the analgesic actions were mediated by peripheral κ-receptors, the hyperalgesic effects were either κ-receptor-selective (Walker et al., 1999) or independent of κ- and *N*-methyl-D-aspartic acid receptors (Machelska et al., 1999). Asimadoline was also hyperalgesic in experimental colonic distension model in healthy human volunteers (Delgado-Aros et al., 2003) and tended to enhance postoperative pain in patients undergoing arthroscopic knee surgery (Machelska et al., 1999). In contrast, in preclinical models of visceral inflammatory pain (Gebhart et al., 2000), barostat-induced colonic distension in patients with irritable bowel syndrome (IBS) (Delvaux et al., 2004), and in phase 2b IBS trial (Mangel et al., 2008; Mangel and Williams, 2010), asimadoline was reported to decrease

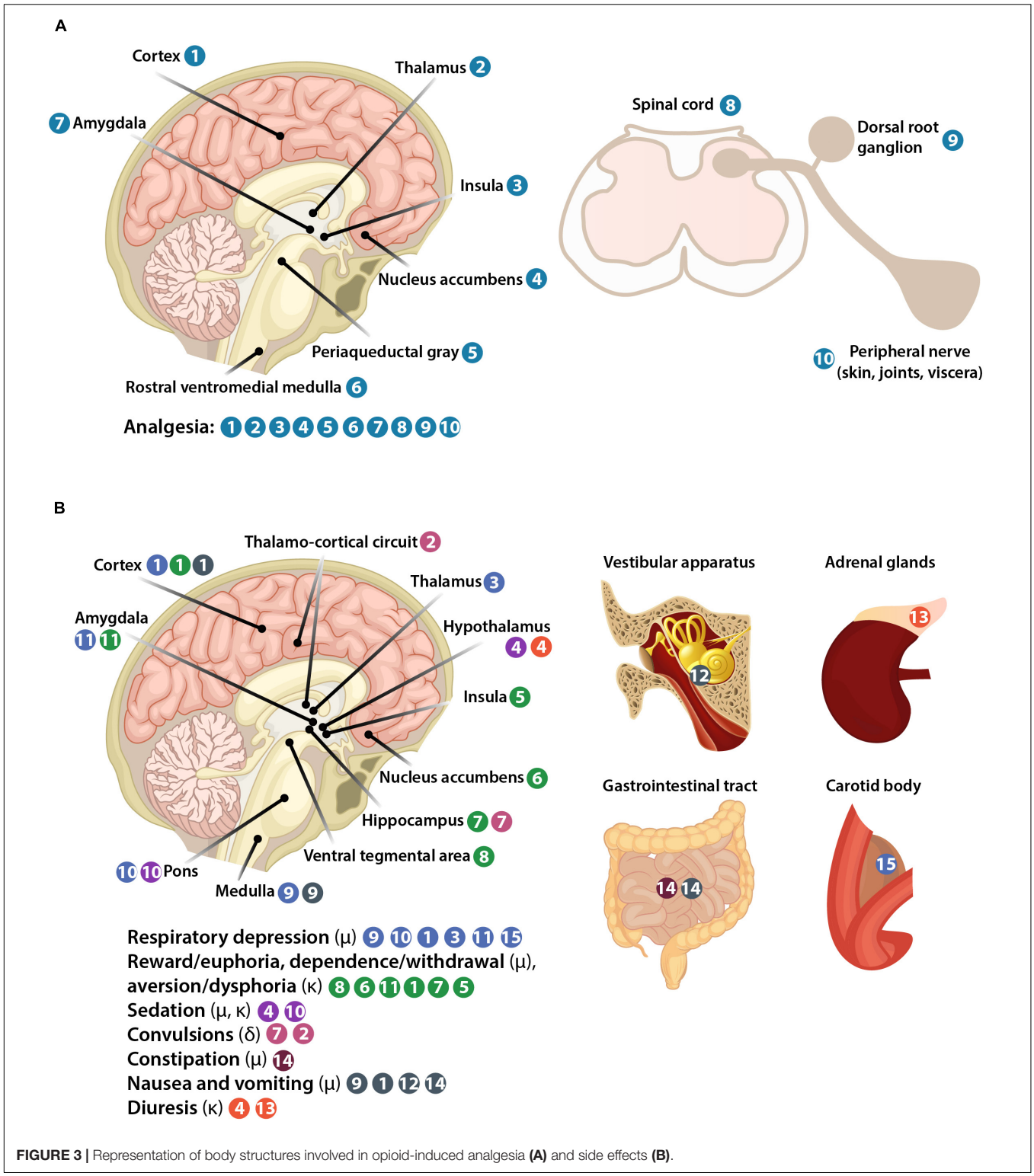

FIGURE 3 | Representation of body structures involved in opioid-induced analgesia **(A)** and side effects **(B)**.

pain. It produced some side effects, which could be of CNS (sedation, headache, dizziness) or both CNS and peripheral origin (diuresis), albeit at higher than analgesic doses (Mangel and Hicks, 2012). These results led to the conclusion that in contrast to somatic pain, asimadoline may be efficacious in the visceral pain, and it is now developed for management of diarrhea-predominant IBS with moderate-to-severe pain (Mangel and Hicks, 2012; Foxx-Orenstein, 2016).

CR845 is a tetrapeptide currently under development by Cara Therapeutics (Stamford, CT, United States) for postoperative

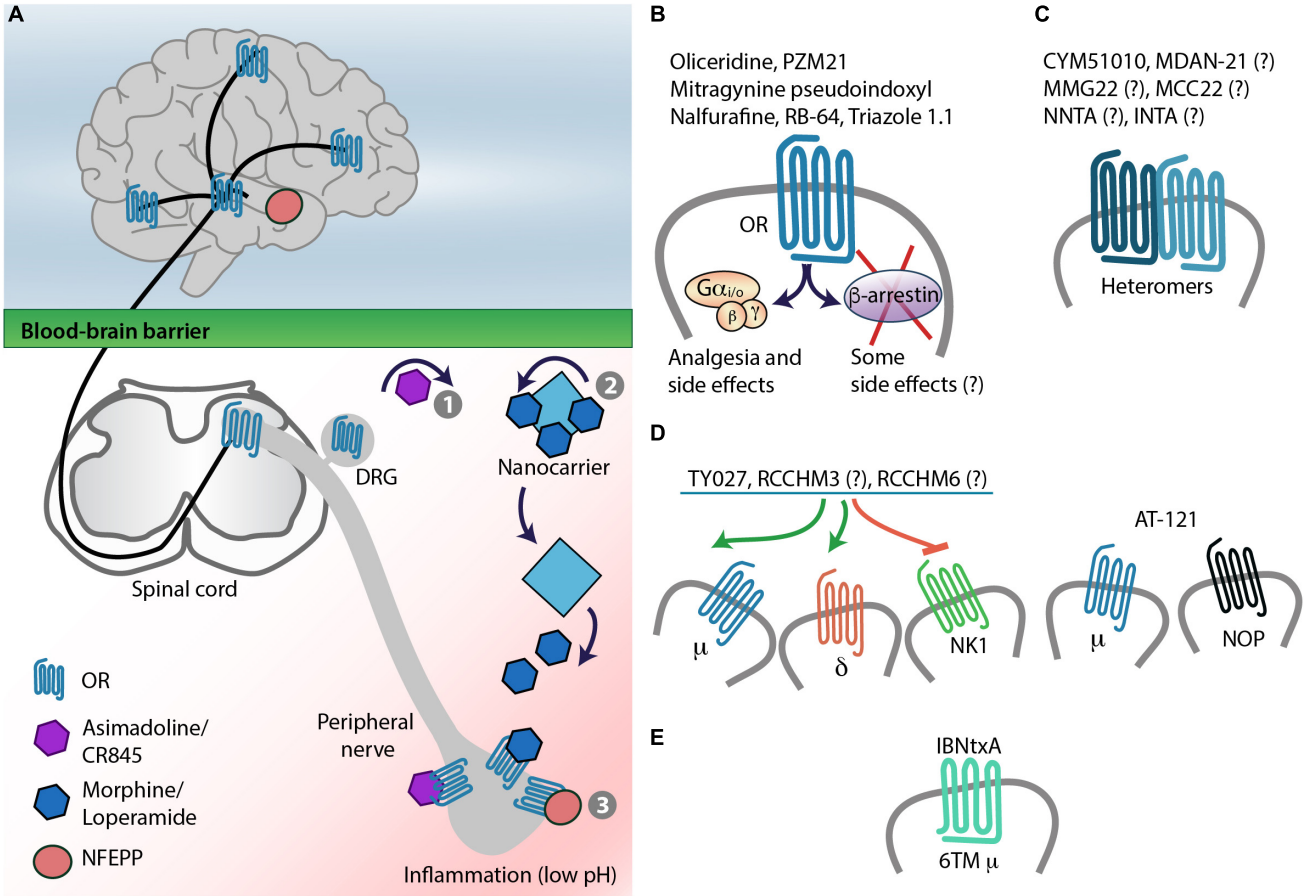

FIGURE 4 | Strategies for safer pain control – targeting opioid receptors. **(A)** Targeting opioid receptors (OR) in peripheral painful tissue by chemical modification of agonists, which results in their decreased blood-brain barrier penetration (1), nanocarrier-based opioid delivery to inflamed tissue (2), or by low pH-dependent OR activation (3). **(B)** Biased agonism: This approach aims at targeting OR–G protein signaling without activation of β-arrestins, which were considered to mediate opioid-induced side effects, but not analgesia. This might need reconsideration, since G proteins not only mediate analgesia but also side effects (see also **Figure 2A**). **(C)** Targeting heteromers. **(D)** Development of multifunctional ligands, which act as μ- and δ-opioid receptor agonists and NK1 receptor antagonists, or μ- and NOP-receptor agonists. **(E)** Targeting truncated, 6TM domain μ-receptor variants. Question marks indicate that heteromer/multiple receptor selectivity of the ligand was not tested or not confirmed (see also **Tables 2**, **3**).

and osteoarthritis pain. Its analgesic effects were reported in animal models of pancreatitis, abdominal, inflammatory, and neuropathic pain. Completed phase 2 clinical trials stated that CR845 attenuated postoperative pain after laparoscopic hysterectomy and in some patients after bunionectomy, and it was well tolerated with repetitive dosing over 2 weeks in patients with osteoarthritis of knee or hip. The side effects were considered mild and, similar to asimadoline, they included dizziness, headache, and diuresis. However, these data were only presented in abstracts, press releases, and at the ClinicalTrials.gov website (Albert-Vartanian et al., 2016), and thus, independent, peer-reviewed studies will be essential to verify these findings.

Nanocarrier-Based Approaches

A promising strategy to alter the pharmacokinetic profile and improve therapeutic effects of drugs is the use of nanoparticles as drug carriers. Nanoparticles are defined as molecules of 1–100 nm in at least one dimension, and examples include liposomes, micelles, and polymer-based particles. They have been widely examined for tumor-directed delivery of chemotherapeutics to reduce their off-target toxicity (Cheng et al., 2012). Similar strategies have recently been used to deliver opioids to peripheral inflamed tissue (**Figure 4A**). Liposomes conjugated with an antibody to intercellular adhesion molecule-1 (anti-ICAM-1) were employed to mimic the properties of immune cells (Hua and Cabot, 2013). Indeed, it has earlier been shown that similar to selectins and integrins α4 and β2 (Machelska et al., 1998, 2004), ICAM-1 expressed on vascular endothelium mediates the migration of opioid peptide-containing immune cells to peripheral inflamed tissue to locally alleviate pain (Machelska et al., 2002). Accordingly, intravenously injected anti-ICAM-1-conjugated liposomes loaded with μ-receptor agonist loperamide accumulated in inflamed tissue and alleviated mechanical hypersensitivity via local opioid receptors in a rat model of unilateral hind paw inflammation (Hua and Cabot, 2013). In

TABLE 1 | Novel opioid treatments in clinical trials.

Category	Name/Target	Clinical conditions	Effects	Reference
Agonists with reduced CNS access	Asimadoline* (peripheral κ-receptors)	Postoperative pain (knee surgery); randomized, double-blind, placebo-controlled; oral	- Tendency to hyperalgesia - No serious side effects (data not shown)	a
		Healthy volunteers (barostat-induced colonic distension); randomized, double-blind, placebo-controlled; oral	- Hyperalgesia - Side effects comparable to placebo (dizziness, nausea, headache)	b
		IBS (barostat-induced colonic distension); randomized, double-blind, placebo-controlled; oral	- Analgesia - Side effects not reported	c
		IBS; randomized, double-blind, placebo-controlled; oral	- Analgesia in D-IBS - No drug-related side effects in analgesic doses*	d
	CR845# (peripheral κ-receptors)	Postoperative pain (hysterectomy, bunionectomy); oral, i.v.	- Analgesia - Side effects: dizziness, headache, diuresis	e
Biased agonists	Oliceridine (TRV130) (μ-receptors)	Healthy volunteers (cold pain test); randomized, double-blind, placebo-controlled; i.v.	- Analgesia (superior to morphine) - Side effects: vs. morphine, lesser nausea, similar respiratory depression	f
		Postoperative pain (bunionectomy); randomized, double-blind, placebo-controlled; i.v.	- Analgesia (superior to morphine) - Side effects: constipation, nausea, vomiting, dizziness similar to morphine	g
		Postoperative pain (abdominoplasty); randomized, double-blind, placebo-controlled; i.v. PCA	- Analgesia (similar to morphine) - Side effects: lesser nausea and vomiting vs. morphine	h
	Nalfurafine (κ-receptors)	Approved for uremic pruritus in Japan, but not in Europe	Sedation in analgesic doses (not recommended for pain treatment)	i
DENK inhibitors	PL37, PL265 (enkephalin peptidases)	Postoperative pain (PL37), neuropathic and ocular pain (PL265)	Data not available	j, k
Gene therapy	HSV-PENK (enkephalin overexpression in DRG neurons)	Intractable cancer pain; not randomized, not blinded, not placebo-controlled; intradermal	- Analgesia vs. pre-injection - Side effects: transient and mild injection site erythema and pruritus, body temperature elevation	l
		Intractable cancer pain; randomized, double-blind, placebo-controlled; intradermal	Data not available	m
Agonists with low rate CNS entry	NKTR-181 (μ-receptors)	Osteoarthritis and low back pain; randomized, double-blind, placebo-controlled; oral	Data not available	n

*D-IBS, diarrhea-predominant irritable bowel syndrome; I.v., intravenous; PCA, patient-controlled analgesia. *Currently under development for D-IBS with moderate-to-severe pain. Sedation, headache, dizziness, diuresis – in higher than D-IBS analgesic dose (Mangel et al., 2008; Mangel and Williams, 2010; Foxx-Orenstein, 2016). #Currently under development for postoperative and osteoarthritis pain. Data in abstracts, press releases, ClinicalTrials.gov; No published peer-reviewed trials. (a) Machelska et al., 1999; (b) Delgado-Aros et al., 2003; (c) Delvaux et al., 2004; (d) Mangel et al., 2008; (e) Albert-Vartanian et al., 2016; (f) Soergel et al., 2014; (g) Viscusi et al., 2016; (h) Singla et al., 2017; (i) Inui, 2012; (j) Roques et al., 2012; (k) http://www.pharmaleads.com; (l) Fink et al., 2011; (m) ClinicalTrials.gov NCT01291901; (n) ClinicalTrials.gov NCT02367820 and NCT02362672.*

the same model, analgesic effects were exerted by loperamide-encapsulated liposomal gel applied topically on the inflamed paw (Iwaszkiewicz and Hua, 2014). In both cases, anti-inflammatory effects were also observed, and all actions of loperamide-loaded liposomes were superior to either conventional loperamide or loperamide gel, respectively. However, in the rat model of polyarthritis, despite producing analgesia, loperamide liposomal gel unexpectedly exacerbated arthritis (**Table 2**). As the opioid receptor-selectivity has not been tested, the mechanisms of these actions are currently unclear (Hua et al., 2017).

Another nanocarrier-based approach utilized hyperbranched, dendritic polyglycerols (PG) to deliver morphine to peripheral inflamed tissue. Morphine was covalently bound to PG via cleavable ester linker sensitive to esterases and low pH (González-Rodríguez et al., 2017). The rationale was that due to its high molecular weight and hydrophilicity, such PG-morphine injected intravenously will not cross the BBB, but will selectively extravasate from leaky blood vessels characteristic of inflamed tissue. The local low pH and leukocyte esterases will then trigger the release of morphine from PG-morphine to ameliorate pain (Fleige et al., 2012; Nehoff et al., 2014). Indeed, in contrast to morphine, intravenous PG-morphine exclusively produced analgesia via peripheral opioid receptors in painful tissue without sedation and constipation, in a rat model of unilateral hind

TABLE 2 | Novel opioid treatments in preclinical models of pathological pain.

Category	Name/Target	Experimental conditions	Effects	Reference
Nanocarrier agonist delivery	anti-ICAM-1 conjugated liposomes loaded with loperamide (μ-receptors in peripheral inflamed tissue)	- CFA hind paw inflammation - Paw pressure test - I.v. or gel on inflamed paw - Blinding (+), R (+), SSE (–)	- Analgesia - Decreased paw volume - Side effects not evaluated	a, b
		- CFA polyarthritis - Paw pressure test - Gel on inflamed paws - Blinding (+), R (+), SSE (–)	- Analgesia - Exacerbated arthritis: higher paw volume, pannus, angiogenesis	c
	PG-morphine (μ-receptors in peripheral inflamed tissue)	- CFA hind paw inflammation - Paw pressure test - Into inflamed paw, i.v. - Blinding (+), R (+), SSE (–)	- Analgesia - No sedation, constipation; 2-fold higher than analgesic doses	d
pH-sensitive receptor activation	**NFEPP** (μ-receptors in peripheral inflamed tissue)	- CFA hind paw inflammation - Hind paw incision - CCI neuropathy - Paw pressure, von Frey, Hargreaves tests - Into inflamed paw, i.v., s.c. - Blinding (+), R (–), SSE (+)	- Analgesia - No sedation, constipation, motor impairment, reward (CPP), respiratory depression (naïve rats); 10-fold higher than analgesic doses	e, f
	FF3 (μ-receptors in peripheral inflamed tissue)	- CFA hind paw inflammation - Hind paw incision - CCI neuropathy - Paw pressure, von Frey, Hargreaves tests - I.v., s.c. - Blinding (+), R (–), SSE (+)	- Analgesia - Sedation, constipation, motor impairment, reward (CPP), respiratory depression (naïve rats); 2.5–10-fold higher than analgesic doses	g
Heteromer bivalent ligands	MMG22 μ-agonist–mGluR5-antagonist (putative μ–mGluR5)	- Lipopolysaccharide (LPS) systemic inflammation - CFA hind paw inflammation - Bone cancer - SNI neuropathy - Tail-flick, von Frey tests - Supraspinal, spinal - Blinding (–,+)*, R (–), SSE (–)	- Analgesia; μ–mGluR5 selectivity not confirmed - No analgesic tolerance, no respiratory depression after spinal injection (LPS or naïve mice); lower than analgesic doses	h, i
	MCC22 μ-agonist–CCR5-antagonist (putative μ–CCR5)	- Sickle disease - von Frey test - Intraperitoneal - Blinding (+), R (+), SSE (–)	- Analgesia; μ–CCR5 selectivity not tested - No analgesic tolerance	j
Multifunctional ligands (μ- and δ-agonists and NK1 receptor antagonists)	**TY027** (CNS μ-, δ-, and NK1 receptors)	- SNL neuropathy - Paw pressure, von Frey, Hargreaves tests - Supraspinal, spinal, i.v. - Blinding (only ferrets), R (only ferrets), SSE (–)	- Analgesia - No constipation, reward (CPP), analgesic tolerance, withdrawal (teeth chattering, wet-dog shakes, diarrhea, weight loss) (naïve rats), vomiting (naïve ferrets); up to 5-fold lower than analgesic doses	k
	RCCHM3, RCCHM6 (CNS μ-, δ-, and NK1 receptors)	- CCI neuropathy - von Frey, cold plate tests - Spinal - Blinding (–), R (–), SSE (–)	- Analgesia - Side effects not evaluated	l
μ-Receptor splice variant agonists	**IBNtxA** (CNS 6TM μ-receptors)	- CFA hind paw inflammation - Zymosan ankle inflammation - SNI neuropathy - von Frey test, facial grimacing; S.c. - Blinding (–,+)*, R (+), SSE (–)	- Analgesia - Less constipation, no reward (CPP), respiratory depression, withdrawal (jumping); analgesic or 2.5-fold higher doses	m, n

(Continued)

TABLE 2 | Continued

Category	Name/Target	Experimental conditions	Effects	Reference
Gene therapy	HSV-μ-receptors (overexpressed μ-receptors in DRG neurons)	- SNL neuropathy - von Frey, Hargreaves tests - Into ipsilateral paw - Blinding (+), R (+), SSE (–)	- Reduced basal von Frey sensitivity - Enhanced morphine - and loperamide-analgesia - Side effects not evaluated	o
Endomorphin-1 analog	**Analog 4** (ZH853) (CNS μ-receptors)	- CFA hind paw inflammation - Hind paw incision - SNI neuropathy - Paw pressure, von Frey, Hargreaves tests - Oral, spinal, i.v., s.c. - Blinding (+), R (–,+)*, SSE (–)	- Analgesia - Less analgesic tolerance, motor impairment, reward (CPP, SA), respiratory depression (naïve mice or rats); 2-fold lower or analgesic doses	p, q

*CCI, chronic constriction injury; CFA, complete Freund's adjuvant; I.v., intravenous; R, randomization; SA, self-administration; S.c., subcutaneous; SNI, spared nerve injury; SNL, spinal nerve ligation; SSE, sample size estimation; TM, transmembrane domain. *Each sign refers to the corresponding reference (in the citation order). Marked in bold are compounds currently the most comprehensively examined (pathological pain models, various side effects) and showing promising results. (a) Hua and Cabot, 2013; (b) Iwaszkiewicz and Hua, 2014; (c) Hua et al., 2017; (d) González-Rodríguez et al., 2017; (e) Spahn et al., 2017; (f) Rodriguez-Gaztelumendi et al., 2018; (g) Spahn et al., 2018; (h) Akgün et al., 2013; (i) Peterson et al., 2017; (j) Cataldo et al., 2018; (k) Largent-Milnes et al., 2013; (l) Starnowska et al., 2017; (m) Majumdar et al., 2011; (n) Wieskopf et al., 2014; (o) Klein et al., 2018; (p) Zadina et al., 2016; (q) Feehan et al., 2017.*

paw inflammation (**Table 2**). Consistent with these actions, free morphine was only measured in inflamed paw tissue, but not in the contralateral, non-inflamed paw tissue, blood, and brain (González-Rodríguez et al., 2017). Together, although polyglycerols are biocompatible (Kainthan et al., 2006), the organ toxicity and broader side effect profile, including abuse potential and effects on respiration of PG-morphine need to be investigated to strengthen the clinical applicability of this strategy.

Painful Tissue-Specific Opioid Receptor Activation

Recent studies explored the opioid receptor–ligand interactions that are specific to pathological painful conditions such as acidosis (pH 5–7 vs. 7.4 in non-inflamed tissue) (Spahn et al., 2017). An agonist designed to fulfill such requirements could freely distribute throughout the whole body, including the brain, but would only activate opioid receptors in peripheral inflamed tissue (Del Vecchio et al., 2017) (**Figure 4A**). This has been achieved by lowering the dissociation constant (p*K*a) of the μ-receptor agonist fentanyl to the acidic pH. Accordingly, fluorination of fentanyl (p*K*a 8.43) by computer simulations resulted in a design of a novel compound NFEPP [(±)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide] with a p*K*a 6.8, which can only be protonated, and thus bind to receptors, at lower than physiological pH. Indeed, *in vitro* experiments confirmed that NFEPP bound to and activated μ-receptors only at acidic pH, whereas fentanyl was active at both acidic and physiological pH. Importantly, unlike fentanyl, intravenously applied NFEPP produced analgesia by activation of opioid receptors exclusively in peripheral injured tissue in rat models of unilateral hind paw inflammation or surgical incision (Spahn et al., 2017), sciatic nerve injury-induced neuropathy, and abdominal pain (Rodriguez-Gaztelumendi et al., 2018). Furthermore, NFEPP did not induce respiratory depression, sedation, motor impairment, reward (assessed by conditioned place preference; CPP), and constipation, even at doses 10-fold higher than the most effective analgesic doses (Spahn et al., 2017) (**Table 2**). As this compound will not be an option for patients with CNS inflammation, it represents a promising analgesic for pain conditions associated with peripheral tissue damage, which needs to be demonstrated in clinical trials.

Interestingly, another fentanyl derivative FF3 ((±)-*N*-[1-(2-fluoro-2-phenylethyl)piperidine-4-yl]-*N*-phenyl propionamide) with a higher than NFEPP's p*K*a, 7.22 (but still lower than that of fentanyl), produced injury-restricted analgesia in rat models of inflammatory, surgical, neuropathic, and abdominal pain, similarly to NFEPP. However, unlike NFEPP, FF3 induced side effects, including respiratory depression, sedation, motor impairment, reward, and constipation, at 2.5–10-fold higher than analgesic doses (**Table 2**). These results suggest that a ligand's p*K*a should be close to the pH of injured tissue to obtain analgesia without side effects (Spahn et al., 2018).

BIASED AGONISM

Background

The concept of biased agonism (or functional selectivity) is based on the ability of different ligands of the same receptor to stabilize various receptor active states, which leads to the activation of diverse signaling pathways – a biased agonist preferentially activates one signaling pathway over another. Some biased agonists of GPCRs, including opioid receptors, might activate G protein-mediated pathway, whereas others might involve β-arrestin-2. The role of β-arrestin-2 was first examined in the μ-receptor function using β-arrestin-2 knockout mice. These studies used naïve mice, without pathological pain, and reported that morphine induced more efficacious and prolonged analgesia in acute heat pain tests, absent (in hot plate test) or delayed (in tail-flick test) analgesic tolerance, and decreased constipation and respiratory depression

in β-arrestin-2 knockout compared to wild-type mice (Bohn et al., 1999, 2000, 2002; Raehal et al., 2005). Whereas naloxone-precipitated morphine withdrawal was unchanged (Bohn et al., 2000), morphine-induced hypothermia and reward (CPP) were substantially enhanced in β-arrestin-2 knockout mice (Bohn et al., 1999, 2003) (**Figure 2B**). Of note, analgesic tolerance and naloxone-precipitated withdrawal following injection of other μ-receptor agonists such as fentanyl, oxycodone, and methadone did not differ between β-arrestin-2 knockout and wild-type mice (Raehal and Bohn, 2011). Intriguingly, opposite effects were observed using GRK3 knockout mice, who showed weaker acute analgesic tolerance to fentanyl, oxycodone, and methadone, but not to morphine (Melief et al., 2010). Further work of that group indicated that analgesic tolerance to fentanyl involves GRK3/arrestin and c-Jun N-terminal kinase-2 belonging to the MAPK family, whereas tolerance to morphine also involves this kinase, but in GRK3/arrestin-independent manner (Kuhar et al., 2015). It is unclear whether these contradictory results relate to different actions mediated by GRK3 and β-arrestin-2 in response to μ-receptor activation, or to other, unknown alterations resulting from knockout strategies, which cannot be excluded, since GRKs and β-arrestins interact with many GPCRs, not only with opioid receptors (Reiter et al., 2012). Regardless of the discrepancies, these findings suggest that GRK3 and β-arrestin-2 are not essential for side effects exerted by μ-agonists, and that β-arrestin-2 might actually be required for dampening the reinforcement/abuse potential of morphine. Nevertheless, the following efforts focused on design of agonists without or with minimal β-arrestin-2 recruitment properties, but with bias toward G protein-mediated signaling (**Figure 4B**).

It is currently accepted that biased agonism occurs at all three opioid receptors (Al-Hasani and Bruchas, 2011; Siuda et al., 2017). There is *in vitro* evidence that δ-receptors can adopt distinct receptor conformations in response to different agonists, and that agonist-dependent δ-receptor trafficking and different arrestin isoform recruitment may have behavioral implications (Vicente-Sanchez and Pradhan, 2017). However, no biased δ-receptor agonists with a potential distinction between analgesic actions and undesirable effects such as convulsions have been developed so far. Therefore, the following sections focus on μ- and κ-opioid receptors.

μ-Receptor Biased Ligands

The first G protein-biased μ-receptor agonist was oliceridine (formerly TRV130) (DeWire et al., 2013) and initially it was classified as potent analgesic with reduced side effect profile (Kingwell, 2015). However, closer analysis of the data and subsequent studies appear less consistent. In mice, oliceridine produced similarly effective analgesia in acute heat pain test, but less constipation compared to morphine. Both agonists also exerted comparable analgesia in a short-lasting (24 h) post-operative pain model in rats. Respiratory function was not affected by either opioid at the most effective analgesic doses, but it was to a similar degree diminished by approximately 2.5-fold (oliceridine) or 4-fold (morphine) higher doses in naïve rats (DeWire et al., 2013). Subsequent study in rodents confirmed oliceridine-induced analgesia and lack of analgesic tolerance in acute heat pain test, but also reported robust constipation and abuse-related behavior in intracranial self-stimulation (ICSS) assay (Altarifi et al., 2017) (**Table 3**). In healthy human volunteers, compared to morphine, oliceridine exerted superior analgesia in experimental cold pain test, less severe nausea, and comparable degree, but shorter-lasting respiratory depression, which paralleled the time-course of its analgesic effect (Soergel et al., 2014). In phase 2 trial examining patients undergoing bunionectomy, oliceridine produced greater post-operative pain relief, but similar to morphine side effects characterized by the percentage of patients experiencing constipation, nausea, vomiting, and dizziness, as well as by the severity and number of these events (Viscusi et al., 2016). The most recent phase 2b study in patients undergoing abdominoplasty reported comparable rescue analgesic use and reduction in pain intensity, but significantly lower percentage of patients experiencing nausea and vomiting following oliceridine vs. morphine treatment. Whereas earlier clinical trials used fixed-dose design, in that latest study opioids were delivered on an as-needed basis via patient-controlled analgesia (Singla et al., 2017) (**Table 1**). Together, as all so far performed pre-clinical and clinical studies consistently showed analgesia induced by oliceridine, its side effect profile appears more variable across the studies with most reporting comparable to morphine adverse actions. Additional limitation is the abuse liability of oliceridine (Altarifi et al., 2017) and possibly of other G protein-biased μ-receptor agonists (Bohn et al., 2003).

G protein-biased ligands with μ-receptor agonistic activity, but also affinities to other opioid receptors were later described. PZM21 was initially characterized as μ-receptor agonist with κ-receptor antagonistic activity, and no β-arrestin-2 recruitment. It was reported to produce analgesia in acute heat pain test and in short-lasting (30 min) hind paw inflammation in mice, but no respiratory depression and rewarding (CPP) properties, and less constipation than morphine. However, the side effects were examined in equivalent or lower than the most effective analgesic doses (Manglik et al., 2016). Furthermore, in contrast to that report, a recent study re-examining PZM21 found that it induced respiratory depression similarly to morphine. Additionally, following repeated administration, tolerance developed to PZM21-induced analgesia but not to respiratory depression (Hill et al., 2018) (**Table 3**).

Mitragynine pseudoindoxyl is a derivative of the natural product mitragynine, which *in vitro* preferentially activated G protein without β-arrestin-2 recruitment, and acted as μ-receptor agonist as well as δ- and κ-receptor antagonist. *In vivo* it produced μ-receptor-mediated analgesia in acute heat pain test, delayed analgesic tolerance, lesser constipation, naloxone-precipitated withdrawal and respiratory depression, and no reward compared to morphine or aversion compared to the κ-receptor agonist U50,488H (CPP/conditioned place aversion; CPA). The doses of mitragynine pseudoindoxyl used to examine side effects were higher than ED_{50}, but still

TABLE 3 | Novel opioids tested in animals without pathological pain.

Category	Name/Receptor selectivity	Experimental conditions*	Effects#	Reference
Biased ligands	Oliceridine (TRV130) (μ-agonist)	- Tail-flick; S.c. - Blinding (–), R (+), SSE (–)	- Analgesia - No analgesic tolerance - Robust constipation, reward (ICSS)	a
	PZM21 (μ-agonist; also κ-antagonist *in vitro*)	- Tail-flick, hot plate - Hind paw inflammation (30 min); S.c. - Blinding (only hot plate), R (–), SSE (–)	- Analgesia (not in tail-flick) - Less constipation, no respiratory depression, reward (CPP)	b
		- Hot plate; S.c., i.p. - Blinding (+), R (+), SSE (+)	- Analgesia - Respiratory depression - Tolerance to analgesia, but not to respiratory depression; side effects in analgesic or 2-fold higher doses	c
	Mitragynine pseudoindoxyl (μ-agonist; also δ-, κ-antagonist *in vitro*)	- Tail-flick - S.c., oral, supraspinal - Blinding (–), R (–), SSE (–)	- Analgesia - Less constipation, withdrawal (jumping), respiratory depression, no reward, aversion (CPP/CPA)	d
	RB-64 (κ-agonist)	- Hot plate; S.c. - Blinding (–), R (–), SSE (–)	- Analgesia - No sedation, motor impairment, aversion/anhedonia in ICSS - Robust aversion in CPA	e
	Triazole 1.1 (κ-agonist)	- Tail-flick; S.c., i.p. - Blinding (–), R (–), SSE (+)	- Analgesia - No sedation, aversion (ICSS)	f
Heteromer ligands	CYM51010 (μ–δ heteromer agonist)	- Tail-flick - S.c., i.p., spinal - Blinding (–), R (–), SSE (–)	- Analgesia (partially reversed by μ–δ-specific antibody) - Less analgesic tolerance, diarrhea, body weight loss; No change in jumping, teeth chattering, tremor	g
	MDAN-21 bivalent μ-agonist–δ-antagonist (μ–δ heteromers)	- Tail-flick - I.v., supraspinal - Blinding (–), R (–), SSE (–)	- Analgesia (μ–δ selectivity not tested) - No analgesic tolerance, withdrawal (jumping), reward (CPP)	h,i
	NNTA (monovalent agonist of putative μ–κ heteromers)	- Tail-flick - I.v., supraspinal, spinal - Blinding (–), R (–), SSE (–)	- Analgesia (μ–κ selectivity not tested) - No analgesic tolerance, withdrawal (jumping), reward (CPP) - Strong aversion (CPA)	j
	INTA (monovalent agonist of putative μ–κ and/or δ–κ heteromers)	- Tail-flick - S.c., supraspinal, spinal - Blinding (–), R (–), SSE (–)	- Analgesia (μ–κ or δ–κ selectivity not tested) - No analgesic tolerance, aversion (CPA) - Strong reward (CPP)	k
Multifunctional ligands	AT-121 (μ- and NOP-agonist)	- Rhesus monkeys - Naïve and capsaicin - Tail immersion - S.c. - Blinding (+), R (–), SSE (–)	- Analgesia (μ- and NOP-selective) - No analgesic tolerance, scratching, reward (SA), respiratory depression, withdrawal (increased respiration, heart rate, arterial pressure); up to 10-fold higher than analgesic doses	l
Ligands with low rate CNS entry	NKTR-181 (μ-agonist)	- Hot plate - Writhing test - Oral - Blinding, R (+; but not for SA and rigidity), SSE (–)	- Analgesia; receptor selectivity and action site not tested - No reward (SA), mild muscle rigidity and motor impairment at the most effective analgesic doses	m

*I.p., intraperitoneal; I.v., intravenous; R, randomization; SA, self-administration; S.c., subcutaneous; SSE, sample size estimation. *Experiments were performed in mice or rats, unless otherwise stated. #Side effects were tested in analgesic or lower doses, unless otherwise stated. (a) Altarifi et al., 2017; (b) Manglik et al., 2016; (c) Hill et al., 2018; (d) Váradi et al., 2016; (e) White et al., 2015; (f) Brust et al., 2016; (g) Gomes et al., 2013; (h) Daniels et al., 2005; (i) Lenard et al., 2007; (j) Yekkirala et al., 2011; (k) Le Naour et al., 2014; (l) Ding et al., 2018; (m) Miyazaki et al., 2017.*

substantially lower than the most effective analgesic doses (**Table 3**). Additionally, the relative contribution of its μ-receptor agonist/δ- and κ-receptor antagonist activity and G protein bias to the improved side effect profile is unclear (Váradi et al., 2016).

A recent paper suggested that just the occurrence of biased signaling might be insufficient, and the degree of bias or bias factor (which quantitatively defines the preference for one signaling pathway over another) closer predicts the opioid therapeutic window (i.e., the separation of doses that produce analgesia and doses that produce side effects). Thus, the higher the G protein bias factor the better the therapeutic window, as calculated for several μ-receptor agonists by comparing respiratory depression and analgesia. Generally, the authors found a correlation between the bias factor and therapeutic window. Nevertheless, it is difficult to clearly define the best bias factor, since it strongly depended and substantially varied with the *in vitro* assays and conditions (e.g., cell line, native tissue, mouse vs. human μ-receptors, signaling pathway type). Similarly, the therapeutic window varied with the pain tests (tail-flick or hot plate) and respiratory depression parameters (arterial oxygen saturation or breath rate). For example, for the most G protein-biased compound SR17018, the G protein bias factor varied from 40 to 102 and therapeutic window for respiratory depression vs. analgesia ranged from 26 to 105 (Schmid et al., 2017); the bias factor of 3 was calculated for oliceridine (DeWire et al., 2013). Furthermore, the correlation between the bias factor and therapeutic window in pathological pain models and for other opioid side effects (constipation, reward, physical dependence) is unknown.

κ-Receptor Biased Ligands

Nalfurafine (previously TRK-820), first synthetized and characterized in the late 1990s, is a κ-receptor agonist with particularly strong G protein bias at human κ-receptors (bias factor of 300 vs. 7 for rat κ-receptors) (Schattauer et al., 2017). It was initially described as efficacious analgesic and antipruritic with favorable side effect profile; however, a recent study demonstrated its aversive/anhedonic effects (in ICSS assay) in rats (Lazenka et al., 2018). Furthermore, it produced severe sedation at analgesic doses in humans, but lower doses decreased pruritus without severe side effects. Nalfurafine is currently used in Japan for the treatment of uremic pruritus in individuals undergoing hemodialysis (Inui, 2012), but was not approved in Europe, and it is not recommended for the treatment of pain (Inui, 2012)[1] (**Table 1**).

RB-64 is a derivative of salvinorin A, an active psychotropic ingredient of a plant *Salvia divinorum*, with a G protein/β-arrestin-2 bias factor of 96 (vs. 3 for typical κ-agonist U69593) (White et al., 2015). It produced κ-receptor-selective analgesia in acute heat pain test, but did not induce sedation, motor impairment, and aversion/anhedonia in ICSS assay compared to U69593 and salvinorin A; however, it was aversive in the CPA paradigm (**Table 3**). Additionally, U69593 and salvinorin A produced similar analgesia, sedation, and aversion in wild-type and β-arrestin-2 knockout mice, and only motor impairment was slightly lesser in the latter. These data suggest that analgesia and most side effects induced by κ-opioids are mediated by G protein-, but not by β-arrestin-2-dependent signaling. Thus, although RB-64 did not recruit β-arrestin-2 *in vitro* (White et al., 2015), it is unclear whether the lack of β-arrestin-2 signaling indeed account for its effects *in vivo*.

Another G protein-biased κ-receptor agonist triazole 1.1 (with G protein/β-arrestin-2 bias factor of 28) produced similar degree analgesia in acute heat pain test and an anti-pruritic activity, but did not decrease dopamine release in the striatum and did not possess sedative and aversive properties (in ICSS test) compared to classic κ-receptor agonist U50,488H (Brust et al., 2016). Still, the fact that triazole 1.1 was not tested in chronic pathological pain models and the analgesic doses from acute pain tests were used to examine side effects pose some limitations (**Table 3**).

In summary, the idea to separate desirable and undesirable opioid actions by biased agonists stimulated pain research in the last decades. As analgesic effects of μ- and κ-receptor biased agonists were examined in animals without pain or in very short-lasting inflammation (30 min–24 h) (**Table 3**), it will be essential to use animal models of pathological pain to closer reflect clinical conditions. To broaden therapeutic window, it is desirable to also use doses exceeding the most effective analgesic doses for testing adverse actions, since fatal effects result from overdosing, as in case of respiratory arrest. The potential abuse liability of G protein-biased μ-receptor agonists, as in case of oliceridine, even in the absence of other side effects, must be seriously considered. Clearly, the addictive properties of opioids have led to their misuse and abuse, which resulted in the opioid crisis worldwide (Abdel-Hamid et al., 2016; Novak et al., 2016; Volkow et al., 2018). Although bias factor depends on experimental conditions and cannot be used as an absolute predictor of the ligand action, the degree of bias is often emphasized, but even very high bias factor does not guarantee the absence of side effects, as in case of nalfurafine. Moreover, it was not always clear whether *in vivo* actions of biased agonists indeed resulted from G protein bias and the lack of β-arrestin-2 engagement, or from a complex pharmacological profile or yet unidentified pathways, as in case of mitragynine pseudoindoxyl and RB-64. Considering these issues, including uncertain mechanistic basis for action of biased agonists, it needs to be acknowledged that opioid-mediated side effects do involve G protein-dependent signaling (see Introduction and **Figure 2A**).

HETEROMERS, BIVALENT AND MULTIFUNCTIONAL LIGANDS

Heteromers are defined as complexes composed of at least two functional receptor units (protomers) and having different

[1]https://www.ema.europa.eu/documents/medicine-qa/withdrawal-marketing-authorisation-application-winfuran-nalfurafine_en.pdf

biochemical properties than the individual units. Additional criteria include the colocalization and physical interaction of protomers, and the ability to alter heteromer action by heteromer-specific reagents (Gomes et al., 2016). Heteromers might thus potentially exhibit new pharmacology and represent a novel therapeutic target (**Figure 4C**). *In vitro* studies in heterologous cells indicated heteromerization between opioid receptors to form μ–δ, μ–κ, and δ–κ heteromers, as well as between opioid and other receptors to form heteromers such as μ-opioid–gastrin-releasing peptide receptor, μ-opioid–metabotropic glutamate receptor 5 (mGluR5), μ-opioid–chemokine receptor 5 (CCR5), μ-opioid–neurokinin 1 (NK1) receptor, μ-opioid–cannabinoid 1, and δ-opioid–cannabinoid 1 receptor. There is only scarce evidence that such complexes exist in endogenous systems, and only μ–δ heteromers appear to fulfill the criteria required for heteromerization in native tissue (Gomes et al., 2016). For example, using μ–δ heteromer-specific antibody, this heteromer was detected in cultured DRG neurons and in various pain-related brain areas in mice (Gupta et al., 2010), although some authors question the co-expression of μ- and δ-receptors in DRG neurons (Wang et al., 2018). Screening of a small molecule library identified CYM51010 as the μ–δ heteromer agonist. This compound produced analgesia in acute heat pain test, which was partially reversed by μ–δ heteromer antibody. Compared to morphine, CYM51010 induced lesser analgesic tolerance and less severe diarrhea and body weight loss, but did not improve other signs of naloxone-precipitated withdrawal (jumping, teeth chattering, paw tremor, whole body tremor) (Gomes et al., 2013) (**Table 3**). A bivalent ligand comprising a μ-receptor agonist (oxymorphone-derived ligand, oxymorphamine) linked to a δ-receptor antagonist (naltrindole) by a 21-atom spacer (MDAN-21) was designed as a putative μ–δ heteromer ligand. This is based on earlier studies reporting attenuation of morphine-induced side effects by blocking δ-receptor function (Gendron et al., 2016). MDAN-21 produced analgesia in the acute heat pain test, but did not induce acute tolerance, naloxone-precipitated jumping, and reward (in CPP assay) compared to morphine in mice; nevertheless, the μ–δ heteromer-selectivity of MDAN-21 action was not shown (Daniels et al., 2005; Lenard et al., 2007) (**Table 3**).

To target other putative heteromers, several compounds of different chemistry have been generated. Examples of bivalent ligands are MMG22 and MCC22, which exert agonistic action at μ-receptors and antagonistic activity at various receptors mediating pain. The former was designed to target μ–mGluR5 heteromers, as it consists of μ-agonist (oxymorphamine) and mGluR5 antagonist (metoxy-2-methyl-6-(phenylethynyl)-pyridine) connected by a 22-atom spacer. Compared to morphine or the individual pharmacophores, MMG22 was more potent, but similarly efficacious in mouse models of inflammatory, bone cancer (Akgün et al., 2013), and neuropathic pain (Peterson et al., 2017). However, as the latter study showed that MMG22 acted at μ-receptors and mGluR5 as separate monomers rather than heteromers (Peterson et al., 2017), and the examination of side effects was very limited (Akgün et al., 2013), a rigorous evaluation of a broad adverse effect spectrum will be essential to justify the utility of this compound. MCC22 comprises a μ-agonist (oxymorphamine) and CCR5 antagonist (TAK-220) linked by a 22-atom spacer, and was designed to act at μ–CCR5 heteromers. Compared to morphine, MCC22 ameliorated tactile hypersensitivity with similar efficacy, but higher potency and of longer duration, without inducing tolerance, in a mouse model of sickle cell disease. The assessment of receptor specificity and other than tolerance side effects awaits further research (Cataldo et al., 2018) (**Table 2**).

Monovalent molecules *N*-naphthoyl-β-naltrexamine (NNTA) and *N*-2′-indolylnaltrexamine (INTA) were developed to target heteromers containing κ-receptors, probably because κ-receptor activation does not induce reward. NNTA designed to act at μ–κ heteromers produced analgesia in acute heat pain test, little tolerance, and no naloxone-precipitated jumping. It also did not induce reward at half-maximal analgesic doses, but exerted strong aversion at maximal analgesic doses (in CPP/CPA paradigm), which is consistent with the pharmacology of mixed κ-receptor agonist/μ-receptor antagonist opioid class (Yekkirala et al., 2011). INTA designed to target μ–κ and/or δ–κ heteromers did not induce acute analgesic tolerance in heat pain test, and was not aversive, but produced robust reward (Le Naour et al., 2014). It is still unclear whether NNTA and INTA exert the respective heteromer-selective effects, and their aversive or rewarding properties are clear drawbacks (**Table 3**).

Multifunctional ligands are designed to interact with two or more receptors, but heteromers are not necessary their primary target. The advantages of such multitarget, single compounds over a co-administration of several, each receptor-selective ligands, include easier pharmacokinetics and the lack of potential drug–drug interactions. A known, clinically used example of such compound is buprenorphine, which is a partial agonist at μ- and nociceptin/orphanin FQ peptide (NOP) receptors, and a weak antagonist at κ- and δ-receptors. Buprenorphine is predominately applied for the treatment of opioid dependency, as it exhibits ceiling effect for respiratory depression, which diminishes the likelihood of respiratory arrest, and has reduced abuse liability (probably due to its partial μ-receptor agonistic activity) and diminished aversive properties (possibly due to its antagonistic κ-receptor activity). It appears that the majority of clinical trials in cancer pain patients were observational, of poor quality, and with a high risk of bias. Similarly, good quality, randomized studies in neuropathic pain patients are needed. In randomized trials of postoperative or osteoarthritis pain, buprenorphine was concluded to produce fewer respiratory complications, but equivalent analgesia to other opioids (Davis et al., 2018), although the reason for the lack of ceiling analgesic effects in contrast to respiratory depression is unclear, and other studies reported high rate of drop-out due to nausea/vomiting (Fishman and Kim, 2018). Together, as buprenorphine is successfully used for opioid maintenance therapy, the evidence for its analgesic superiority over other opioids in clinical setting appears moderate and more good quality comparative studies are needed (Davis et al., 2018).

Examples of new multifunctional ligands tested in preclinical studies are peptides Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-Bn(CF3)2 (TY027), RCCHM3, and RCCHM6, which exert μ-

and δ-opioid receptor agonistic and NK1 receptor antagonistic activity (**Figure 4D**). In a very comprehensive study, TY027 injected supraspinally, spinally, or intravenously reversed neuropathy-induced heat and tactile hypersensitivity. In contrast to morphine, TY027 did not produce analgesic tolerance, reward (in CPP test), naloxone-precipitated withdrawal (teeth chattering, wet-dog shakes, diarrhea, weight loss), did not inhibit gastrointestinal transit (in mice or rats), and did not cause retching/vomiting (in ferrets), although the doses were up to 5-fold lower than the most effective analgesic doses. Additionally, TY027 was shown to act as opioid receptor agonist and NK1 receptor antagonist *in vivo* (Largent-Milnes et al., 2013). RCCHM3 and RCCHM6 were efficacious in ameliorating neuropathy-induced tactile and cold hypersensitivity in mice, but the receptor selectivity and side effects were not examined (Starnowska et al., 2017) (**Table 2**).

Additionally, a bifunctional partial agonist at μ- and NOP receptors, AT-121, has been recently developed. The rationale is based on earlier studies reporting synergistic analgesic actions of morphine and NOP receptor agonists, as well as reduced dopamine release and attenuation of rewarding effects of μ-agonists by NOP receptor agonists (Toll et al., 2016). In rhesus monkeys, subcutaneously injected AT-121 did not induce scratching, but produced comparable to morphine analgesia, which was reversed by opioid and NOP receptor antagonists in an acute heat pain test. AT-121 also reversed capsaicin-induced sensitivity measured by the same test. Unlike oxycodone, it lacked reinforcing effects in self-administration paradigm, and partially attenuated reinforcing action of oxycodone. Unlike heroin, AT-121 at 10 times the analgesic doses did not compromise respiratory and cardiovascular function (respiration rate, minute volume, heart rate, mean arterial pressure). These parameters were also unchanged after injection of the antagonists, indicating a lack of antagonist-precipitated withdrawal in AT-121-treated monkeys. Moreover, following repeated administration (twice daily for 4 weeks), in contrast to morphine, AT-121 did not produce analgesic tolerance in the heat pain test (Ding et al., 2018) (**Table 3**). Although understandably, the numbers of monkeys per group were low and the chronic pain could not be examined, these conditions present some limitations. Additionally, since NOP receptors are very widely distributed throughout the nervous system and peripheral tissues, other potential side effects produced by NOP receptor agonists, including motor disturbance, memory impairment, and gastrointestinal complications, need to be considered (Mogil and Pasternak, 2001; Toll et al., 2016).

Together, of all opioid receptor heteromers described in heterologous systems *in vitro*, the μ–δ heteromer might be present and function *in vivo*. However, more research would be needed to develop selective ligands, test them in pathological pain models and in a broad range of side effect tests to justify the targeting of μ–δ heteromer as improved pain therapy. Of numerous ligands designed to simultaneously act at various receptors, TY027 has been thoroughly examined, showed analgesic efficacy in pathological pain and promising side effect profile.

μ-RECEPTOR SPLICE VARIANTS

Alternative splicing is a genetic regulation that takes place during gene expression when particular exons (transcriptional sequences) of a gene are either included or excluded from the final mRNA, which may result in generation of multiple protein isoforms (Black, 2003). Among opioid receptors, the alternative splicing of μ-receptor coding exons has been extensively examined and the generation of multiple splice variants in mice, rats, and humans has been revealed. In addition to classic full-length, seven-transmembrane (7TM) domain μ-receptor variants, there are also exon 1-associated truncated 1TM domain variants and exon 11-associated truncated 6TM domain variants. Depending on the species, two to five 1TM domain and 6TM domain variants have been described (Pasternak and Pan, 2013). Several of these variants have been detected in the mouse brain, spinal cord, and DRG at the mRNA level (Pasternak and Pan, 2013; Wieskopf et al., 2014), and some of them were examined by immunohistochemistry, but difficulties associated with the specificity of antibodies preclude the convincing evidence on their expression at the protein level (Pasternak and Pan, 2013). It has been suggested that 1TM domain variants do not bind ligands, but function as molecular chaperones that facilitate expression of the 7TM domain μ-receptor and thereby enhance morphine analgesia. In contrast, 6TM domain variants appear to possess distinct pharmacology characterized by the use of a compound iodobenzoylnaltrexamide (IBNtxA) (**Figure 4E**). Radiolabeled IBNtxA-binding sites were detected in the brain membrane homogenates in wild-type mice and mice lacking 7TM domain μ-, δ-, and κ-opioid receptors, but were absent in exon 11 knockout mice (Majumdar et al., 2011). Systemically applied IBNtxA diminished spontaneous inflammatory pain and mechanical hypersensitivity in inflammatory and neuropathic pain models in wild-type mice, whereas the effects in the latter two models were absent in exon 11-lacking mice (Wieskopf et al., 2014). Compared to morphine, IBNtxA at analgesic or higher doses exerted lesser constipation, and did not produce respiratory depression, naloxone-precipitated jumping, and CPP reward (Majumdar et al., 2011) (**Table 2**). Experiments in μ-receptor knockout mice reconstituted with 6TM domain variants confirmed their contribution to IBNtxA-induced analgesia (in the acute heat pain test) (Lu et al., 2018). It is still unclear what cellular mechanisms underlie analgesic effects and improved side effect profile of 6TM domain variants, and whether these variants are functional in humans. The complexity of this system is additionally implied by animal studies describing excitatory cellular actions of 6TM domain variants and enhanced heat sensitivity following repetitive injections of IBNtxA in naïve mice (Convertino et al., 2015; Samoshkin et al., 2015).

TARGETING ENDOGENOUS OPIOID PEPTIDES

Enkephalinase Inhibitors

Enhancing the activity of endogenous opioid peptides as natural agonists of opioid receptors represents an intrinsic pain

control. Opioid peptides, including β-endorphin, enkephalins and dynorphins are expressed in neurons in pain-relevant regions of the central or peripheral nervous system as well as in immune cells accumulating in peripheral painful tissue (Fields, 2004; Stein and Machelska, 2011). Hence, targeting endogenous opioids at the site of their native expression may diminish the risk of off-site, unphysiological actions. Electrical stimulation of periventricular/periaqueductal gray matter and thalamus, or activation of immune cells in peripheral inflamed tissue (by surgery-related stress or local application of opioid peptide-releasing agents) alleviates pathological pain involving endogenous opioids in humans (Stein et al., 1993; Bittar et al., 2005; Likar et al., 2007). Notably, immune cell-derived opioid peptides exerted additive/synergistic analgesic action with peripherally (intra-articularly) applied morphine in patients with postoperative pain (Stein et al., 1996; Likar et al., 2004), which may be related to the activation of leukocyte opioid receptors (Celik et al., 2016). Nonetheless, opioid peptides are rapidly enzymatically degraded, and the best characterized enzymes are aminopeptidase N (APN; also known as CD13) and neutral endopeptidase (NEP; also known as neprilysin, CD10, or enkephalinase). Among opioid peptides, the predominant substrates of APN and NEP are Met- and Leu-enkephalin, but dynorphin A 1-17 can also be inactivated. Both enzymes are functional in the CNS, peripheral nerves, and immune cells, and their blockade prevented opioid peptide degradation (Bourgoin et al., 1986; Le Guen et al., 2003; Schreiter et al., 2012). Since the actions of both peptidases are complementary, their concomitant blockade is most efficient, which led to the development of dual APN and NEP inhibitors, now known as dual enkephalinase (DENK) inhibitors (**Figure 5A**). Over the last four decades, numerous DENK inhibitors have been synthetized and found to alleviate inflammatory, neuropathic, abdominal, cancer, and postoperative pain, when applied intravenously, orally, or into inflamed tissue in animal models (Roques et al., 2012; Schreiter et al., 2012). Compared to morphine, DENK inhibitors in analgesic or higher doses produced no or less severe side effects, including tolerance, naloxone-precipitated withdrawal, reward (CPP, ICSS), respiratory depression, and constipation (Noble and Roques, 2007). Within the last decade, DENK inhibitors developed by Pharmaleads (Paris, France) for the treatment of postoperative pain (PL37) or neuropathic and ocular pain (PL265) entered clinical trials, but the data are not yet available (Roques et al., 2012)[2] (**Table 1**).

Gene Therapy

Gene therapy (or gene transfer) is based on the introduction of DNA or RNA encoding a protein of interest, and offers a possibility of the protein long-term expression in native tissue. For *in vivo* delivery of genes encoding enkephalin precursor proenkephalin (PENK) or β-endorphin precursor proopiomelanocortin (POMC), different vectors have been used, including plasmids, non-replicating adenoviruses, adeno-associated viruses, and herpes simplex virus (HSV), as well as non-plasmid and non-viral DNA vectors (e.g., MIDGE; minimalistic, immunologically defined gene expression vector). There are numerous preclinical studies, in which these PENK- or POMC-encoding vectors were applied intramuscularly, on the spinal cord, intra-articularly, or into the skin/subcutaneous tissue, which resulted in enhanced expression of the respective peptides (Met/Leu-enkephalin or β-endorphin) in the corresponding tissue. Consequently, these treatments led to attenuation of mechanical and heat hypersensitivity in models of inflammatory, neuropathic, or cancer pain, mediated by spinal or peripheral opioid receptors; these analgesic effects were rather modest, but in some cases persisted for several weeks (Machelska et al., 2009; Simonato et al., 2013; Goss et al., 2014; Hu et al., 2016; Klein et al., 2018). Since this strategy targets peripheral and spinal cord tissue, the opioid side effects mostly mediated in the brain are not anticipated, but this has not been verified. Compared to non-viral vectors, viral vectors have higher transfection efficacy, which is attributed to the natural ability of viruses to infect and express their genes in host cells. However, viral vectors can potentially cause toxicity and inflammation, which depends on treatment conditions (e.g., dosing, route of application), although based on so far available data, HSV vectors inoculated into the skin are predicted to be safe (Wolfe et al., 2009; Simonato et al., 2013; Goss et al., 2014). The first phase 1 clinical trial testing this strategy employed HSV-based vector encoding human PENK injected intradermally (into the pain-corresponding dermatomes) in terminally ill patients with intractable cancer pain. The treatment was well tolerated and no serious adverse events were observed. Over the 4-month follow-up, the treatment-emergent adverse effects (injection site erythema and pruritus, and body temperature elevation) were transient and judged of mild severity. The study was very small (four or fewer patients per group), not blinded and not placebo-controlled, but also reported a dose-related decrease of pain (up to 4 weeks post-treatment) as the secondary outcome (Fink et al., 2011). A phase 2, randomized, double-blind, placebo-controlled, multicenter study testing HSV-encoding PENK in patients with intractable malignant pain has been completed, but the data are not yet released (ClinicaleTrials.gov NCT01291901) (**Table 1**). Based on the corresponding pre-clinical studies it is anticipated that HSV-encoding PENK is taken up by cutaneous terminals of peripheral sensory neurons and axonally transported to their cell bodies in DRG, where PENK is processed to enkephalins. The enkephalins can be then transported toward peripheral and central DRG neuron terminals, released and respectively activate peripheral and spinal opioid receptors to provide analgesia (Antunes Bras et al., 1998, 2001; Goss et al., 2001; Klein et al., 2018) (**Figure 5B**). Similar strategy can also be used to enhance expression of opioid receptors. For example, HSV-encoding μ-receptors applied to mouse hind paw elevated μ-receptor-immunoreactivity in epidermal skin fibers, DRG cells, and dorsal horn spinal cord, alleviated basal mechanical hypersensitivity, and enhanced analgesic effects of morphine and peripherally acting loperamide injected systemically in a neuropathic pain model (**Table 2**). Surprisingly and not clarified yet, combined treatment with HSV-encoding μ-receptors and HSV-encoding PENK was ineffective (Klein et al., 2018).

[2]http://www.pharmaleads.com/

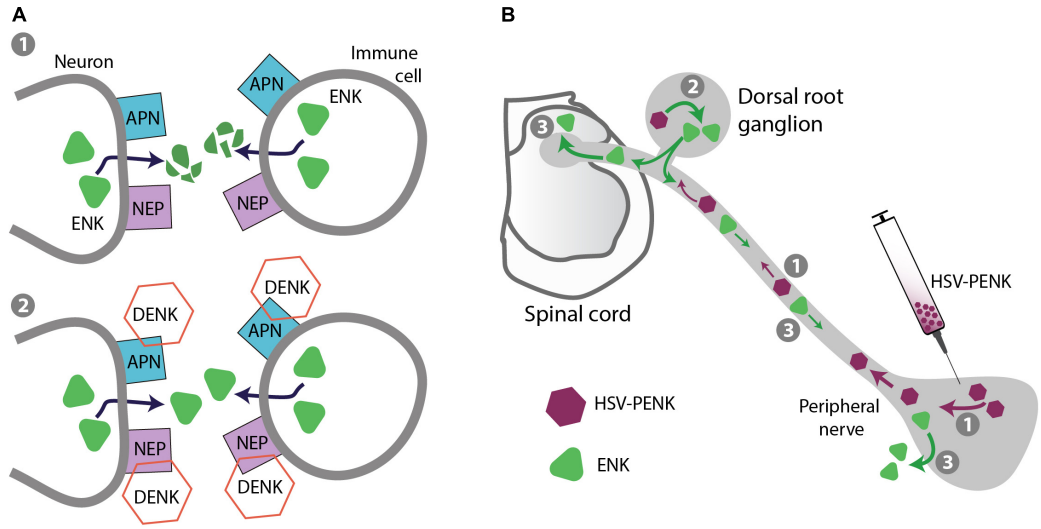

FIGURE 5 | Strategies for safer pain control – targeting endogenous opioid peptides. **(A)** Prevention of opioid peptide degradation. (1) Opioid peptides, including enkephalins (ENK) are degraded by APN and NEP expressed on neurons (central and peripheral) and immune cells in inflamed tissue. (2) DENK inhibitors block APN and NEP, and prevent ENK degradation to locally alleviate pain. **(B)** Gene transfer to enhance opioid peptide production in native tissue. As an example, HSV vector encoding ENK precursor PENK injected into peripheral tissue is taken up by peripheral terminals of dorsal root ganglion (DRG) neurons and transported to their cell bodies in DRG (1), where PENK is processed to ENK (2). ENK is then transported to peripheral and central DRG neuron terminals (3), released, and respectively activates peripheral and spinal opioid receptors to produce analgesia.

OTHER APPROACHES

Abuse-Deterrent Opioid Formulations

Currently clinically used opioids have been modified to obtain abuse-deterrent formulations, and several of such substances have been approved by the Food and Drug Administration (Becker and Fiellin, 2017). The general aim was to make these new formulations difficult to inhale or inject, and to get a high from. This has been attempted by means of physical or chemical barriers to hinder crushing, chewing, or solubilization of pills, as in case of modifications of morphine (MorphaBond ER, Arymo ER), oxycodone (OxyContin, RoxyBond, Xtampza ER), and hydrocodone (Vantrela ER, Hyslinga ER). Alternatively, opioid receptor agonists were combined with antagonists, as in case of Embeda (morphine and naltrexone), Troxyca ER (oxycodone and naltrexone), or Targiniq ER (oxycodone and naloxone) (Becker and Fiellin, 2017; Salwan et al., 2018). However, these strategies have not proved successful in preventing opioid abuse. To overcome the obstacles associated with hindering the misuse of these formulations they were taken at higher doses or replaced with other opioids having a higher abuse liability such as heroin or fentanyl (Cicero and Ellis, 2015; Becker and Fiellin, 2017; Curfman et al., 2018; Salwan et al., 2018).

Agonists With Low Rate CNS Entry

Nektar Therapeutics (San Francisco, CA, United States) has synthetized and been testing a compound NKTR-181, a μ-receptor agonist with a low rate influx across the BBB, proposing that such substance should have lower abuse potential compared to drugs with rapid CNS entry. The slow CNS entry has been achieved by addition of a polyethylene glycol functional group to morphine-like (morphinan) pharmacophore. NKTR-181 produced analgesia in naïve animals in acute heat pain test and in acetic acid-induced writhing model, but the μ-receptor-selectivity and the action site (central, peripheral) have not been examined. Its side effect profile was improved compared to oxycodone, although at the most effective analgesic doses NKTR-181 induced mild muscle rigidity and motor impairment. Compared to cocaine and oxycodone, it did not produce reward in self-administration paradigm (Miyazaki et al., 2017) (**Table 3**). In healthy, non-physically dependent recreational opioid users, single oral application of NKTR-181 (in doses used in ongoing phase 3 trials) induced significantly lower drug liking effects (indicative of lower abuse potential) and smaller changes in the pupil diameter (indicative of less robust CNS actions) relative to oxycodone. Still, the effects of the highest NKTR-181 dose used were significantly higher vs. placebo (Webster et al., 2018). The compound is considered to be resistant to physical or chemical tampering, albeit the data were not shown (Miyazaki et al., 2017), and the possibility of taking it at high doses to achieve high CNS levels cannot be excluded, as in case of abuse-deterrent opioids. The company sponsored completed phase 2 trial in patients with osteoarthritis (NCT02367820) and phase 3 trial in patients with chronic low back pain (NCT02362672; both at ClinicalTrials.gov), but the peer reviewed data are not available yet (**Table 1**).

Endomorphin Analogs

Endomorphin-1 and endomorphin-2 are additional endogenous opioid peptides, although (in contrast to endorphins, enkephalins, and dynorphins) their precursor has not been

identified so far (Zadina et al., 1997). Both endomorphins are highly selective at μ-receptors and exerted analgesia with reduced side effects relative to conventional opioids in some preclinical studies. Due to their poor metabolic stability, numerous endomorphin analogs with potentially improved pharmacological properties have been developed (Gu et al., 2017). Example is a recently characterized cyclized, D-amino acid-containing endomorphin 1 peptide analog termed analog 4 or ZH853 (Tyr-c-[D-Lys-Trp-Phe-Glu]-Gly-NH2). This compound alleviated heat and mechanical hypersensitivity in models of neuropathic, inflammatory, and postoperative pain following spinal or intravenous injections. Relative to morphine, the analog 4-induced analgesia was equally effective but longer-lasting (Feehan et al., 2017). Moreover, in contrast to morphine, analog 4 produced lesser analgesic tolerance, no motor impairment, respiratory depression, and reward (in CPP and self-administration paradigms), and did not induce spinal glia activation (Zadina et al., 2016), although side effects were mostly tested using similar or lower than analgesic doses (Feehan et al., 2017) (**Table 2**). Whereas the results appear promising, considering that the compound crosses the BBB and activates μ-receptors in the brain (Zadina et al., 2016), it will be important to elucidate the mechanistic basis for its improved side effect profile.

Allosteric Modulators

Allosteric modulators are ligands that bind the allosteric site of the receptor (i.e., the site that does not bind orthosteric ligands such as endogenous and standard exogenous ligands) and can modulate (positively or negatively) the effect of the orthosteric ligand without eliciting activity on its own. For example, it is anticipated that positive allosteric modulators will enhance the activity of endogenous opioid peptides, maintain their temporal and spatial action, and potentially limit the off-target adverse effects. Although several such compounds have been characterized *in vitro*, their utility *in vivo* is yet to be determined (Remesic et al., 2017).

CONCLUSION

Conventional opioids are the most effective painkillers, but they also produce adverse affects. Additionally, their prolonged use leads to addiction, which limits the effectiveness of pain therapy and has resulted in a worldwide opioid epidemic (Abdel-Hamid et al., 2016; Novak et al., 2016; Volkow et al., 2018). Therefore, the search for opioids with improved side effect profile and low abuse liability is undisputed. Several novel treatments targeting peripheral κ-receptors (asimadoline, CR845), endogenous opioid peptides (DENK inhibitors, HSV-PENK), and agonist with a low rate CNS entry (NKTR-181) are under development and are tested in clinical trials, but not all results are available yet (**Table 1**) and it remains to be seen whether they enter clinical practice. The G protein-biased agonism as a safer pain therapy needs to be verified, since opioid-induced adverse actions are mediated by G proteins (**Figure 2A**), and there are increasing numbers of studies that report biased agonist-induced constipation, sedation, respiratory depression, and addiction (Inui, 2012; Soergel et al., 2014; Altarifi et al., 2017; Hill et al., 2018) (**Tables 1**, **3**). Encouragingly, there are several new opioids examined in preclinical studies, which are comprehensively characterized in various pathological pain models and methods assessing a wide spectrum of side effects, and show promising results. They include an agonist sensitive to low pH characteristic of painful tissue (NFEPP), ligands targeting multiple receptors (TY027) or μ-receptor splice variants (IBNtxA), and endomorphin-1 analog (analog 4 or ZH853) (**Table 2**). Nevertheless, several aspects are still open such as mechanistic basis of analgesia and improved side effect profile (IBNtxA, endomorphin-1 analog), the need for replication of the initial findings (NFEPP, TY027), and examination of their clinical efficacy. Although there are no preclinical assays that ideally reflect pain in humans, the pathological pain models involving tissue damage and lasting for days or weeks (**Table 2**) closer resemble clinical conditions than the tests inducing pain lasting for seconds in naïve animals (Mogil, 2009) (**Table 3**). Additionally, even though there is an increasing awareness of the importance of the rigorous study design and performance, including blinding, randomization, and sample size estimation (Kilkenny et al., 2010; Berg, 2017), many animal studies still do not adhere to these requirements (**Tables 2**, **3**). It is thus critical that all these aspects are considered when the clinical translation of preclinical studies is judged. Finally, it is crucial to recognize the multifactorial biopsychosocial etiology of chronic pain and that it requires a multidisciplinary management comprising not only pharmacologic, but also psychological, and physiotherapeutic approaches (Scascighini et al., 2008; Stein and Kopf, 2009). Pharmacologic treatment alone is insufficient and will always carry a risk of unwanted behaviors, as seen by the shifting trends in pain management and the addiction landscape toward alternative opioid (e.g., loperamide) and non-opioid (e.g., gabapentin, pregabalin), but also potentially dangerous medications (Throckmorton et al., 2018).

AUTHOR CONTRIBUTIONS

HM wrote the manuscript. MÖC prepared the figures. HM and MÖC approved the final version of the manuscript.

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REFERENCES

Abdel-Hamid, I. A., Andersson, K. E., Waldinger, M. D., and Anis, T. H. (2016). Tramadol abuse and sexual function. *Sex. Med. Rev.* 4, 235–246. doi: 10.1016/j.sxmr.2015.10.014

Akgün, E., Javed, M. I., Lunzer, M. M., Smeester, B. A., Beitz, A. J., and Portoghese, P. S. (2013). Ligands that interact with putative MOR-mGluR5 heteromer in mice with inflammatory pain produce potent antinociception. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11595–11599. doi: 10.1073/pnas.1305461110

Albert-Vartanian, A., Boyd, M. R., Hall, A. L., Morgado, S. J., Nguyen, E., Nguyen, V. P., et al. (2016). Will peripherally restricted kappa-opioid receptor agonists (pKORAs) relieve pain with less opioid adverse effects and abuse potential? *J. Clin. Pharm. Ther.* 41, 371–382. doi: 10.1111/jcpt.12404

Al-Hasani, R., and Bruchas, M. R. (2011). Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* 155, 1363–1381. doi: 10.1097/ALN.0b013e318238bba6

Altarifi, A. A., David, B., Muchhala, K. H., Blough, B. E., Akbarali, H., and Negus, S. S. (2017). Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. *J. Psychopharmacol.* 31, 730–739. doi: 10.1177/0269881116689257

Antonijevic, I., Mousa, S. A., Schäfer, M., and Stein, C. (1995). Perineurial defect and peripheral opioid analgesia in inflammation. *J. Neurosci.* 15, 165–172. doi: 10.1523/JNEUROSCI.15-01-00165.1995

Antunes Bras, J., Becker, C., Bourgoin, S., Lombard, M., Cesselin, F., Hamon, M., et al. (2001). Met-enkephalin is preferentially transported into the peripheral processes of primary afferent fibres in both control and HSV1-driven proenkephalin a overexpressing rats. *Neuroscience* 103, 1073–1083. doi: 10.1016/S0306-4522(01)00034-3

Antunes Bras, J. M., Epstein, A. L., Bourgoin, S., Hamon, M., Cesselin, F., and Pohl, M. (1998). Herpes simplex virus 1-mediated transfer of preproenkephalin A in rat dorsal root ganglia. *J. Neurochem.* 70, 1299–1303. doi: 10.1046/j.1471-4159.1998.70031299.x

Barber, A., Bartoszyk, G. D., Bender, H. M., Gottschlich, R., Greiner, H. E., Harting, J., et al. (1994). A pharmacological profile of the novel, peripherally-selective kappa-opioid receptor agonist, EMD 61753. *Br. J. Pharmacol.* 113, 1317–1327. doi: 10.1111/j.1476-5381.1994.tb17142.x

Barber, A., and Gottschlich, R. (1997). Novel developments with selective, non-peptidic kappa-opioid receptor agonists. *Expert Opin. Investig. Drugs* 6, 1351–1368. doi: 10.1517/13543784.6.10.1351

Becker, W. C., and Fiellin, D. A. (2017). Abuse-deterrent opioid formulations - putting the potential benefits into perspective. *N. Engl. J. Med.* 376, 2103–2105. doi: 10.1056/NEJMp1701553

Berg, J. (2017). Measuring and managing bias. *Science* 357:849. doi: 10.1126/science.aap7679

Bittar, R. G., Kar-Purkayastha, I., Owen, S. L., Bear, R. E., Green, A., Wang, S., et al. (2005). Deep brain stimulation for pain relief: a meta-analysis. *J. Clin. Neurosci.* 12, 515–519. doi: 10.1016/j.jocn.2004.10.005

Black, D. L. (2003). Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.* 72, 291–336. doi: 10.1146/annurev.biochem.72.121801.161720

Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J., and Caron, M. G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408, 720–723. doi: 10.1038/35047086

Bohn, L. M., Gainetdinov, R. R., Sotnikova, T. D., Medvedev, I. O., Lefkowitz, R. J., Dykstra, L. A., et al. (2003). Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice. *J. Neurosci.* 23, 10265–10273. doi: 10.1523/JNEUROSCI.23-32-10265.2003

Bohn, L. M., Lefkowitz, R. J., and Caron, M. G. (2002). Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. *J. Neurosci.* 22, 10494–10500. doi: 10.1523/JNEUROSCI.22-23-10494.2002

Bohn, L. M., Lefkowitz, R. J., Gainetdinov, R. R., Peppel, K., Caron, M. G., and Lin, F. T. (1999). Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286, 2495–2498. doi: 10.1126/science.286.5449.2495

Boué, J., Basso, L., Cenac, N., Blanpied, C., Rolli-Derkinderen, M., Neunlist, M., et al. (2014). Endogenous regulation of visceral pain via production of opioids by colitogenic CD4(+) T cells in mice. *Gastroenterology* 146, 166–175. doi: 10.1053/j.gastro.2013.09.020

Bourgoin, S., Le Bars, D., Artaud, F., Clot, A. M., Bouboutou, R., Fournie-Zaluski, M. C., et al. (1986). Effects of kelatorphan and other peptidase inhibitors on the in vitro and in vivo release of methionine-enkephalin-like material from the rat spinal cord. *J. Pharmacol. Exp. Ther.* 238, 360–366.

Bruchas, M. R., Macey, T. A., Lowe, J. D., and Chavkin, C. (2006). Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin dependent in neurons and astrocytes. *J. Biol. Chem.* 281, 18081–18089. doi: 10.1074/jbc.M513640200

Bruijnzeel, A. W. (2009). Kappa-opioid receptor signaling and brain reward function. *Brain Res. Rev.* 62, 127–146. doi: 10.1016/j.brainresrev.2009.09.008

Brust, T. F., Morgenweck, J., Kim, S. A., Rose, J. H., Locke, J. L., Schmid, C. L., et al. (2016). Biased agonists of the kappa opioid receptor suppress pain and itch without causing sedation or dysphoria. *Sci. Signal.* 9:ra117.

Burks, T. F. (1990). Central nervous system regulation of gastrointestinal motility. *Ann. N. Y. Acad. Sci.* 597, 36–42. doi: 10.1111/j.1749-6632.1990.tb16156.x

Cai, Q., Qiu, C. Y., Qiu, F., Liu, T. T., Qu, Z. W., Liu, Y. M., et al. (2014). Morphine inhibits acid-sensing ion channel currents in rat dorsal root ganglion neurons. *Brain Res.* 1554, 12–20. doi: 10.1016/j.brainres.2014.01.042

Cataldo, G., Lunzer, M. M., Olson, J. K., Akgün, E., Belcher, J. D., Vercellotti, G. M., et al. (2018). The bivalent ligand MCC22 potently attenuates nociception in a murine model of sickle cell disease. *Pain* 159, 1382–1391. doi: 10.1097/j.pain.0000000000001225

Celik, M. Ö, Labuz, D., Henning, K., Busch-Dienstfertig, M., Gaveriaux-Ruff, C., Kieffer, B. L., et al. (2016). Leukocyte opioid receptors mediate analgesia via Ca(2+)-regulated release of opioid peptides. *Brain Behav. Immun.* 57, 227–242. doi: 10.1016/j.bbi.2016.04.018

Che, T., Majumdar, S., Zaidi, S. A., Ondachi, P., McCorvy, J. D., Wang, S., et al. (2018). Structure of the nanobody-stabilized active state of the kappa opioid receptor. *Cell* 172, 55 e15–67.e15. doi: 10.1016/j.cell.2017.12.011

Cheng, Z., Al Zaki, A., Hui, J. Z., Muzykantov, V. R., and Tsourkas, A. (2012). Multifunctional nanoparticles: cost versus benefit of adding targeting and imaging capabilities. *Science* 338, 903–910. doi: 10.1126/science.1226338

Christie, M. J. (2008). Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *Br. J. Pharmacol.* 154, 384–396. doi: 10.1038/bjp.2008.100

Chung, S., Weber, F., Zhong, P., Tan, C. L., Nguyen, T. N., Beier, K. T., et al. (2017). Identification of preoptic sleep neurons using retrograde labelling and gene profiling. *Nature* 545, 477–481. doi: 10.1038/nature22350

Cicero, T. J., and Ellis, M. S. (2015). Abuse-deterrent formulations and the prescription opioid abuse epidemic in the United States: lessons learned from OxyContin. *JAMA Psychiatry* 72, 424–430. doi: 10.1001/jamapsychiatry.2014.3043

Convertino, M., Samoshkin, A., Gauthier, J., Gold, M. S., Maixner, W., Dokholyan, N. V., et al. (2015). μ-Opioid receptor 6-transmembrane isoform: a potential therapeutic target for new effective opioids. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 62, 61–67. doi: 10.1016/j.pnpbp.2014.11.009

Corder, G., Tawfik, V. L., Wang, D., Sypek, E. I., Low, S. A., Dickinson, J. R., et al. (2017). Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nat. Med.* 23, 164–173. doi: 10.1038/nm.4262

Cunha, T. M., Roman-Campos, D., Lotufo, C. M., Duarte, H. L., Souza, G. R., Verri, W. A. Jr., et al. (2010). Morphine peripheral analgesia depends on activation of the PI3Kgamma/AKT/nNOS/NO/KATP signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* 107, 4442–4447. doi: 10.1073/pnas.0914733107

Curfman, G. D., Beletsky, L., and Sarpatwari, A. (2018). Benefits, limitations, and value of abuse-deterrent opioids. *JAMA Intern. Med.* 178, 131–132. doi: 10.1001/jamainternmed.2017.7259

Daniels, D. J., Lenard, N. R., Etienne, C. L., Law, P. Y., Roerig, S. C., and Portoghese, P. S. (2005). Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc. Natl. Acad. Sci. U.S.A.* 102, 19208–19213. doi: 10.1073/pnas.0506627102

Davis, M. P., Pasternak, G., and Behm, B. (2018). Treating chronic pain: an overview of clinical studies centered on the buprenorphine option. *Drugs* 78, 1211–1228. doi: 10.1007/s40265-018-0953-z

Del Vecchio, G., Spahn, V., and Stein, C. (2017). Novel opioid analgesics and side effects. *ACS Chem. Neurosci.* 8, 1638–1640. doi: 10.1021/acschemneuro.7b00195

Delgado-Aros, S., Chial, H. J., Camilleri, M., Szarka, L. A., Weber, F. T., Jacob, J., et al. (2003). Effects of a κ-opioid agonist, asimadoline, on satiation and GI

motor and sensory function in humans. *Am. J. Physiol. Gastrointest. Liver Physiol.* 284, G558–G566. doi: 10.1152/ajpgi.00360.2002

Delvaux, M., Beck, A., Jacob, J., Bouzamondo, H., Weber, F. T., and Frexinos, J. (2004). Effect of asimadoline, a kappa opioid agonist, on pain induced by colonic distension in patients with irritable bowel syndrome. *Aliment. Pharmacol. Ther.* 20, 237–246. doi: 10.1111/j.1365-2036.2004.01922.x

Dembla, S., Behrendt, M., Mohr, F., Goecke, C., Sondermann, J., Schneider, F. M., et al. (2017). Anti-nociceptive action of peripheral mu-opioid receptors by G-beta-gamma protein-mediated inhibition of TRPM3 channels. *eLife* 6:e26280. doi: 10.7554/eLife.26280

DeWire, S. M., Yamashita, D. S., Rominger, D. H., Liu, G., Cowan, C. L., Graczyk, T. M., et al. (2013). A G protein-biased ligand at the μ-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J. Pharmacol. Exp. Ther.* 344, 708–717. doi: 10.1124/jpet.112.201616

Ding, H., Kiguchi, N., Yasuda, D., Daga, P. R., Polgar, W. E., Lu, J. J., et al. (2018). A bifunctional nociceptin and mu opioid receptor agonist is analgesic without opioid side effects in nonhuman primates. *Sci. Transl. Med.* 10:eaar3483. doi: 10.1126/scitranslmed.aar3483

Dripps, I. J., Boyer, B. T., Neubig, R. R., Rice, K. C., Traynor, J. R., and Jutkiewicz, E. M. (2018). Role of signalling molecules in behaviours mediated by the δ opioid receptor agonist SNC80. *Br. J. Pharmacol.* 175, 891–901. doi: 10.1111/bph.14131

Ehrich, J. M., Messinger, D. I., Knakal, C. R., Kuhar, J. R., Schattauer, S. S., Bruchas, M. R., et al. (2015). Kappa opioid receptor-induced aversion requires p38 MAPK activation in VTA dopamine neurons. *J. Neurosci.* 35, 12917–12931. doi: 10.1523/JNEUROSCI.2444-15.2015

Endres-Becker, J., Heppenstall, P. A., Mousa, S. A., Labuz, D., Oksche, A., Schäfer, M., et al. (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol. Pharmacol.* 71, 12–18. doi: 10.1124/mol.106.026740

Evans, C. J., Keith, D. E. Jr., Morrison, H., Magendzo, K., and Edwards, R. H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* 258, 1952–1955. doi: 10.1126/science.1335167

Feehan, A. K., Morgenweck, J., Zhang, X., Amgott-Kwan, A. T., and Zadina, J. E. (2017). Novel endomorphin analogs are more potent and longer-lasting analgesics in neuropathic, inflammatory, postoperative, and visceral pain relative to morphine. *J. Pain* 18, 1526–1541. doi: 10.1016/j.jpain.2017.08.007

Fields, H. (2004). State-dependent opioid control of pain. *Nat. Rev. Neurosci.* 5, 565–575. doi: 10.1038/nrn1431

Fink, D. J., Wechuck, J., Mata, M., Glorioso, J. C., Goss, J., Krisky, D., et al. (2011). Gene therapy for pain: results of a phase I clinical trial. *Ann. Neurol.* 70, 207–212. doi: 10.1002/ana.22446

Fishman, M. A., and Kim, P. S. (2018). Buprenorphine for chronic pain: a systemic review. *Curr. Pain Headache Rep.* 22:83. doi: 10.1007/s11916-018-0732-2

Fleige, E., Quadir, M. A., and Haag, R. (2012). Stimuli-responsive polymeric nanocarriers for the controlled transport of active compounds: concepts and applications. *Adv. Drug Deliv. Rev.* 64, 866–884. doi: 10.1016/j.addr.2012.01.020

Foxx-Orenstein, A. E. (2016). New and emerging therapies for the treatment of irritable bowel syndrome: an update for gastroenterologists. *Therap. Adv. Gastroenterol.* 9, 354–375. doi: 10.1177/1756283X16633050

Galligan, J. J., and Akbarali, H. I. (2014). Molecular physiology of enteric opioid receptors. *Am. J. Gastroenterol. Suppl.* 2, 17–21. doi: 10.1038/ajgsup.2014.5

Gavériaux-Ruff, C. (2013). Opiate-induced analgesia: contributions from mu, delta and kappa opioid receptors mouse mutants. *Curr. Pharm. Des.* 19, 7373–7381. doi: 10.2174/13816128194214010516372 7

Gebhart, G. F., Su, X., Joshi, S., Ozaki, N., and Sengupta, J. N. (2000). Peripheral opioid modulation of visceral pain. *Ann. N. Y. Acad. Sci.* 909, 41–50. doi: 10.1111/j.1749-6632.2000.tb06675.x

Gendron, L., Cahill, C. M., von Zastrow, M., Schiller, P. W., and Pineyro, G. (2016). Molecular pharmacology of δ-opioid receptors. *Pharmacol. Rev.* 68, 631–700. doi: 10.1124/pr.114.008979

Gold, M. S., and Levine, J. D. (1996). DAMGO inhibits prostaglandin E2-induced potentiation of a TTX-resistant Na+ current in rat sensory neurons in vitro. *Neurosci. Lett.* 212, 83–86. doi: 10.1016/0304-3940(96)12791-9

Gomes, I., Ayoub, M. A., Fujita, W., Jaeger, W. C., Pfleger, K. D., and Devi, L. A. (2016). G protein-coupled receptor heteromers. *Annu. Rev. Pharmacol. Toxicol.* 56, 403–425. doi: 10.1146/annurev-pharmtox-011613-135952

Gomes, I., Fujita, W., Gupta, A., Saldanha, S. A., Negri, A., Pinello, C. E., et al. (2013). Identification of a μ-δ opioid receptor heteromer-biased agonist with antinociceptive activity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12072–12077. doi: 10.1073/pnas.1222044110

González-Rodríguez, S., Quadir, M. A., Gupta, S., Walker, K. A., Zhang, X., Spahn, V., et al. (2017). Polyglycerol-opioid conjugate produces analgesia devoid of side effects. *eLife* 6:e27081. doi: 10.7554/eLife.2708

Goss, J. R., Krisky, D., Wechuck, J., and Wolfe, D. (2014). Herpes simplex virus-based nerve targeting gene therapy in pain management. *J. Pain Res.* 7, 71–79. doi: 10.2147/JPR.S36619

Goss, J. R., Mata, M., Goins, W. F., Wu, H. H., Glorioso, J. C., and Fink, D. J. (2001). Antinociceptive effect of a genomic herpes simplex virus-based vector expressing human proenkephalin in rat dorsal root ganglion. *Gene Ther.* 8, 551–556. doi: 10.1038/sj.gt.3301430

Granier, S., Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Weis, W. I., et al. (2012). Structure of the δ-opioid receptor bound to naltrindole. *Nature* 485, 400–404. doi: 10.1038/nature11111

Greco, M. A., Fuller, P. M., Jhou, T. C., Martin-Schild, S., Zadina, J. E., Hu, Z., et al. (2008). Opioidergic projections to sleep-active neurons in the ventrolateral preoptic nucleus. *Brain Res.* 1245, 96–107. doi: 10.1016/j.brainres.2008.09.043

Gu, Z. H., Wang, B., Kou, Z. Z., Bai, Y., Chen, T., Dong, Y. L., et al. (2017). Endomorphins: promising endogenous opioid peptides for the development of novel analgesics. *Neurosignals* 25, 98–116. doi: 10.1159/000484909

Gupta, A., Mulder, J., Gomes, I., Rozenfeld, R., Bushlin, I., Ong, E., et al. (2010). Increased abundance of opioid receptor heteromers after chronic morphine administration. *Sci. Signal.* 3:ra54. doi: 10.1126/scisignal.2000807

Hackel, D., Krug, S. M., Sauer, R. S., Mousa, S. A., Böcker, A., Pflücke, D., et al. (2012). Transient opening of the perineurial barrier for analgesic drug delivery. *Proc. Natl. Acad. Sci. U.S.A.* 109, E2018–E2027. doi: 10.1073/pnas.1120800109

Hassan, A. H., Ableitner, A., Stein, C., and Herz, A. (1993). Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. *Neuroscience* 55, 185–195. doi: 10.1016/0306-4522(93)90465-R

Hill, R., Disney, A., Conibear, A., Sutcliffe, K., Dewey, W., Husbands, S., et al. (2018). The novel μ-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. *Br. J. Pharmacol.* 175, 2653–2661. doi: 10.1111/bph.14224

Hu, C., Cai, Z., Lu, Y., Cheng, X., Guo, Q., Wu, Z., et al. (2016). Nonviral vector plasmid DNA encoding human proenkephalin gene attenuates inflammatory and neuropathic pain-related behaviors in mice. *Neurosci. Lett.* 634, 87–93. doi: 10.1016/j.neulet.2016.09.040

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician* 16, E199–E216.

Hua, S., Dias, T. H., Pepperall, D. G., and Yang, Y. (2017). Topical loperamide-encapsulated liposomal gel increases the severity of inflammation and accelerates disease progression in the adjuvant-induced model of experimental rheumatoid arthritis. *Front. Pharmacol.* 8:503. doi: 10.3389/fphar.2017.00503

Huang, W., Manglik, A., Venkatakrishnan, A. J., Laeremans, T., Feinberg, E. N., Sanborn, A. L., et al. (2015). Structural insights into μ-opioid receptor activation. *Nature* 524, 315–321. doi: 10.1038/nature14886

Imam, M. Z., Kuo, A., Ghassabian, S., and Smith, M. T. (2017). Progress in understanding mechanisms of opioid-induced gastrointestinal adverse effects and respiratory depression. *Neuropharmacology* 131, 238–255. doi: 10.1016/j.neuropharm.2017.12.032

Ingram, S. L., and Williams, J. T. (1994). Opioid inhibition of Ih via adenylyl cyclase. *Neuron* 13, 179–186. doi: 10.1016/0896-6273(94)90468-5

Inui, S. (2012). Nalfurafine hydrochloride for the treatment of pruritus. *Expert Opin. Pharmacother.* 13, 1507–1513. doi: 10.1517/14656566.2012.693164

Iwaszkiewicz, K. S., and Hua, S. (2014). Development of an effective topical liposomal formulation for localized analgesia and anti-inflammatory actions in the complete freund's adjuvant rodent model of acute inflammatory pain. *Pain Physician* 17, E719–E735.

Jagla, C., Martus, P., and Stein, C. (2014). Peripheral opioid receptor blockade increases postoperative morphine demands–a randomized, double-blind, placebo-controlled trial. *Pain* 155, 2056–2062. doi: 10.1016/j.pain.2014.07.011

Jutkiewicz, E. M., Baladi, M. G., Folk, J. E., Rice, K. C., and Woods, J. H. (2006). The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid agonists in rats: comparison with pentylenetetrazol. *J. Pharmacol. Exp. Ther.* 317, 1337–1348. doi: 10.1124/jpet.105.095810

Kainthan, R. K., Janzen, J., Levin, E., Devine, D. V., and Brooks, D. E. (2006). Biocompatibility testing of branched and linear polyglycidol. *Biomacromolecules* 7, 703–709. doi: 10.1021/bm0504882

Kalso, E., Smith, L., McQuay, H. J., and Andrew Moore, R. (2002). No pain, no gain: clinical excellence and scientific rigour–lessons learned from IA morphine. *Pain* 98, 269–275. doi: 10.1016/S0304-3959(02)00019-2

Kapusta, D. R. (1995). Opioid mechanisms controlling renal function. *Clin. Exp. Pharmacol. Physiol* 22, 891–902. doi: 10.1111/j.1440-1681.1995.tb02324.x

Kastin, A. J., and Pan, W. (2010). Concepts for biologically active peptides. *Curr. Pharm. Des.* 16, 3390–3400. doi: 10.2174/138161210793563491

Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C. G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. U.S.A.* 89, 12048–12052. doi: 10.1073/pnas.89.24.12048

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., and Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 8:e1000412. doi: 10.1371/journal.pbio.1000412

Kingwell, K. (2015). Pioneering biased ligand offers efficacy with reduced on-target toxicity. *Nat. Rev. Drug Discov.* 14, 809–810. doi: 10.1038/nrd4784

Klein, A. H., Mohammad, H. K., Ali, R., Peper, B., Wilson, S. P., Raja, S. N., et al. (2018). Overexpression of μ-opioid receptors in peripheral afferents, but not in combination with enkephalin, decreases neuropathic pain behavior and enhances opioid analgesia in mouse. *Anesthesiology* 128, 967–983. doi: 10.1097/ALN.0000000000002063

Koob, G. F., and Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology* 35, 217–238. doi: 10.1038/npp.2009.110

Kuhar, J. R., Bedini, A., Melief, E. J., Chiu, Y. C., Striegel, H. N., and Chavkin, C. (2015). Mu opioid receptor stimulation activates c-Jun N-terminal kinase 2 by distinct arrestin-dependent and independent mechanisms. *Cell. Signal.* 27, 1799–1806. doi: 10.1016/j.cellsig.2015.05.019

Labuz, D., Schmidt, Y., Schreiter, A., Rittner, H. L., Mousa, S. A., and Machelska, H. (2009). Immune cell-derived opioids protect against neuropathic pain in mice. *J. Clin. Invest.* 119, 278–286. doi: 10.1172/JCI36246

Land, B. B., Bruchas, M. R., Schattauer, S., Giardino, W. J., Aita, M., Messinger, D., et al. (2009). Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19168–19173. doi: 10.1073/pnas.0910705106

Largent-Milnes, T. M., Brookshire, S. W., Skinner, D. P. Jr., Hanlon, K. E., Giuvelis, D., Yamamoto, T., et al. (2013). Building a better analgesic: multifunctional compounds that address injury-induced pathology to enhance analgesic efficacy while eliminating unwanted side effects. *J. Pharmacol. Exp. Ther.* 347, 7–19. doi: 10.1124/jpet.113.205245

Law, P. Y., Wong, Y. H., and Loh, H. H. (2000). Molecular mechanisms and regulation of opioid receptor signaling. *Annu. Rev. Pharmacol. Toxicol.* 40, 389–430. doi: 10.1146/annurev.pharmtox.40.1.389

Lazenka, M. L., Moerke, M. J., Townsend, E. A., Freeman, K. B., Carroll, F. I., and Negus, S. S. (2018). Dissociable effects of the kappa opioid receptor agonist nalfurafine on pain/itch-stimulated and pain/itch-depressed behaviors in male rats. *Psychopharmacology* 235, 203–213. doi: 10.1007/s00213-017-4758-7

Le, Guen S, Mas, Nieto M, Canestrelli, C., Chen, H., Fournié-Zaluski, M. C., Cupo, A., et al. (2003). Pain management by a new series of dual inhibitors of enkephalin degrading enzymes: long lasting antinociceptive properties and potentiation by CCK2 antagonist or methadone. *Pain* 104, 139–148. doi: 10.1016/S0304-3959(02)00486-4

Le Naour, M., Lunzer, M. M., Powers, M. D., Kalyuzhny, A. E., Benneyworth, M. A., Thomas, M. J., et al. (2014). Putative kappa opioid heteromers as targets for developing analgesics free of adverse effects. *J. Med. Chem.* 57, 6383–6392. doi: 10.1021/jm500159d

Lenard, N. R., Daniels, D. J., Portoghese, P. S., and Roerig, S. C. (2007). Absence of conditioned place preference or reinstatement with bivalent ligands containing μ-opioid receptor agonist and δ-opioid receptor antagonist pharmacophores. *Eur. J. Pharmacol.* 566, 75–82. doi: 10.1016/j.ejphar.2007.02.040

Li, Y., and van den Pol, A. N. (2008). Mu-opioid receptor-mediated depression of the hypothalamic hypocretin/orexin arousal system. *J. Neurosci.* 28, 2814–2819. doi: 10.1523/JNEUROSCI.5447-07.2008

Likar, R., Mousa, S. A., Philippitsch, G., Steinkellner, H., Koppert, W., Stein, C., et al. (2004). Increased numbers of opioid expressing inflammatory cells do not affect intra-articular morphine analgesia. *Br. J. Anaesth.* 93, 375–380. doi: 10.1093/bja/aeh222

Likar, R., Mousa, S. A., Steinkellner, H., Koppert, W., Philippitsch, G., Stein, C., et al. (2007). Involvement of intra-articular corticotropin-releasing hormone in postoperative pain modulation. *Clin. J. Pain* 23, 136–142. doi: 10.1097/01.ajp.0000210954.93878.0d

Liu, J. G., and Anand, K. J. (2001). Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence. *Brain Res. Brain Res. Rev.* 38, 1–19. doi: 10.1016/S0165-0173(01)00057-1

Lu, Z., Xu, J., Xu, M., Rossi, G. C., Majumdar, S., Pasternak, G. W., et al. (2018). Truncated μ-opioid receptors with 6 transmembrane domains are essential for opioid analgesia. *Anesth. Analg.* 126, 1050–1057. doi: 10.1213/ANE.0000000000002538

Machelska, H., Brack, A., Mousa, S. A., Schopohl, J. K., Rittner, H. L., Schäfer, M., et al. (2004). Selectins and integrins but not platelet-endothelial cell adhesion molecule-1 regulate opioid inhibition of inflammatory pain. *Br. J. Pharmacol.* 142, 772–780. doi: 10.1038/sj.bjp.0705837

Machelska, H., Cabot, P. J., Mousa, S. A., Zhang, Q., and Stein, C. (1998). Pain control in inflammation governed by selectins. *Nat. Med.* 4, 1425–1428. doi: 10.1038/4017

Machelska, H., Mousa, S. A., Brack, A., Schopohl, J. K., Rittner, H. L., Schafer, M., et al. (2002). Opioid control of inflammatory pain regulated by intercellular adhesion molecule-1. *J. Neurosci.* 22, 5588–5596. doi: 10.1523/JNEUROSCI.22-13-05588.2002

Machelska, H., Pflüger, M., Weber, W., Piranvisseh-Völk, M., Daubert, J. D., Dehaven, R., et al. (1999). Peripheral effects of the kappa-opioid agonist EMD 61753 on pain and inflammation in rats and humans. *J. Pharmacol. Exp. Ther.* 290, 354–361.

Machelska, H., Schroff, M., Oswald, D., Binder, W., Sitte, N., Mousa, S. A., et al. (2009). Peripheral non-viral MIDGE vector-driven delivery of beta-endorphin in inflammatory pain. *Mol. Pain* 5:72. doi: 10.1186/1744-8069-5-7

Majumdar, S., Grinnell, S., Le Rouzic, V., Burgman, M., Polikar, L., Ansonoff, M., et al. (2011). Truncated G protein-coupled mu opioid receptor MOR-1 splice variants are targets for highly potent opioid analgesics lacking side effects. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19778–19783. doi: 10.1073/pnas.1115231108

Mangel, A. W., Bornstein, J. D., Hamm, L. R., Buda, J., Wang, J., Irish, W., et al. (2008). Clinical trial: asimadoline in the treatment of patients with irritable bowel syndrome. *Aliment. Pharmacol. Ther.* 28, 239–249. doi: 10.1111/j.1365-2036.2008.03730.x

Mangel, A. W., and Hicks, G. A. (2012). Asimadoline and its potential for the treatment of diarrhea-predominant irritable bowel syndrome: a review. *Clin. Exp. Gastroenterol.* 5, 1–10. doi: 10.2147/CEG.S23274

Mangel, A. W., and Williams, V. S. (2010). Asimadoline in the treatment of irritable bowel syndrome. *Expert Opin. Investig. Drugs* 19, 1257–1264. doi: 10.1517/13543784.2010.515209

Manglik, A., Lin, H., Aryal, D. K., McCorvy, J. D., Dengler, D., Corder, G., et al. (2016). Structure-based discovery of opioid analgesics with reduced side effects. *Nature* 537, 185–190. doi: 10.1038/nature19112

Melief, E. J., Miyatake, M., Bruchas, M. R., and Chavkin, C. (2010). Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11608–11613. doi: 10.1073/pnas.1000751107

Mestek, A., Hurley, J. H., Bye, L. S., Campbell, A. D., Chen, Y., Tian, M., et al. (1995). The human mu opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C. *J. Neurosci.* 15, 2396–2406. doi: 10.1523/JNEUROSCI.15-03-02396.1995

Miyazaki, T., Choi, I. Y., Rubas, W., Anand, N. K., Ali, C., Evans, J., et al. (2017). NKTR-181: a novel mu-opioid analgesic with inherently low abuse potential. *J. Pharmacol. Exp. Ther.* 363, 104–113. doi: 10.1124/jpet.117.243030

Mogil, J. S. (2009). Animal models of pain: progress and challenges. *Nat. Rev. Neurosci.* 10, 283–294. doi: 10.1038/nrn2606

Mogil, J. S., and Pasternak, G. W. (2001). The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol. Rev.* 53, 381–415.

Montandon, G., Ren, J., Victoria, N. C., Liu, H., Wickman, K., Greer, J. J., et al. (2016). G-protein-gated inwardly rectifying potassium channels modulate respiratory depression by opioids. *Anesthesiology* 124, 641–650. doi: 10.1097/ALN.0000000000000984

Mousa, S. A., Cheppudira, B. P., Shaqura, M., Fischer, O., Hofmann, J., Hellweg, R., et al. (2007). Nerve growth factor governs the enhanced ability of opioids to suppress inflammatory pain. *Brain* 130, 502–513. doi: 10.1093/brain/awl330

Mousa, S. A., Shaqura, M., Al-Madol, M., Tafelski, S., Khalefa, B. I., Shakibaei, M., et al. (2017). Accessibility of axonal G protein coupled mu-opioid receptors requires conceptual changes of axonal membrane targeting for pain modulation. *J. Control. Release* 268, 352–363. doi: 10.1016/j.jconrel.2017.10.016

Nehoff, H., Parayath, N. N., Domanovitch, L., Taurin, S., and Greish, K. (2014). Nanomedicine for drug targeting: strategies beyond the enhanced permeability and retention effect. *Int J Nanomed.* 22, 2539–2555. doi: 10.2147/IJN.S47129

Nestler, E. J., and Aghajanian, G. K. (1997). Molecular and cellular basis of addiction. *Science* 278, 58–63. doi: 10.1126/science.278.5335.58

Noble, F., and Roques, B. P. (2007). Protection of endogenous enkephalin catabolism as natural approach to novel analgesic and antidepressant drugs. *Expert Opin. Ther. Targets* 11, 145–159. doi: 10.1517/14728222.11.2.145

Novak, S. P., Håkansson, A., Martinez-Raga, J., Reimer, J., Krotki, K., and Varughese, S. (2016). Nonmedical use of prescription drugs in the European Union. *BMC Psychiatry* 16:274. doi: 10.1186/s12888-016-0909-3

Ocaña, M., Cendán, C. M., Cobos, E. J., Entrena, J. M., and Baeyens, J. M. (2004). Potassium channels and pain: present realities and future opportunities. *Eur. J. Pharmacol.* 500, 203–219. doi: 10.1016/j.ejphar.2004.07.026

Pasternak, G. W., and Pan, Y. X. (2013). Mu opioids and their receptors: evolution of a concept. *Pharmacol. Rev.* 65, 1257–1317. doi: 10.1124/pr.112.007138

Pattinson, K. T. (2008). Opioids and the control of respiration. *Br. J. Anaesth.* 100, 747–758. doi: 10.1093/bja/aen094

Peterson, C. D., Kitto, K. F., Akgün, E., Lunzer, M. M., Riedl, M. S., Vulchanova, L., et al. (2017). Bivalent ligand that activates mu opioid receptor and antagonizes mGluR5 receptor reduces neuropathic pain in mice. *Pain* 158, 2431–2441. doi: 10.1097/j.pain.0000000000001050

Porreca, F., and Ossipov, M. H. (2009). Nausea and vomiting side effects with opioid analgesics during treatment of chronic pain: mechanisms, implications, and management options. *Pain. Med.* 10, 654–662. doi: 10.1111/j.1526-4637.2009.00583.x

Raehal, K. M., and Bohn, L. M. (2011). The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* 60, 58–65. doi: 10.1016/j.neuropharm.2010.08.003

Raehal, K. M., Walker, J. K., and Bohn, L. M. (2005). Morphine side effects in betaarrestin 2 knockout mice. *J. Pharmacol. Exp. Ther.* 314, 1195–1201. doi: 10.1124/jpet.105.087254

Reiter, E., Ahn, S., Shukla, A. K., and Lefkowitz, R. J. (2012). Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. *Annu. Rev. Pharmacol. Toxicol.* 52, 179–197. doi: 10.1146/annurev.pharmtox.010909.105800

Remesic, M., Hruby, V. J., Porreca, F., and Lee, Y. S. (2017). Recent advances in the realm of allosteric modulators for opioid receptors for future therapeutics. *ACS Chem. Neurosci.* 8, 1147–1158. doi: 10.1021/acschemneuro.7b00090

Rittner, H. L., Brack, A., Machelska, H., Mousa, S. A., Bauer, M., Schäfer, M., et al. (2001). Opioid peptide-expressing leukocytes: identification, recruitment, and simultaneously increasing inhibition of inflammatory pain. *Anesthesiology* 95, 500–508. doi: 10.1097/00000542-200108000-00036

Rivière, P. J. (2004). Peripheral kappa-opioid agonists for visceral pain. *Br. J. Pharmacol.* 141, 1331–1334. doi: 10.1038/sj.bjp.0705763

Rodriguez-Gaztelumendi, A., Spahn, V., Labuz, D., Machelska, H., and Stein, C. (2018). Analgesic effects of a novel pH-dependent μ-opioid receptor agonist in models of neuropathic and abdominal pain. *Pain* 159, 2277–2284. doi: 10.1097/j.pain.0000000000001328

Roeckel, L. A., Utard, V., Reiss, D., Mouheiche, J., Maurin, H., Robé, A., et al. (2017). Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Sci. Rep.* 7:10406. doi: 10.1038/s41598-017-11120-4

Roques, B. P., Fournie-Zaluski, M. C., and Wurm, M. (2012). Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nat. Rev. Drug Discov.* 11, 292–310. doi: 10.1038/nrd3673

Salwan, A. J., Hagemeier, N. E., and Harirforoosh, S. (2018). Abuse-deterrent opioid formulations: a key ingredient in the recipe to prevent opioid disasters? *Clin. Drug Investig.* 38, 573–577. doi: 10.1007/s40261-018-0651-3

Samoshkin, A., Convertino, M., Viet, C. T., Wieskopf, J. S., Kambur, O., Marcovitz, J., et al. (2015). Structural and functional interactions between six-transmembrane μ-opioid receptors and β2-adrenoreceptors modulate opioid signaling. *Sci. Rep.* 5:18198. doi: 10.1038/srep18198

Sawynok, J., and Liu, J. (2014). Contributions of peripheral, spinal, and supraspinal actions to analgesia. *Eur. J. Pharmacol.* 734, 114–121. doi: 10.1016/j.ejphar.2014.04.006

Scascighini, L., Toma, V., Dober-Spielmann, S., and Sprott, H. (2008). Multidisciplinary treatment for chronic pain: a systematic review of interventions and outcomes. *Rheumatology* 47, 670–678. doi: 10.1093/rheumatology/ken021

Schattauer, S. S., Kuhar, J. R., Song, A., and Chavkin, C. (2017). Nalfurafine is a G-protein biased agonist having significantly greater bias at the human than rodent form of the kappa opioid receptor. *Cell. Signal.* 32, 59–65. doi: 10.1016/j.cellsig.2017.01.016

Schmid, C. L., Kennedy, N. M., Ross, N. C., Lovell, K. M., Yue, Z., Morgenweck, J., et al. (2017). Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell* 171, 1165–1175. doi: 10.1016/j.cell.2017.10.035

Schreiter, A., Gore, C., Labuz, D., Fournie-Zaluski, M. C., Roques, B. P., Stein, C., et al. (2012). Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue. *FASEB J.* 26, 5161–5171. doi: 10.1096/fj.12-208678

Seseña, E., Vega, R., and Soto, E. (2014). Activation of μ-opioid receptors inhibits calcium-currents in the vestibular afferent neurons of the rat through a cAMP dependent mechanism. *Front. Cell. Neurosci.* 8:90. doi: 10.3389/fncel.2014.00090

Simonato, M., Bennett, J., Boulis, N. M., Castro, M. G., Fink, D. J., Goins, W. F., et al. (2013). Progress in gene therapy for neurological disorders. *Nat. Rev. Neurol.* 9, 277–291. doi: 10.1038/nrneurol.2013.56

Simonin, F., Gavériaux-Ruff, C., Befort, K., Matthes, H., Lannes, B., Micheletti, G., et al. (1995). kappa-Opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7006–7010. doi: 10.1073/pnas.92.15.7006

Singla, N., Minkowitz, H. S., Soergel, D. G., Burt, D. A., Subach, R. A., Salamea, M. Y., et al. (2017). A randomized, Phase IIb study investigating oliceridine (TRV130), a novel μ-receptor G-protein pathway selective (μ-GPS) modulator, for the management of moderate to severe acute pain following abdominoplasty. *J. Pain Res.* 10, 2413–2424. doi: 10.2147/JPR.S137952

Siuda, E. R., Carr, R. III, Rominger, D. H., and Violin, J. D. (2017). Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. *Curr. Opin. Pharmacol.* 32, 77–84. doi: 10.1016/j.coph.2016.11.007

Snyder, L. M., Chiang, M. C., Loeza-Alcocer, E., Omori, Y., Hachisuka, J., Sheahan, T. D., et al. (2018). Kappa opioid receptor distribution and function in primary afferents. *Neuron* 99, 1274.e6–1288.e6. doi: 10.1016/j.neuron.2018.08.044

Soergel, D. G., Subach, R. A., Burnham, N., Lark, M. W., James, I. E., Sadler, B. M., et al. (2014). Biased agonism of the μ-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: a randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain* 155, 1829–1835. doi: 10.1016/j.pain.2014.06.011

Sondergaard, K. B., and Gislason, G. (2017). NSAIDs and cardiac arrest: non-steroidal anti-inflammatory drug use is associated with increased risk of out-of-hospital cardiac arrest: a nationwide case-time-control study. *Eur. Heart J.* 38, 1788–1789. doi: 10.1093/eurheartj/ehx267

Spahn, V., Del Vecchio, G., Labuz, D., Rodriguez-Gaztelumendi, A., Massaly, N., Temp, J., et al. (2017). A nontoxic pain killer designed by modeling of pathological receptor conformations. *Science* 355, 966–969. doi: 10.1126/science.aai8636

Spahn, V., Del Vecchio, G., Rodriguez-Gaztelumendi, A., Temp, J., Labuz, D., Kloner, M., et al. (2018). Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist. *Sci. Rep.* 8:8965. doi: 10.1038/s41598-018-27313-4

Starnowska, J., Costante, R., Guillemyn, K., Popiolek-Barczyk, K., Chung, N. N., Lemieux, C., et al. (2017). Analgesic properties of opioid/nk1 multitarget ligands with distinct in vitro profiles in naive and chronic constriction injury mice. *ACS Chem. Neurosci.* 8, 2315–2324. doi: 10.1021/acschemneuro.7b00226

Stein, C. (2016). Opioid receptors. *Annu. Rev. Med.* 67, 433–451. doi: 10.1146/annurev-med-062613-093100

Stein, C., Hassan, A. H., Lehrberger, K., Giefing, J., and Yassouridis, A. (1993). Local analgesic effect of endogenous opioid peptides. *Lancet* 342, 321–324. doi: 10.1016/0140-6736(93)91471-W

Stein, C., Hassan, A. H., Przewłocki, R., Gramsch, C., Peter, K., and Herz, A. (1990). Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5935–5939. doi: 10.1073/pnas.87.15.5935

Stein, C., and Jagla, C. (2014). Methylnaltrexone and opioid analgesia. *Pain* 155, 2722–2723. doi: 10.1016/j.pain.2014.09.008

Stein, C., and Kopf, A. (2009). "Anesthesia and treatment for chronic pain," in *Miller's Anesthesia*. 17th Edn, ed. R. D. Miller (Philadelphia, PA: Elsevier), 1797–1818.

Stein, C., and Machelska, H. (2011). Modulation of peripheral sensory neurons by the immune system: implications for pain therapy. *Pharmacol. Rev.* 63, 860–881. doi: 10.1124/pr.110.003145

Stein, C., Pflüger, M., Yassouridis, A., Hoelzl, J., Lehrberger, K., Welte, C., et al. (1996). No tolerance to peripheral morphine analgesia in presence of opioid expression in inflamed synovia. *J. Clin. Invest.* 98, 793–799. doi: 10.1172/JCI118852

Stein, C., Schäfer, M., and Machelska, H. (2003). Attacking pain at its source: new perspectives on opioids. *Nat. Med.* 9, 1003–1008. doi: 10.1038/nm908

Throckmorton, D. C., Gottlieb, S., and Woodcock, J. (2018). The FDA and the next wave of drug abuse - proactive pharmacovigilance. *N. Engl. J. Med.* 379, 205–207. doi: 10.1056/NEJMp1806486

Toll, L., Bruchas, M. R., Calo', G., Cox, B. M., and Zaveri, N. T. (2016). Nociceptin/orphanin FQ receptor structure, signaling, ligands, functions, and interactions with opioid systems. *Pharmacol. Rev.* 68, 419–457. doi: 10.1124/pr.114.009209

Váradi, A., Marrone, G. F., Palmer, T. C., Narayan, A., Szabó, M. R., Le Rouzic, V., et al. (2016). Mitragynine/corynantheidine pseudoindoxyls as opioid analgesics with mu agonism and delta antagonism, which do not recruit β-arrestin-2. *J. Med. Chem.* 59, 8381–8397. doi: 10.1021/acs.jmedchem.6b00748

Vetter, I., Wyse, B. D., Monteith, G. R., Roberts-Thomson, S. J., and Cabot, P. J. (2006). The mu opioid agonist morphine modulates potentiation of capsaicin-evoked TRPV1 responses through a cyclic AMP-dependent protein kinase A pathway. *Mol. Pain* 2:22.

Vicente-Sanchez, A., and Pradhan, A. A. (2017). Ligand-directed signaling at the delta opioid receptor. *Handb. Exp. Pharmacol.* [Epub ahead of print]. doi: 10.1007/164_2017_39

Viscusi, E. R., Webster, L., Kuss, M., Daniels, S., Bolognese, J. A., Zuckerman, S., et al. (2016). A randomized, phase 2 study investigating TRV130, a biased ligand of the μ-opioid receptor, for the intravenous treatment of acute pain. *Pain* 157, 264–272. doi: 10.1097/j.pain.0000000000000363

Volkow, N., Benveniste, H., and McLellan, A. T. (2018). Use and misuse of opioids in chronic pain. *Annu. Rev. Med.* 69, 451–465. doi: 10.1146/annurev-med-011817-044739

Waldhoer, M., Bartlett, S. E., and Whistler, J. L. (2004). Opioid receptors. *Annu. Rev. Biochem.* 73, 953–990. doi: 10.1146/annurev.biochem.73.011303.073940

Walker, J. S., Catheline, G., Guilbaud, G., and Kayser, V. (1999). Lack of cross-tolerance between the antinociceptive effects of systemic morphine and asimadoline, a peripherally selective κ-opioid agonist, in CCI-neuropathic rats. *Pain* 83, 509–516. doi: 10.1016/S0304-3959(99)00158-X

Wang, D., Tawfik, V. L., Corder, G., Low, S. A., François, A., Basbaum, A. I., et al. (2018). Functional divergence of delta and mu opioid receptor organization in CNS pain circuits. *Neuron* 98, 90–108. doi: 10.1016/j.neuron.2018.03.002

Webster, L., Henningfield, J., Buchhalter, A. R., Siddhanti, S., Lu, L., Odinecs, A., et al. (2018). Human abuse potential of the new opioid analgesic molecule NKTR-181 compared with oxycodone. *Pain Med.* 19, 307–318. doi: 10.1093/pm/pnw344

Weibel, R., Reiss, D., Karchewski, L., Gardon, O., Matifas, A., Filliol, D., et al. (2013). Mu opioid receptors on primary afferent nav1.8 neurons contribute to opiate-induced analgesia: insight from conditional knockout mice. *PLoS One* 8:e74706. doi: 10.1371/journal.pone.0074706

Welsch, P., Üçeyler, N., Klose, P., Walitt, B., and Häuser, W. (2018). Serotonin and noradrenaline reuptake inhibitors (SNRIs) for fibromyalgia. *Cochrane Database. Syst. Rev.* 2:CD010292. doi: 10.1002/14651858.CD010292.pub2

White, K. L., Robinson, J. E., Zhu, H., DiBerto, J. F., Polepally, P. R., Zjawiony, J. K., et al. (2015). The G protein-biased κ-opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities in vivo. *J. Pharmacol. Exp. Ther.* 352, 98–109. doi: 10.1124/jpet.114.216820

Wieskopf, J. S., Pan, Y. X., Marcovitz, J., Tuttle, A. H., Majumdar, S., Pidakala, J., et al. (2014). Broad-spectrum analgesic efficacy of IBNtxA is mediated by exon 11-associated splice variants of the mu-opioid receptor gene. *Pain* 155, 2063–2070. doi: 10.1016/j.pain.2014.07.014

Wolfe, D., Mata, M., and Fink, D. J. (2009). A human trial of HSV-mediated gene transfer for the treatment of chronic pain. *Gene Ther.* 16, 455–460. doi: 10.1038/gt.2009.17

Yaksh, T. L. (1997). Pharmacology and mechanisms of opioid analgesic activity. *Acta Anaesthesiol. Scand.* 41, 94–111. doi: 10.1111/j.1399-6576.1997.tb04623.x

Yekkirala, A. S., Lunzer, M. M., McCurdy, C. R., Powers, M. D., Kalyuzhny, A. E., Roerig, S. C., et al. (2011). N-naphthoyl-beta-naltrexamine (NNTA), a highly selective and potent activator of μ/kappa-opioid heteromers. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5098–5103. doi: 10.1073/pnas.1016277108

Yudin, Y., and Rohacs, T. (2018). Inhibitory Gi/O-coupled receptors in somatosensory neurons: potential therapeutic targets for novel analgesics. *Mol. Pain* 14:1744806918763646. doi: 10.1177/1744806918763646

Zadina, J. E., Hackler, L., Ge, L. J., and Kastin, A. J. (1997). A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 386, 499–502. doi: 10.1038/386499a0

Zadina, J. E., Nilges, M. R., Morgenweck, J., Zhang, X., Hackler, L., and Fasold, M. B. (2016). Endomorphin analog analgesics with reduced abuse liability, respiratory depression, motor impairment, tolerance, and glial activation relative to morphine. *Neuropharmacology* 105, 215–227. doi: 10.1016/j.neuropharm.2015.12.024

Zeng, C., Gao, S. G., Cheng, L., Luo, W., Li, Y. S., Tu, M., et al. (2013). Single-dose intra-articular morphine after arthroscopic knee surgery: a meta-analysis of randomized placebo-controlled studies. *Arthroscopy* 29, 1450–1458. doi: 10.1016/j.arthro.2013.04.005

Zöllner, C., Mousa, S. A., Fischer, O., Rittner, H. L., Shaqura, M., Brack, A., et al. (2008). Chronic morphine use does not induce peripheral tolerance in a rat model of inflammatory pain. *J. Clin. Invest.* 118, 1065–1073. doi: 10.1172/JCI25911

Zöllner, C., Shaqura, M. A., Bopaiah, C. P., Mousa, S., Stein, C., and Schafer, M. (2003). Painful inflammation-induced increase in mu-opioid receptor binding and G-protein coupling in primary afferent neurons. *Mol. Pharmacol.* 64, 202–210. doi: 10.1124/mol.64.2.202

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The Nociceptin Receptor (NOP) Agonist AT-312 Blocks Acquisition of Morphine- and Cocaine-Induced Conditioned Place Preference in Mice

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Treatment of drug addiction remains an unmet medical need due to the dearth of approved pharmacotherapies. There are no approved treatments for cocaine addiction, whereas the current opioid crisis has revealed the stark reality of the limited options to treat prescription and illicit opioid abuse. Preclinical studies in rodents and nonhuman primates have shown that orphanin FQ/nociceptin (N/OFQ), the endogenous ligand for the nociceptin opioid receptor (NOP) reduces the rewarding effects of several abused substances, including opioids, psychostimulants and alcohol. A few nonpeptide small-molecule NOP agonists have also shown efficacy in attenuating the rewarding effects of various abused drugs. We previously demonstrated that a high affinity small-molecule NOP agonist AT-312 selectively reduced the rewarding effects of ethanol in the conditioned place preference paradigm in mice. In the present study, we examined if AT-312 (3 mg/kg, i.p. or s.c. respectively), would alter the rewarding action of morphine (7.5 mg/kg, s.c.) or cocaine (15 mg/kg, i.p.). The effect of AT-312 on morphine- and cocaine-induced motor stimulation was also assessed on the conditioning days. The role of the NOP receptor in the effects of AT-312 was further confirmed by conducting the place conditioning experiments in NOP knockout mice and compared to their wild-type controls. Our results showed that AT-312 significantly reduced the acquisition of morphine and cocaine CPP in wild-type mice but not in mice lacking NOP receptors. AT-312 also suppressed morphine-induced and completely abolished cocaine-induced motor stimulation in NOP wild-type mice, but not in NOP knockout mice. These results show that small-molecule NOP receptor agonists have promising efficacy for attenuating the rewarding effects of morphine and cocaine, and may have potential as pharmacotherapy for opioid and psychostimulant addiction or for treating polydrug addiction.

Keywords: morphine, cocaine, conditioned place preference, AT-312, NOP agonist, NOP receptor knockout, polydrug addiction, addiction pharmacotherapy

INTRODUCTION

Addiction pharmacotherapy remains an area severely in need of new approaches and new targets, given that there are no approved therapies for cocaine addiction and the limited suboptimal options available for those addicted to opioids fueling the opioid crises. Addiction to more than one drug is also quite prevalent, but there are few, if any, appropriate pharmacotherapies that can address polydrug addiction effectively. Most addictive substances cause an increase in dopamine release in the mesolimbic areas of the reward circuitry, albeit through different mechanisms. Cocaine blocks the dopamine transporter and increases dopamine levels in the nucleus accumbens (NAc) (1–3); whereas opioid drugs increase dopamine levels in the VTA and NAc through their action at the mu opioid receptor (4, 5). Even the endogenous opioid peptides, beta-endorphin and enkephalins increase the activity of the mesolimbic dopaminergic neurons and stimulate dopamine release in the NAc (6). In fact, the endogenous opioid system has been shown to be involved in the rewarding actions of other drugs of abuse such as cocaine and alcohol (7–9). Therefore, approaches that inhibit the dopaminergic transmission in the mesolimbic circuitry may be useful for reducing rewarding effects of many different addictive drugs.

The endogenous peptide, nociceptin/orphanin FQ (N/OFQ), acting through the nociceptin opioid peptide (NOP) receptor, is present in several areas of the brain associated with reward and stress pathways such as the ventral tegmental area, prefrontal cortex, amygdala and lateral hypothalamus (10, 11). The NOP receptor and its endogenous ligand N/OFQ are the fourth members of the opioid family of G protein-coupled receptors mu, delta and kappa and their endogenous ligands endorphins, enkephalins and dynorphin (12, 13). Unlike the classical opioid ligands, however, N/OFQ has a broad inhibitory effect on multiple neurotransmitter systems involved in drug reward and has been shown to decrease drug-induced dopamine levels in the nucleus accumbens (14, 15). Intracerebroventricular (i.c.v.) administration of N/OFQ has been shown to block morphine-induced dopamine release in the NAc (14, 16–19). We and others have shown that central administration of the N/OFQ peptide decreases cocaine-induced dopamine release in the NAc in rats (20, 21). Consistent with these observations, i.c.v. administration of N/OFQ blocks the rewarding effects of morphine, cocaine and alcohol in animal models of drug reward such as the conditioned place preference (CPP) (17, 22–24). Targeting the NOP-N/OFQ system is therefore a potential approach to reduce the rewarding effects of multiple abused substances and develop pharmacotherapy to treat addiction to various drugs and possibly polydrug addiction (25–28).

Some effort along these lines has been expended with few synthetic small-molecule NOP agonists, producing equivocal results. The nonpeptide small-molecule NOP agonist Ro 64-6198 was reported to block acquisition and reinstatement of morphine-induced CPP after intraperitoneal (i.p.) administration (29), and the expression, acquisition and reinstatement of alcohol-induced CPP in mice (24). However, i.p. Ro 64-6198 was also reported to produce a place preference in rats through a purported dopaminergic mechanism, and not through an opioid or NOP-related mechanism (30). A different NOP agonist Ro 65-6570 on the other hand, blocked opioid- and cocaine-induced CPP in rats in the same study, and this effect was reversed by a NOP antagonist J-113397, confirming the role of the NOP receptor in the anti-rewarding actions of Ro 65-6570 (30). NOP agonist SCH221510 was shown to reduce opioid (remifentanil) self-administration in rats only when administered intracisternally but not systemically (31). We recently reported that SCH221510 showed a modest inhibition of ethanol-induced CPP in mice at high doses given i.p. (32). On the other hand, in the same study, we showed that a novel and selective NOP agonist AT-312 showed a significant and robust inhibition of ethanol CPP in mice at doses lower than that of SCH221510. Moreover, this effect was absent in mice lacking the NOP receptor, confirming the NOP-targeted inhibition of ethanol CPP by AT-312 (32).

The aims of this study were to determine the efficacy of AT-312 in attenuating the rewarding effects of other drugs of abuse such as morphine and cocaine. To confirm the pharmacological mechanism of the effect of AT-312, we conducted the CPP experiments in mice lacking the NOP receptor and their wild-type littermates. In addition, we also characterized the effect of AT-312 on the locomotor stimulation produced by morphine or cocaine in wild-type and NOP knockout mice.

MATERIALS AND METHODS

Subjects

Mice lacking the NOP receptor and their wild-type controls, fully backcrossed on a C57BL/6J mouse strain, were bred in house, and weaned at the age of 21–24 days, prior to genotyping. Mice between the ages of 2–4 months were used for these experiments. Mice were housed 2–4 per cage with free access to laboratory chow and water and maintained under a 12 h light/12 h dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, CA) and were in accord with the NIH Guide for the Use and Care of Animal in Research.

Drugs

AT-312 ((1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol) was synthesized at Astraea Therapeutics. The details of the synthesis and the *in vitro* pharmacological profile of AT-312 at the opioid receptors have been previously reported (32). AT-312 was dissolved in 1–2% DMSO and then diluted to the desired concentration with 0.5% aqueous hydroxypropylcellulose (HPC) and injected subcutaneously (s.c.) or intraperitoneally (i.p.) at a dose of 3 mg/kg in a volume of 0.1 ml/10 g of body weight. The dose of AT-312 was selected based on our previous study (32). Controls received 0.1 ml/10 g of body weight of the appropriate vehicle (1–2% DMSO in 0.5% of HPC). Morphine sulfate and cocaine hydrochloride were obtained from NIDA Drug Supply and dissolved in normal saline. The doses of morphine and cocaine are as their salt forms.

Experimental Procedures

Effect of AT-312 on Morphine-Induced CPP in Wild-Type and NOP Knockout Mice

A detailed description of the CPP apparatus and procedure is given elsewhere (33). Briefly, female mice lacking NOP receptor and their wild-type controls were tested for baseline place preference on day 1, in which each mouse was placed in the neutral central chamber and allowed to freely explore the CPP chambers for 15 min. The amount of time that mice spent in each conditioning chamber was recorded. There was no initial preference for any of the CPP chambers, i.e., the CPP paradigm was unbiased. The following day, mice were treated with either vehicle or AT-312 (3 mg/kg, i.p.) followed, 5 min later, by morphine or saline (morphine vehicle) (7.5 mg/kg, s.c.) and confined to the vehicle-paired chamber (VPCh; if they received vehicle and then saline) or drug-paired chamber (DPCh; if they received vehicle or AT-312 before morphine) for 60 min. The dose of AT-312 and morphine, the conditioning time, and the time of AT-312 administration with respect to morphine administration were based on our previous studies (32, 34). The CPP procedure was carried out in a counterbalanced manner, in which some animals were treated with vehicle or AT-312 followed by morphine in the morning and some in the afternoon. If they received vehicle or AT-312 followed by morphine in the morning, they received vehicle followed by saline in the afternoon on that day. If they received vehicle followed by saline in the morning, they received conditioning with vehicle or AT-312 followed by morphine in the afternoon. Locomotor activity, measured as distance traveled, was also recorded on each conditioning day. The twice daily conditioning continued for three consecutive days on days 2–4. Mice were then tested for post conditioning place preference on day 5 in a drug-free state, as described for day 1.

Effect of AT-312 on Cocaine-Induced CPP in Wild-Type and NOP Knockout Mice

We also assessed the effect of AT-312 on cocaine-induced CPP in mice. The CPP procedure was the same as described above for morphine except that male mice were used for this experiment. On conditioning days, mice were treated with vehicle or AT-312 (3 mg/kg, s.c.) and 5 min later with cocaine (15 mg/kg, i.p.), and then confined to the DPCh for 30 min. Other mice received vehicle and 5 min later saline and were confined to the VPCh for 30 min. In the afternoon, mice received the alternative treatment (e.g., vehicle followed by saline; if they were treated with vehicle or AT-312 followed by cocaine) and were confined to the opposite conditioning chamber (e.g., chamber with the rod floor if mice received the other treatment in the chamber with mesh floor) for 30 min. This twice-daily conditioning lasted for 3 consecutive days and mice were tested for post conditioning place preference on day 5, as described above.

Data Analysis

Values represent mean (± S.E.M.) of the amount of time (sec) that mice spent in the CPP chambers on the preconditioning (day 1, D1) and postconditioning (day 5, D5) test days or the distance traveled (cm) during the conditioning days. Data were analyzed using three-way repeated measures analysis of variance (ANOVA) followed by the *post-hoc* Tukey's test. The factors were pretreatment (i.e., vehicle vs. AT-312) and treatment (saline vs. cocaine) and time (day 1 and day 5). A $P < 0.05$ was considered significant.

RESULTS

AT-312 Reduced CPP Induced by Morphine in Wild-Type but Not NOP Knockout Mice

Figure 1 shows the amount of time (sec) that mice lacking NOP and their wild-type controls spent in the conditioning chambers before (day 1, D1) and after (day 5, D5) conditioning. A three-way ANOVA of the data in wild-type mice showed a significant effect of treatment [$F_{1, 40} = 14.88$; $P < 0.001$] and a significant interaction between time and treatment [$F_{1, 40} = 16.61$; $P < 0.001$] and a trend toward an interaction between time, pretreatment and treatment [$F_{1, 40} = 3.09$; $P < 0.09$]. The Tukey's *post-hoc* test revealed that morphine induced a robust CPP response in vehicle-treated control mice, as evidenced by a significant ($P < 0.0001$) increase in the amount of time that mice spent in the drug-paired chamber (DPCh) compared to vehicle-paired chamber (VPCh) on the postconditioning test day (D5; **Figure 1**; upper panel; compare DPCh vs. VPCh for the Veh-Mor group). However, this response was abolished in mice treated with AT-312 in conjunction with morphine on each conditioning day (**Figure 1**, upper panel; compare DPCh vs. VPCh for the AT-Mor group). In contrast, AT-312 did not alter morphine-induced CPP in mice lacking NOP (**Figure 1**, lower panel). The Tukey *post-hoc* test revealed a significant increase in the amount of time that mice of each pretreatment group (vehicle or AT-312 pretreated) spent in the DPCh vs. VPCh on day 5 ($P < 0.05$). These results indicate that AT-312 blocked the acquisition of morphine-induced CPP via the NOP receptor.

AT-312 Reduced Morphine-Induced Motor Stimulation During Conditioning in Wild-Type but Not in NOP Knockout Mice

We found that AT-312 reduced the motor stimulatory effect of morphine in wild-type mice (**Figure 2**, upper right panel) but not in mice lacking NOP receptor (**Figure 2**, lower right panel). Three-way ANOVA of the data in wild-type mice revealed a significant effect of pretreatment [$F_{(1, 60)} = 4.03$, $P < 0.05$] and a significant effect of treatment [$F_{(1, 60)} = 62.11$, $P < 0.0001$] but no significant interaction between pretreatment, treatment and time [$F_{(2, 60)} = 0.11$, $P > 0.05$]. The Tukey's *post-hoc* test revealed that vehicle-pretreated mice traveled significantly greater distances in the chamber conditioned with morphine compared to saline on each conditioning day ($P < 0.05$; **Figure 2**, upper left panel). However, this response was attenuated in wild-type mice pretreated with AT-312 as evidenced by no significant difference in distance traveled after morphine treatment compared to saline in this group ($P > 0.05$; **Figure 2**, upper right panel). On the other hand, the *post-hoc* test showed that mice lacking NOP receptors conditioned with morphine traveled significantly more distance compared to saline-conditioned animals on each conditioning day (**Figure 2**, lower left panel). However, this response was not reduced in NOP knockout mice treated with AT-312 prior to

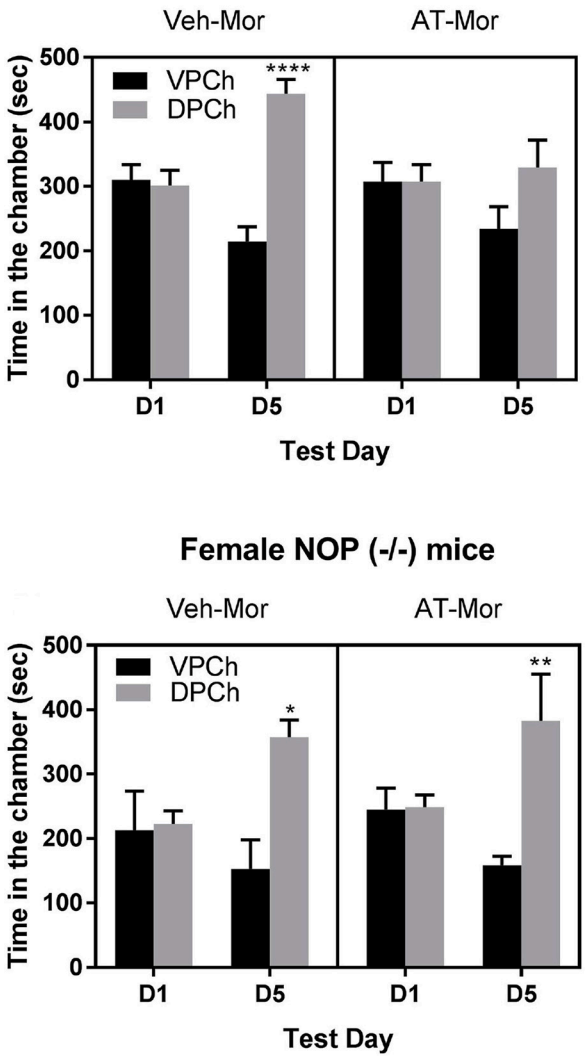

FIGURE 1 | Effects of AT-312 on morphine-induced CPP in mice lacking NOP and their wild-type littermates/controls. Mice ($n = 6$ mice per treatment for each genotype) were tested for baseline place preference on day 1, conditioned with morphine (7.5 mg/kg, s.c.) in the presence and absence of AT-312 (3 mg/kg, i.p.) on days 2–4 and then tested for CPP on day 5. Vehicle or AT-312 was given 5 min before morphine on each conditioning day to wild-type **(Upper)** and knockout **(Lower)** mice. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$, a significant increase in the amount of time that mice spent in the drug-paired chamber (DPCh) compared to vehicle-paired chamber (VPCh) on this day.

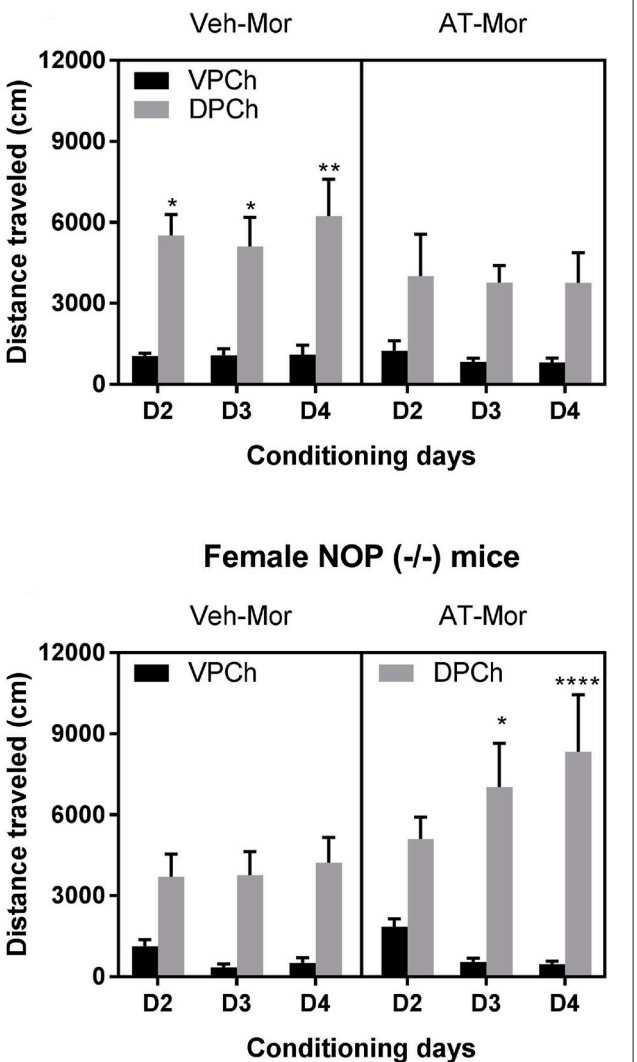

FIGURE 2 | Effects of AT-312 on morphine-induced motor stimulation during the conditioning days in the CPP chambers in mice lacking NOP and their wild-type littermates/controls. On each conditioning day, wild-type **(Upper)** and NOP knockout **(Lower)** mice were treated with vehicle or AT-312 (3 mg/kg; i.p.; $n = 6$ mice per treatment for each genotype) 5 min before morphine (7.5 mg/kg, s.c.) and distance traveled by mice in the morphine-paired chamber (DPCh) was recorded for 60 min each day. Distance traveled by the mice in the saline-paired chamber (VPCh) was also recorded, in which mice were injected with vehicle and 5 min later with saline. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$, a significant increase in distance traveled by mice in the DPCh vs. their respective VPCh on this day.

morphine (**Figure 2**, lower right panel). Interestingly, AT-312 not only did not reduce morphine-induced motor stimulation in the NOP knockout mice but it appeared that the stimulatory action of morphine was increased in these mice in the presence of AT-312. (**Figure 2**, lower right panel, compare day 2 vs. day 4) However, this response was not different than that in the Veh-Mor knockout mice ($P > 0.05$). Overall, these results suggest the involvement of the NOP receptors in the suppressive effect of AT-312 on the motor stimulation produced by morphine.

AT-312 Reduced Acquisition of Cocaine CPP in Wild-Type but Not NOP Knockout Mice

Figure 3 shows the amount of time that wild-type (upper panel) and knockout (lower panel) mice spent in the vehicle-paired chamber (VPCh) and drug-paired chamber (DPCh) on the preconditioning (D1) and postconditioning (D5) test days.

Three-way repeated measures ANOVA of the data in wild-type mice showed a significant effect of treatment [$F_{(1, 96)} = 11.35$; $P < 0.001$], a significant effect of pretreatment × treatment interaction [$F_{(1, 96)} = 4.96$: $P < 0.03$] and a significant interaction between time × pretreatment × treatment [$F_{(1, 96)} = 6.93$; $P < 0.01$]. The Tukey's *post-hoc* test showed that cocaine induced a robust CPP in vehicle-treated control, as evidenced by a significant increase in the amount of time that mice spent in the DPCh compared to VPCh on the postconditioning day (**Figure 3**, upper left panel; compare DPCh vs. VPCh on day 5 for the Veh-Coc group). In contrast, this response was abolished in mice treated with AT-312 prior to cocaine on each conditioning day (**Figure 3**, upper right panel; compare DPCh vs. VPCh on day 5 in the AT-Coc group). On the other hand, AT-312 had no effect on the place preference induced by cocaine in mice lacking NOP (**Figure 3**, lower left panel). Three-way ANOVA showed a significant effect of time × treatment interaction [$F_{1, 40} = 12.62$, $P < 0.001$] but no significant interaction between pretreatment × treatment [$F_{1, 40} = 0.01$; $P > 0.05$] and no significant interaction between pretreatment × treatment and time [$F_{1,40} = 0.05$; $P > 0.05$]. The Tukey's *post-hoc* test revealed a significant increase in the amount of time that mice spent in DPCh compared to VPCh on the postconditioning day regardless of the pretreatment ($P < 0.05$). Overall, these results indicate that AT-312 blocked cocaine-induced CPP via the NOP receptor.

Figure 4 shows the effect of AT-312 on the motor stimulatory action of cocaine in wild-type (upper panel) and NOP knockout (lower panel) mice. Three-way ANOVA revealed a significant interaction between pretreatment and treatment [$F_{(1, 60)} = 43.9$, $P < 0.0001$] but no significant time × treatment [$F_{(2, 60)} = 0.63$; $P > 0.05$] or time × pretreatment × treatment interaction [$F_{(2, 60)} = 0.65$; $P > 0.05$]. *Post-hoc* analyses of the data showed that cocaine increases motor activity compared to saline in wild-type mice (**Figure 4**, upper left panel; compare distance traveled between DPCh and VPCh in mice pretreated with vehicle and conditioned with cocaine, i.e., Veh-Coc group). This response was completely blocked in wild-type mice pretreated with AT-312 (**Figure 4**, upper right panel; compare distance traveled in the DPCh vs. VPCh in mice AT-Coc group). In contrast, AT-312 failed to alter the motor stimulatory action of cocaine in mice lacking NOP (**Figure 4**, lower right panel). Three-way ANOVA revealed a significant effect of treatment [$F_{(1, 60)} = 82.02$; $P < 0.0001$] but no significant interaction between pretreatment and treatment [$F_{(1, 60)} = 0.12$; $P > 0.05$] or time × pretreatment × treatment interaction [$F_{(2, 60)} = 0.43$; $P > 0.05$]. The *post-hoc* test showed a significant increase in distance traveled by cocaine in mice lacking NOP regardless of the pretreatment (**Figure 4**, lower panel). Together, these results suggest that AT-312 abolished the motor stimulatory action of cocaine in wild-type but not knockout mice.

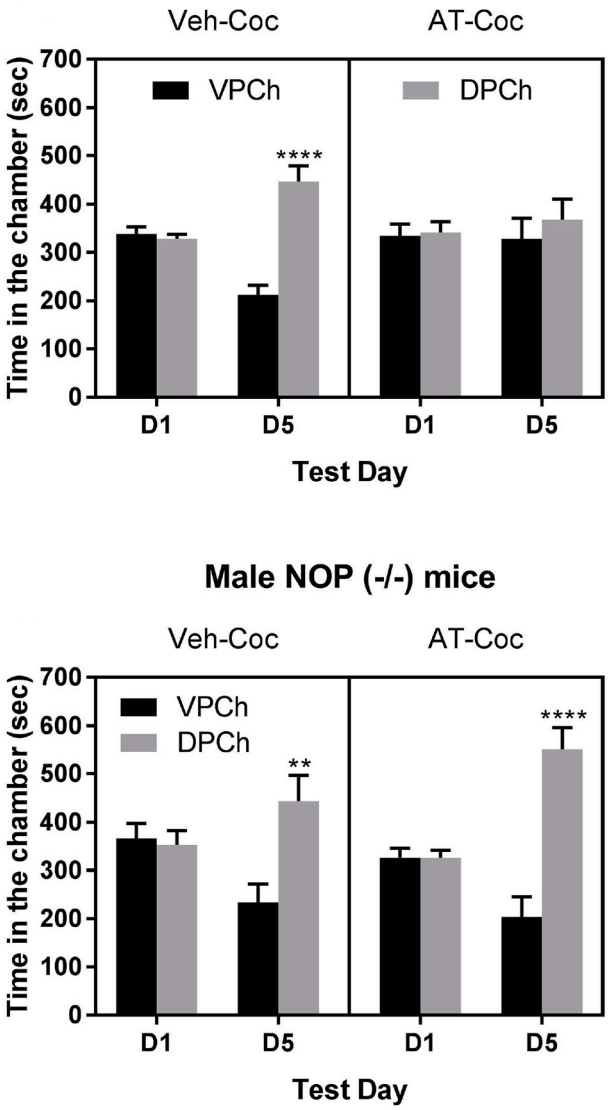

FIGURE 3 | Effect of AT-312 on cocaine-induced CPP in wild-type (**Upper**; $n = 13$ mice per treatment) or knockout (**Lower**; $n = 10$ mice per treatment) mice. Data are mean (±SEM) of the amount of time that mice spent in the vehicle-paired chamber (VPCh) and drug-paired chamber (DPCh) on the preconditioning (D1) and postconditioning (D5) test days. $^{**}P < 0.01$; $^{****}P < 0.0001$, a significant increase in the amount of time that mice spent in the DPCh vs. its respective VPCh on D5.

DISCUSSION

The main findings of the present study are (i) AT-312 significantly attenuates acquisition of CPP to morphine and cocaine in the CPP paradigm; (ii) AT-312 attenuates morphine and cocaine CPP through its action at the NOP receptor, as this attenuation is absent in mice lacking the NOP receptor; and (iii) AT-312 blocks morphine- and cocaine-induced locomotor stimulation.

Previous studies have shown that N/OFQ reduces the rewarding action of morphine (17, 35), raising the possibility that nonpeptide NOP agonists may reduce the rewarding effects of opioids. Indeed, the selective small-molecule NOP agonist Ro 64-6198 blocked the acquisition and reinstatement of morphine CPP in mice (29). A chemically-related NOP agonist Ro 65-6570

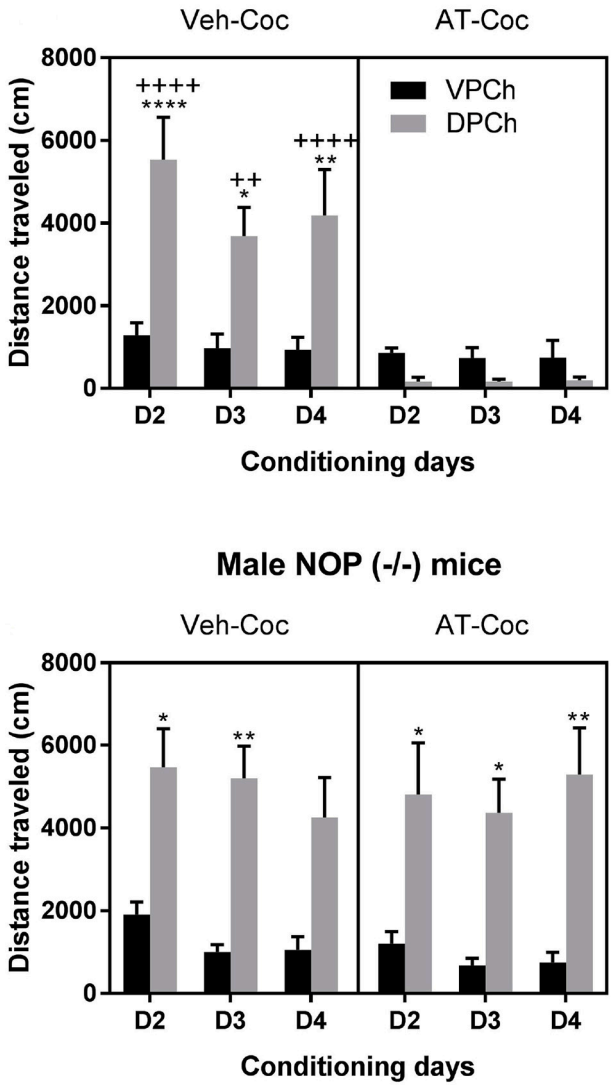

FIGURE 4 | Effects of AT-312 on cocaine-induced motor stimulation during conditioning in wild-type and knockout mice. Motor activity was measured in a subset of animals ($n = 6$ mice per genotype for each treatment) and the experiment was repeated three times ($n = 2$ mice of each genotype per treatment for each cohort). On each conditioning day, wild-type **(Upper)** and NOP knockout **(Lower)** mice were treated with vehicle or AT-312 (3 mg/kg) 5 min before cocaine (15 mg/kg, i.p.) and distance traveled by mice in the drug-paired chamber (DPCh) was recorded for 30 min each day. The distance traveled by mice in the saline-paired chamber (VPCh) was also recorded, in which mice were injected with vehicle and 5 min later with saline. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$, a significant increase in distance traveled by mice in the DPCh vs. their respective VPCh on this day. $^{++}P < 0.01$ and $^{++++}P < 0.0001$ vs. DPCh in mice treated with AT-312 followed by cocaine on this day.

demonstrated significant "anti-opiate" effects in rats by reducing CPP of a variety of opiate drugs including morphine, oxycodone, and heroin, particularly when administered within a 15-min (but not 30-min) pretreatment interval. We previously reported that another chemically distinct, and modestly selective NOP agonist AT-202 suppressed the acquisition of morphine CPP in mice, and this effect was reversed by pretreatment with a selective NOP antagonist SB-612111 (36), confirming the NOP receptor as the target for the anti-rewarding effect of AT-202. Results in this study showed that morphine induced a robust acquisition of CPP response in NOP wild-type mice, and this response was significantly reduced in mice pretreated with AT-312 in conjunction with morphine. The inhibitory effect of AT-312 on acquisition of morphine CPP appears to be consistent with that observed with other previously reported NOP agonists. Morphine also showed a robust CPP in mice lacking the NOP receptor; however, pretreatment with AT-312 had no effect on morphine place preference in these mice. This lack of an effect in the NOP knockout mice further confirms that the efficacy of AT-312 in attenuating morphine place preference is through its action at the NOP receptor.

We recently showed that AT-312 is devoid of intrinsic motivational effects and does not induce CPP or CPA in mice (32). Similarly, we have shown that other NOP-selective agonists such as AT-202 is also devoid of intrinsic rewarding effects in the CPP paradigm in mice, as is the NOP partial agonist AT-200 (36). N/OFQ (i.c.v.) was also shown to be devoid of intrinsic rewarding effects (37) and even though it decreases basal dopamine levels in the NAc (14), it shows only a mild conditioned place aversion in mice (23). Among other nonpeptide NOP agonists, Ro 65-6570 showed no intrinsic rewarding effects in the CPP paradigm in rats (30); but, the closely related Ro 64-6198 appeared to induce a place preference in rats in this same study, an effect reversed by dopamine D2 receptor antagonist haloperidol but not by a NOP antagonist or opioid antagonist naloxone (30). Taken together, it appears that nonpeptide NOP agonists lack intrinsic rewarding effects and show selective attenuation of the acquisition of morphine CPP through their action at the NOP receptor.

Opioids and psychostimulant drugs are known to produce hyperlocomotion in rodents. Exogenously administered (i.c.v) N/OFQ (1–10 nmol) inhibits spontaneous locomotor activity in mice (12, 38–40) and rats (41). Icv N/OFQ has also been shown to block the locomotor stimulant effect produced by cocaine in rats (20, 21) although N/OFQ had no effect on morphine-induced locomotor sensitization (22). Small-molecule NOP agonists such as Ro 64-6198 (i.p.) also decreased locomotor activity in mice at 1 and 3 mg/kg doses for 30 min after administration, returning to normal at 60 min (42). One concern with compounds that cause motor sedation is that this may have confounding effects in the place conditioning paradigm. AT-312 was also found to decrease motor activity in wild-type mice in this study. However, since the CPP test is conducted in a drug-free state, we believe that the motor-suppressing effect of the drug is not the reason for the inhibitory effect of AT-312 on the place preference. While AT-312 completely blocked acquisition of morphine CPP, it only modestly reduced the motor stimulatory effect of morphine in wild-type mice (**Figure 2**, upper right panel). This effect was via the NOP receptor because AT-312 did not reduce morphine-induced motor stimulation in NOP knockout mice (**Figure 2**, lower right panel). On the contrary, in NOP knockout mice,

AT-312 pretreatment appeared to enhance the motor stimulatory action of morphine (**Figure 2**, lower right panel). It is tempting to conclude that this effect of AT-312 on morphine locomotor activity in the absence of the NOP receptor, is due to the low level of MOP partial agonist activity of AT-312 (32). However, this may be less likely because the CPP response was comparable between the vehicle and the AT-312-treated groups in the NOP knockout mice (**Figure 1**, lower panel). The use of a MOP receptor antagonist in NOP knockout mice should shed more light in this regard. Together, it appears that AT-312 selectively blocks the rewarding and motor stimulatory actions of morphine through the NOP receptor. Furthermore, this effect is not through a non-selective reduction in the motor activity of mice, as we recently demonstrated that other sedative-hypnotic drugs such as pentobarbital, which robustly reduce motor activity in mice, do not diminish the place preference induced by ethanol, even when administered at motor-suppressive doses (32).

N/OFQ (i.c.v.) has been shown to reduce the rewarding effects of cocaine in mice (23). However, studies with small-molecule NOP agonists thus far have shown inconsistent results in their effects in the place conditioning paradigm in rodents (30, 43, 44). For instance, Sartor and colleagues reported that SR-8993, a highly selective nonpeptide agonist failed to block expression, acquisition or reinstatement of cocaine CPP in mice (43). However, the NOP full agonist Ro 65-6570 significantly attenuated cocaine CPP in rats, and the effect was particularly robust when the pretreatment time was increased to 15-min prior to cocaine injections (30). We observed a complete blockade of cocaine-induced CPP in wild-type mice by AT-312 and this response was abolished in mice lacking NOP, suggesting that the inhibitory action of AT-312 was via the NOP receptor. We also found that AT-312 robustly reduced the motor stimulatory effect of cocaine in wild-type mice, which is consistent with our earlier results using N/OFQ (20, 45). Although we do not know why the previous study with small-molecule NOP agonist SR-8993 failed to show an effect on the rewarding action of cocaine, one cannot rule out the impact of pharmacokinetic profiles and pretreatment intervals, which could be a potential confound in terms of their effectiveness in reducing the rewarding effects of cocaine (43). We reported the pharmacokinetic profile and brain penetration of AT-312 in mice (32) and have observed in our studies that AT-312 works within minutes of administration, and that its peak plasma and brain concentration occur within 30 min. Thus, with our pretreatment interval of 5-min and testing period of 30-min, we observe a robust inhibitory effect of AT-312 on cocaine CPP. It is possible that for NOP agonist SR-8993, the reported pretreatment times of 30 min or 2 h before cocaine administration may have missed the window of effect of this compound on cocaine-induced CPP. Further studies with new chemically unrelated selective NOP agonists are certainly warranted to clear the inconsistencies observed thus far with nonpeptide NOP agonists in cocaine reward.

There are different phases of CPP, the acquisition when the CPP response develops and the expression of the CPP response, when the CPP response is measured. In the present study we only assessed the effect of the NOP agonist on the acquisition of the CPP response and tested animals in a drug-free state to rule out the impact of motor impairment on the expression of the CPP response, given that NOP agonists are known to cause motor suppression. However, further studies are needed to assess the impact of NOP agonists on the expression of the CPP response, on the CPP response once it is fully developed and on extinction and reinstatement processes.

NOP agonists have been shown to impact learning and memory processes. In particular, NOP agonists are known to impair several learning tasks and various types of memory (46–53) whereas NOP knockout mice show enhanced memory (50, 54, 55). Therefore, one might argue that AT-312 decreases acquisition of CPP induced by morphine, cocaine (or alcohol in our previous study) by affecting cognitive processes rather than acting on reward-related processes. However, not all types of memory are affected similarly by activation of the NOP receptor. While N/OFQ has been shown to block acquisition of cocaine- as well as morphine-induced CPP, it failed to reduce acquisition of naloxone-induced conditioned place aversion (23). Likewise, N/OFQ has been reported to reduce the expression of morphine-induced CPP at doses that did not alter the expression of naloxone-induced conditioned place aversion (56).

In summary, these results add to our observations that nonpeptide NOP agonists like AT-312 block the rewarding effects of several abused drugs such as morphine, cocaine and alcohol, and may be a promising approach for opioid and psychostimulant addiction pharmacotherapy. Given that addiction to multiple substances is also quite prevalent and there are limited therapeutic options, NOP receptor agonists may have broad therapeutic utility for treating polydrug addiction.

AUTHOR CONTRIBUTIONS

NZ and KL conceived of the research, supervised the research and wrote the paper. KL analyzed the data. PM and AH conducted the CPP experiments. MM synthesized the test drug.

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REFERENCES

1. Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* (1987) 237:1219–23. doi: 10.1126/science.2820058
2. Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. Cocaine self-administration appears to be mediated by dopamine uptake inhibition. *Prog Neuropsychopharmacol Biol Psychiatry* (1988) 12:233–9. doi: 10.1016/0278-5846(88)90040-1
3. Kuhar MJ, Ritz MC, Sharkey J. Cocaine receptors on dopamine transporters mediate cocaine-reinforced behavior. *NIDA Res Monogr.* (1988) 88:14–22.

4. Wise RA. Opiate reward: sites and substrates. *Neurosci Biobehav Rev.* (1989) 13:129–33. doi: 10.1016/S0149-7634(89)80021-1
5. Fields HL, Margolis EB. Understanding opioid reward. *Trends Neurosci.* (2015) 38:217–25. doi: 10.1016/j.tins.2015.01.002
6. Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA.* (1988) 85:5274–8. doi: 10.1073/pnas.85.14.5274
7. Marquez P, Baliram R, Dabaja I, Gajawada N, Lutfy K. The role of beta-endorphin in the acute motor stimulatory and rewarding actions of cocaine in mice. *Psychopharmacology (Berl)* (2008) 197:443–8. doi: 10.1007/s00213-007-1053-z
8. Nguyen AT, Marquez P, Hamid A, Kieffer B, Friedman TC, Lutfy K. The rewarding action of acute cocaine is reduced in beta-endorphin deficient but not in mu opioid receptor knockout mice. *Eur J Pharmacol.* (2012) 686:50–4. doi: 10.1016/j.ejphar.2012.04.040
9. Tseng A, Nguyen K, Hamid A, Garg M, Marquez P, Lutfy K. The role of endogenous beta-endorphin and enkephalins in ethanol reward. *Neuropharmacology* (2013) 73:290–300. doi: 10.1016/j.neuropharm.2013.06.001
10. Neal CR, Jr., Mansour A, Reinscheid R, Nothacker HP, Civelli O, Akil H, et al. Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: comparison of ORL1 receptor mRNA expression with (125)I-[(14)Tyr]-orphanin FQ binding. *J Comp Neurol.* (1999) 412:563–605.
11. Neal CR, Jr., Mansour A, Reinscheid R, Nothacker HP, Civelli O, Watson SJ, Jr. Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. *J Comp Neurol.* (1999) 406:503–47.
12. Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, et al. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* (1995) 270:792–4. doi: 10.1126/science.270.5237.792
13. Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, et al. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* (1995) 377:532–5. doi: 10.1038/377532a0
14. Murphy NP, Ly HT, Maidment NT. Intracerebroventricular orphanin FQ/nociceptin suppresses dopamine release in the nucleus accumbens of anaesthetized rats. *Neuroscience* (1996) 75:1–4. doi: 10.1016/0306-4522(96)00322-3
15. Schlicker E, Morari M. Nociceptin/orphanin FQ and neurotransmitter release in the central nervous system. *Peptides* (2000) 21:1023–9. doi: 10.1016/S0196-9781(00)00233-3
16. Murphy NP, Maidment NT. Orphanin FQ/nociceptin modulation of mesolimbic dopamine transmission determined by microdialysis. *J Neurochem.* (1999) 73:179–86. doi: 10.1046/j.1471-4159.1999.0730179.x
17. Murphy NP, Lee Y, Maidment NT. Orphanin FQ/nociceptin blocks acquisition of morphine place preference. *Brain Res.* (1999) 832:168–70. doi: 10.1016/S0006-8993(99)01425-0
18. Di Giannuario A, Pieretti S. Nociceptin differentially affects morphine-induced dopamine release from the nucleus accumbens and nucleus caudate in rats. *Peptides* (2000) 21:1125–30. doi: 10.1016/S0196-9781(00)00250-3
19. Di Giannuario A, Pieretti S, Catalani A, Loizzo A. Orphanin FQ reduces morphine-induced dopamine release in the nucleus accumbens: a microdialysis study in rats. *Neurosci Lett.* (1999) 272:183–6. doi: 10.1016/S0304-3940(99)00579-0
20. Lutfy K, Do T, Maidment NT. Orphanin FQ/nociceptin attenuates motor stimulation and changes in nucleus accumbens extracellular dopamine induced by cocaine in rats. *Psychopharmacology (Berl)* (2001) 154:1–7. doi: 10.1007/s002130000609
21. Vazquez-DeRose J, Stauber G, Khroyan TV, Xie XS, Zaveri NT, Toll L. Retrodialysis of N/OFQ into the nucleus accumbens shell blocks cocaine-induced increases in extracellular dopamine and locomotor activity. *Eur J Pharmacol.* (2013) 699:200–6. doi: 10.1016/j.ejphar.2012.11.050
22. Ciccocioppo R, Angeletti S, Sanna PP, Weiss F, Massi M. Effect of nociceptin/orphanin FQ on the rewarding properties of morphine. *Eur J Pharmacol.* (2000) 404:153–9. doi: 10.1016/S0014-2999(00)00590-2
23. Sakoori K, Murphy NP. Central administration of nociceptin/orphanin FQ blocks the acquisition of conditioned place preference to morphine and cocaine, but not conditioned place aversion to naloxone in mice. *Psychopharmacology (Berl)* (2004) 172:129–36. doi: 10.1007/s00213-003-1643-3
24. Kuzmin A, Sandin J, Terenius L, Ogren SO. Acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice: effects of opioid receptor-like 1 receptor agonists and naloxone. *J Pharmacol Exp Ther.* (2003) 304:310–8. doi: 10.1124/jpet.102.041350
25. Zaveri NT. The Nociceptin/Orphanin FQ Receptor (NOP) as a target for drug abuse medications. *Curr Topics Med Chem.* (2011) 11:1151–6. doi: 10.2174/156802611795371341
26. Zaveri NT. Nociceptin opioid receptor (NOP) as a therapeutic target: progress in translation from preclinical research to clinical utility. *J Med Chem.* (2016) 59:7011–28. doi: 10.1021/acs.jmedchem.5b01499
27. Lutfy K, Zaveri NT. The nociceptin receptor as an emerging molecular target for cocaine addiction. *Prog Mol Biol Transl Sci.* (2016) 137:149–81. doi: 10.1016/bs.pmbts.2015.10.003
28. Witkin JM, Statnick MA, Rorick-Kehn LM, Pintar JE, Ansonoff M, Chen Y, et al. The biology of Nociceptin/Orphanin FQ (N/OFQ) related to obesity, stress, anxiety, mood, and drug dependence. *Pharmacol Ther.* (2014) 141:283–99. doi: 10.1016/j.pharmthera.2013.10.011
29. Shoblock JR, Wichmann J, Maidment NT. The effect of a systemically active ORL−1 agonist, Ro 64–6198, on the acquisition, expression, extinction, and reinstatement of morphine conditioned place preference. *Neuropharmacology* (2005) 49:439–46. doi: 10.1016/j.neuropharm.2005.04.008
30. Rutten K, De Vry J, Bruckmann W, Tzschentke TM. Effects of the NOP receptor agonist Ro65–6570 on the acquisition of opiate- and psychostimulant-induced conditioned place preference in rats. *Eur J Pharmacol.* (2010) 645:119–26. doi: 10.1016/j.ejphar.2010.07.036
31. Sukhtankar DD, Lagorio CH, Ko M-C. Effects of the NOP agonist SCH221510 on producing and attenuating reinforcing effects as measured by drug self-administration in rats. *Eur J Pharmacol.* (2014) 745:182–9. doi: 10.1016/j.ejphar.2014.10.029
32. Zaveri NT, Marquez P, Meyer MM, Polgar WE, Hamid A, Lutfy K. A novel and selective nociceptin receptor (NOP) agonist (1-(1-((cis)−4-isopropylcyclohexyl) piperidin−4-yl)−1H-indol−2-yl)methanol (AT−312) decreases acquisition of ethanol-induced conditioned place preference in mice. *Alcohol Clin Exp Res.* (2018) 42:461–71. doi: 10.1111/acer.13575
33. Nguyen AT, Marquez P, Hamid A, Lutfy K. The role of mu opioid receptors in psychomotor stimulation and conditioned place preference induced by morphine−6-glucuronide. *Eur J Pharmacol.* (2012) 682:86–91. doi: 10.1016/j.ejphar.2012.02.021
34. Marquez P, Baliram R, Gajawada N, Friedman TC, Lutfy K. Differential involvement of enkephalins in analgesic tolerance, locomotor sensitization, and conditioned place preference induced by morphine. *Behav Neurosci.* (2006) 120:10–5. doi: 10.1037/0735-7044.120.1.10
35. Ciccocioppo R, Angeletti S, Panocka I, Massi M. Nociceptin/orphanin FQ and drugs of abuse. *Peptides* (2000) 21:1071–80. doi: 10.1016/S0196-9781(00)00245-X
36. Toll L, Khroyan TV, Polgar WE, Jiang F, Olsen C, Zaveri NT. Comparison of the antinociceptive and antirewarding profiles of novel bifunctional nociceptin receptor/mu-opioid receptor ligands: implications for therapeutic applications. *J Pharmacol Exp Ther.* (2009) 331:954–64. doi: 10.1124/jpet.109.157446
37. Devine DP, Reinscheid RK, Monsma FJ, Jr., Civelli O, Akil H. The novel neuropeptide orphanin FQ fails to produce conditioned place preference or aversion. *Brain Res.* (1996) 727:225–9. doi: 10.1016/0006-8993(96)00476-3
38. Noble F, Roques BP. Association of aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by nociceptin/orphanin FQ in mice. *FEBS Lett.* (1997) 401:227–9. doi: 10.1016/S0014-5793(96)01476-7
39. Kuzmin A, Sandin J, Terenius L, Ogren SO. Evidence in locomotion test for the functional heterogeneity of ORL−1 receptors. *Br J Pharmacol.* (2004) 141:132–40. doi: 10.1038/sj.bjp.0705583
40. Rizzi A, Bigoni R, Marzola G, Guerrini R, Salvadori S, Regoli D, et al. Characterization of the locomotor activity-inhibiting effect of nociceptin/orphanin FQ in mice. *Naunyn Schmiedebergs Arch Pharmacol.* (2001) 363:161–5. doi: 10.1007/s002100000358
41. Devine DP, Taylor L, Reinscheid RK, Monsma FJ, Jr., Civelli O, Akil H. Rats rapidly develop tolerance to the locomotor-inhibiting effects of

the novel neuropeptide orphanin FQ. *Neurochem Res.* (1996) 21:1387–96. doi: 10.1007/BF02532380
42. Kotlinska J, Wichmann J, Rafalski P, Talarek S, Dylag T, Silberring J. Non-peptidergic OP4 receptor agonist inhibits morphine antinociception but does not influence morphine dependence. *Neuroreport* (2003) 14:601–4. doi: 10.1097/00001756-200303240-00015
43. Sartor GC, Powell SK, Wiedner HJ, Wahlestedt C, Brothers SP. Nociceptin receptor activation does not alter acquisition, expression, extinction and reinstatement of conditioned cocaine preference in mice. *Brain Res.* (2016) 1632:34–41. doi: 10.1016/j.brainres.2015.11.044
44. Kotlinska J, Wichmann J, Legowska A, Rolka K, Silberring J. Orphanin FQ/nociceptin but not Ro 65–6570 inhibits the expression of cocaine-induced conditioned place preference. *Behav Pharmacol.* (2002) 13:229–35. doi: 10.1097/00008877-200205000-00006
45. Lutfy K, Khaliq I, Carroll FI, Maidment NT. Orphanin FQ/nociceptin blocks cocaine-induced behavioral sensitization in rats. *Psychopharmacology (Berl)* (2002) 164:168–76. doi: 10.1007/s00213-002-1192-1
46. Asth L, Correia N, Lobao-Soares B, De Lima TC, Guerrini R, Calo G, et al. Nociceptin/orphanin FQ induces simultaneously anxiolytic and amnesic effects in the mouse elevated T-maze task. *Naunyn Schmiedebergs Arch Pharmacol.* (2015) 388:33–41. doi: 10.1007/s00210-014-1055-4
47. Goeldner C, Reiss D, Wichmann J, Meziane H, Kieffer BL, Ouagazzal AM. Nociceptin receptor impairs recognition memory via interaction with NMDA receptor-dependent mitogen-activated protein kinase/extracellular signal-regulated kinase signaling in the hippocampus. *J Neurosci.* (2008) 28:2190–8. doi: 10.1523/JNEUROSCI.3711-07.2008
48. Hiramatsu M, Miwa M, Hashimoto K, Kawai S, Nomura N. Nociceptin/orphanin FQ reverses mecamylamine-induced learning and memory impairment as well as decrease in hippocampal acetylcholine release in the rat. *Brain Res.* (2008) 1195:96–103. doi: 10.1016/j.brainres.2007.12.008
49. Kuzmin A, Madjid N, Johansson B, Terenius L, Ogren SO. The nociceptin system and hippocampal cognition in mice: a pharmacological and genetic analysis. *Brain Res.* (2009) 1305(Suppl.):S7–19. doi: 10.1016/j.brainres.2009.09.075
50. Nagai J, Kurokawa M, Takeshima H, Kieffer BL, Ueda H. Circadian-dependent learning and memory enhancement in nociceptin receptor-deficient mice with a novel KUROBOX apparatus using stress-free positive cue task. *J Pharmacol Exp Ther.* (2007) 321:195–201. doi: 10.1124/jpet.106.115121
51. Reiss D, Prinssen EP, Wichmann J, Kieffer BL, Ouagazzal AM. The nociceptin orphanin FQ peptide receptor agonist, Ro64–6198, impairs recognition memory formation through interaction with glutamatergic but not cholinergic receptor antagonists. *Neurobiol Learn Memory* (2012) 98:254–60. doi: 10.1016/j.nlm.2012.09.002
52. Rekik K, Faria Da Silva R, Colom M, Pacifico S, Zaveri NT, Calo G, et al. Activation of nociceptin/orphanin FQ receptors inhibits contextual fear memory reconsolidation. *Neuropharmacology* (2017) 125:39–49. doi: 10.1016/j.neuropharm.2017.07.006
53. Sandin J, Georgieva J, Schott PA, Ogren SO, Terenius L. Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats. *Eur J Neurosci.* (1997) 9:194–7. doi: 10.1111/j.1460-9568.1997.tb01367.x
54. Manabe T, Noda Y, Mamiya T, Katagiri H, Houtani T, Nishi M, et al. Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. *Nature* (1998) 394:577–81. doi: 10.1038/29073
55. Nabeshima T, Noda Y, Mamiya T. The role of nociceptin in cognition. *Brain Res.* (1999) 848:167–73. doi: 10.1016/S0006-8993(99)01906-X
56. Sakoori K, Murphy NP. Expression of morphine-conditioned place preference is more vulnerable than naloxone-conditioned place aversion to disruption by nociceptin in mice. *Neurosci Lett.* (2008) 443:108–12. doi: 10.1016/j.neulet.2008.07.043

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CB1 Agonism Alters Addiction-Related Behaviors in Mice Lacking Mu or Delta Opioid Receptors

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Opioids are powerful analgesics but the clinical utility of these compounds is reduced by aversive outcomes, including the development of affective and substance use disorders. Opioid systems do not function in isolation so understanding how these interact with other neuropharmacological systems could lead to novel therapeutics that minimize withdrawal, tolerance, and emotional dysregulation. The cannabinoid system is an obvious candidate as anatomical, pharmacological, and behavioral studies point to opioid-cannabinoid interactions in the mediation of these processes. The aim of our study is to uncover the role of specific cannabinoid and opioid receptors in addiction-related behaviors, specifically nociception, withdrawal, anxiety, and depression. To do so, we tested the effects of a selective CB1 agonist, arachidonyl-2-chloroethylamide (ACEA), on mouse behavior in tail immersion, naloxone-precipitated withdrawal, light-dark, and splash tests. We examined cannabinoid-opioid interactions in these tests by comparing responses of wildtype (WT) mice to mutant lines lacking either Mu or Delta opioid receptors. ACEA, both acute or repeated injections, had no effect on nociceptive thresholds in WT or Mu knockout (KO) mice suggesting that analgesic properties of CB1 agonists may be restricted to chronic pain conditions. The opioid antagonist, naloxone, induced similar levels of withdrawal in all three genotypes following ACEA treatment, confirming an opioidergic contribution to cannabinoid withdrawal. Anxiety-like responses in the light-dark test were similar across WT and KO lines; neither acute nor repeated ACEA injections modified this behavior. Similarly, administration of the Delta opioid receptor antagonist, naltrindole, alone or in combination with ACEA, did not alter responses of WT mice in the light-dark test. Thus, there may be a dissociation in the effect of pharmacological blockade vs. genetic deletion of Delta opioid receptors on anxiety-like behavior in mice. Finally, our study revealed a biphasic effect of ACEA on depressive-like behavior in the splash test, with a prodepressive state induced by acute exposure, followed by a shift to an anti-depressive state with repeated injections. The initial pro-depressive effect of ACEA was absent in Mu KO mice. In sum, our findings confirm interactions between opioid and cannabinoid systems in withdrawal and reveal reduced depressive-like symptoms with repeated CB1 receptor activation.

Keywords: ACEA, nociception, withdrawal, depression, anxiety, CB1, opioid

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INTRODUCTION

Opioid and cannabinoid systems both play a critical role in a number of addiction-related behaviors, such as analgesia, reward, and emotional processing (1, 2). This commonality of function may reflect colocalization of opioid and cannabinoid receptors in brain regions implicated in each process and/or a common mechanism of receptor activation. In terms of the latter, Mu (MOP), Delta (DOP), and Kappa (KOP) opioid receptors, as well as both cannabinoid receptors (CB1 and CB2), are all coupled to inhibitory G proteins. Similarly, anatomical distribution of the three opioid receptors (3, 4) overlaps with CB1 receptor distribution (1) in many areas of the central nervous system (CNS). In contrast, anatomical localization of CB2 receptors in the CNS is not well studied. Indeed, initial reports described CB2 expression in immune cells (5, 6), although more recent work confirmed central expression of these receptors (7–9). Consequently, the anatomical relationship between CB2 receptors and either CB1 or opioid receptors is not known. Importantly, colocalization of opioid and CB1 receptors has been reported in the spinal cord, a critical site for antinociception (10–12), and in higher brain regions associated with emotional processing (13). This pattern of co-expression suggests that cannabinoid-opioid interactions may mediate behavioral responses related to pain relief and addiction (1, 6, 14), a process that may involve the formation of receptor heteromers (15).

Pharmacological and genetic knockout studies confirm interactive effects of opioid and CB1 receptors in antinociception (16) and behaviors related to addiction (17). For example, pharmacological blockade of either opioid or cannabinoid receptors with selective antagonists attenuates behavioral responses induced by an agonist of the other system (14). In addition, genetic inactivation of MOP receptors, producing knockout (KO) mice, decreases physical dependence induced by chronic administration of cannabinoid agonists (18, 19) and reduces the reinforcing properties of these drugs (18). Conversely, inactivation of CB1 receptors inhibits the rewarding properties of a MOP receptor agonist (19–21).

Most studies examining opioid mechanisms in pain relief focus on MOP receptors because of the potent analgesic properties of MOP agonists, such as morphine. Unfortunately, the therapeutic utility of these compounds is often limited as repeated use can lead to both tolerance and addiction (22). One suggestion for increasing clinical efficacy is to combine MOP and cannabinoid agonists, as this leads to increased analgesia (23–25) with fewer side effects (26). Development of these combination drugs depends on a better understanding of opioid-cannabinoid interaction in antinociception and addiction-related behaviors (e.g., tolerance, withdrawal, and emotional processing). To date, the majority of studies examining behavioral responses to cannabinoid receptor activation used Δ9-Tetrahydrocannabinol (THC), the primary phytocannabinoid in the cannabis plant. Because it is a partial agonist at both CB1 and CB2 receptors, THC cannot dissociate the contribution of either cannabinoid receptor to behavioral and affective processes related to pain management. This is a particularly important issue in pain studies, given recent evidence that CB2 receptors are involved in pathological states, such as neuroinflammation and hypersensitivity (27).

In this study, we explored possible interactions between opioid and cannabinoid systems in the mediation of antinociception and addiction-related behaviors. To do so, we examined the effect of the selective CB1 agonist, arachidonyl-2-chloroethylamide (ACEA) (28), on nociception, tolerance, withdrawal, and emotion-related behaviors in MOP or DOP receptor deficient mice. Nociception and tolerance to this effect were assessed in the tail immersion assay; somatic withdrawal symptoms were measured following an injection of the opiate antagonist, naloxone. We relied on naturalistic behaviors to assess anxiety-like (light-dark box) and depressive-like (splash test) responses in mice. Finally, we tested the consequences of both acute and repeated ACEA treatments on opioid-mediated effects in order to assess putative biphasic properties of this agonist.

Abbreviations: ACEA, arachidonyl-2-chloroethylamide; CNS, central nervous system; DOP, delta opioid; KO, knockout; KOP, kappa opioid; MOP, mu opioid; NLX, naloxone; NTI, naltrindole; THC, Δ9-Tetrahydrocannabinol; WT, wildtype.

MATERIALS AND METHODS

Animals

One hundred and sixty-eight male and female mice lacking MOP or DOP receptors (MOP KO and DOP KO, respectively) and their wildtype controls (12–24 weeks) were group housed (2–5/cage) under standard light, temperature, and humidity conditions (12 h light-dark cycle, 22 ± 2°C, 55 ± 10% humidity) with *ad libitum access* to food and water. Mice were generated by homologous recombination (29, 30). The genetic background of all mice was 50% C57/BL6J:50% 129svPas.

Research was conducted in accordance with the European Communities Council Directive of 22 September 2010 (directive 2010/63/UE), under the guidelines of the Committee for Research and Ethical issues of the International Association for the Study of Pain (31). Experiments were approved by the local ethics committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg CREMEAS), and findings are reported following the ARRIVE Guidelines for experiments involving animals.

Drugs

ACEA (Tocris, Bio-techne, Lille, France) was dissolved in 0.9% saline solution (supplied pre-dissolved in ethanol at 5 mg/ml) to obtain doses of 0.15, 3, and 5 mg/kg. Naloxone (NLX) and Naltrindole (NTI) (Sigma-Aldrich St-Quentin Fallavier, France) were dissolved in saline solution to obtain final doses of 1 and 2.5 mg/kg, respectively. Vehicle (saline or 6% ethanol) injections were used as controls. All drug and vehicle injections were administered ip using 100 μl solution per 10 g bodyweight.

Behavioral Procedures

In each group, approximately equal numbers of male and female mice were used. A total of 22 DOP KO, 49 MOP KO, and 97 WT mice were used. Mice were habituated to the facility and

handled for one week before starting the experiments. Behavioral tests were conducted during the light phase and performed blind to genotype and treatment. ACEA or vehicle was administered 45 min before tail immersion or light-dark tests, 35 min before the splash test, and 90 min before precipitated withdrawal.

Tail Immersion

Thermal nociceptive thresholds were assessed in the tail immersion test by gently restraining mice and immersing ∼2/3 of the tail in a water bath at 47°C. The latency to withdraw the tail was recorded before and after ACEA injections. Acute responses to increasing doses of ACEA were tested in WT and MOP KO mice. The effects of repeated ACEA injections (3 mg/kg) on antinociception were tested in a separate group of WT and MOP KO mice by measuring tail immersion responses on day 1 and day 5 of treatment. Hypersensitivity to repeated treatment was assessed 23 h after the last injection, as described previously (32).

Naloxone-Precipitated Withdrawal From ACEA

To evaluate the role of the opioid system in cannabinoid withdrawal, WT, MOP KO, and DOP KO mice received ACEA injections (3 mg/kg once per day for 5 days) followed by a naloxone injection (1 mg/kg), administered 90 min after the last ACEA injection. Withdrawal behaviors were summed over a 20-min observation period and three separate scores were computed for each animal. First, global withdrawal scores were calculated by summing the following values: jumping x 0.8, wet dogs shakes x 1, paw tremors x 0.35, ptosis x 1.5, teeth chattering x 1.5, body tremors x 1.5, and piloerection x 1.5. Second, a subcategory of somatic signs was calculated by combining the total number of jumps, paw tremors, and wet dog shakes. Third, signs of discomfort reflected the sum of stretching, genital licks, and body tremors (33).

Light-Dark Test

The light-dark test, assessing anxiety-like behavior in rodents, employed an apparatus composed of two compartments (20 × 20 × 25 cm), connected by a tunnel (6 × 16.5 × 20 cm) (34). One compartment was brightly illuminated (>400 lx); the other was dark (7 lx). Mice were placed in the dark compartment and allowed to freely explore the apparatus for 5 min while the time spent in each compartment and the tunnel was recorded. Mice have a natural tendency to avoid lit environments: decreased time spent in the dark compartment is a measure of reduced anxiety (35). WT, MOP KO, and DOP KO mice were tested prior to drug administration (baseline; BL) and then following ACEA injections (3 mg/kg) on days 1 and 3. The role of DOP receptors in ACEA-induced changes in anxiety-like behavior was assessed in WT mice by administering NTI prior to ACEA injections; control groups received saline plus ACEA or NTI plus vehicle.

Splash Test

The splash test (36, 37) consists of vaporizing a 20% sucrose solution on the back fur of mice; mice initiate grooming in response to the solution viscosity. The number of grooming responses (head or body grooming, shakes, and scratches) and the time spent grooming were recorded over 5 min. Repeated stress decreases grooming responses, which is reversed by antidepressant treatment (38), providing an assay for changes in depressive-like behavior in rodents. WT, MOP KO, and DOP KO mice were assessed in the splash test following days 1 and 3 of ACEA treatment (3 mg/kg). As with the light-dark test, WT mice were tested following injections NTI plus ACEA, saline plus ACEA, or NTI plus vehicle.

Data Analyses

Statistical tests were performed using Graphpad Prism® statistical software (Version 6.0; La Jolla, CA, USA). Data from the tail immersion, light-dark, and splash tests were analyzed using a two-way analysis of variance (ANOVA) with genotype as a within subject's factor and dose or day of injection as repeated factors. A 2-way ANOVA (genotype by drug) was used to assess each category of withdrawal score. Subsequent comparisons were conducted using Bonferroni *post hoc* tests. Statistical significance was set at $p < 0.05$.

RESULTS

Body weight was monitored across the entire experiment with daily weights recorded on all drug treatment days. Using body weight prior to the first injection as a baseline, ANOVA revealed no effect of drug (ACEA, NTI, or NTI + ACEA) on body weights at d5 in either WT (ACEA, 97.5% of BL; NTI, 95.1% of BL, NTI+ACEA, 101.8% of BL), MOP KO (Vehicle, 102.7% of BL; ACEA, 100.7% of BL), or DOP KO (Vehicle, 100.6% of BL; ACEA, 97.9 % of BL) mice.

Thermal Nociception

As shown in **Figure 1A**, acute injections of ACEA had no effect on thermal nociception in WT or MOP KO mice across a range of doses [WT: $F_{(3, 32)} = 1.43$, $P = 0.24$; MOP KO: $F_{(3, 31)} = 1.86$, $P = 0.15$]. Although MOP receptors preferentially mediate nociceptive response in mice (30), we did not observe any significant modification of nociceptive thresholds in this genotype, compared to WT mice ($P > 0.05$). We also investigated the effects of repeated subanalgesic doses of ACEA (**Figure 1B**), revealing no drug-induced alteration in thermal nociceptive thresholds in WT mice [$F_{(1, 45)} = 0.04$, $P = 0.15$] and no effect of repeated injections [$F_{(2, 90)} = 1.30$, $P = 0.27$]. MOR KO mice developed hypersensitivity, with decreased thermal nociceptive thresholds after the 1st vehicle and 5th ACEA injections [Drug X Time Interaction: $F_{(2, 44)} = 3.40$, $P < 0.05$]. Finally, there was no effect of chronic ACEA treatment on thermal nociceptive thresholds in either WT or MOP KO mice, measured 23 h after the last injection (**Figure 1C**) [WT: $F_{(1, 45)} = 0.40$, $P = 0.15$; MOP KO: $F_{(1, 22)} = 2.68$, $P = 0.11$].

Naloxone-Precipitated Withdrawal From ACEA

Naloxone induced increased global withdrawal scores in animals chronically treated with ACEA [$F_{(1, 41)} = 28.85$, $P < 0.0001$]. The effect was consistent across WT, MOP KO, and DOP KO mice [$F_{(2, 41)} = 1.74$, $P = 0.16$], with modest changes in all three genotypes treated with vehicle (**Figure 2A**). A more detailed

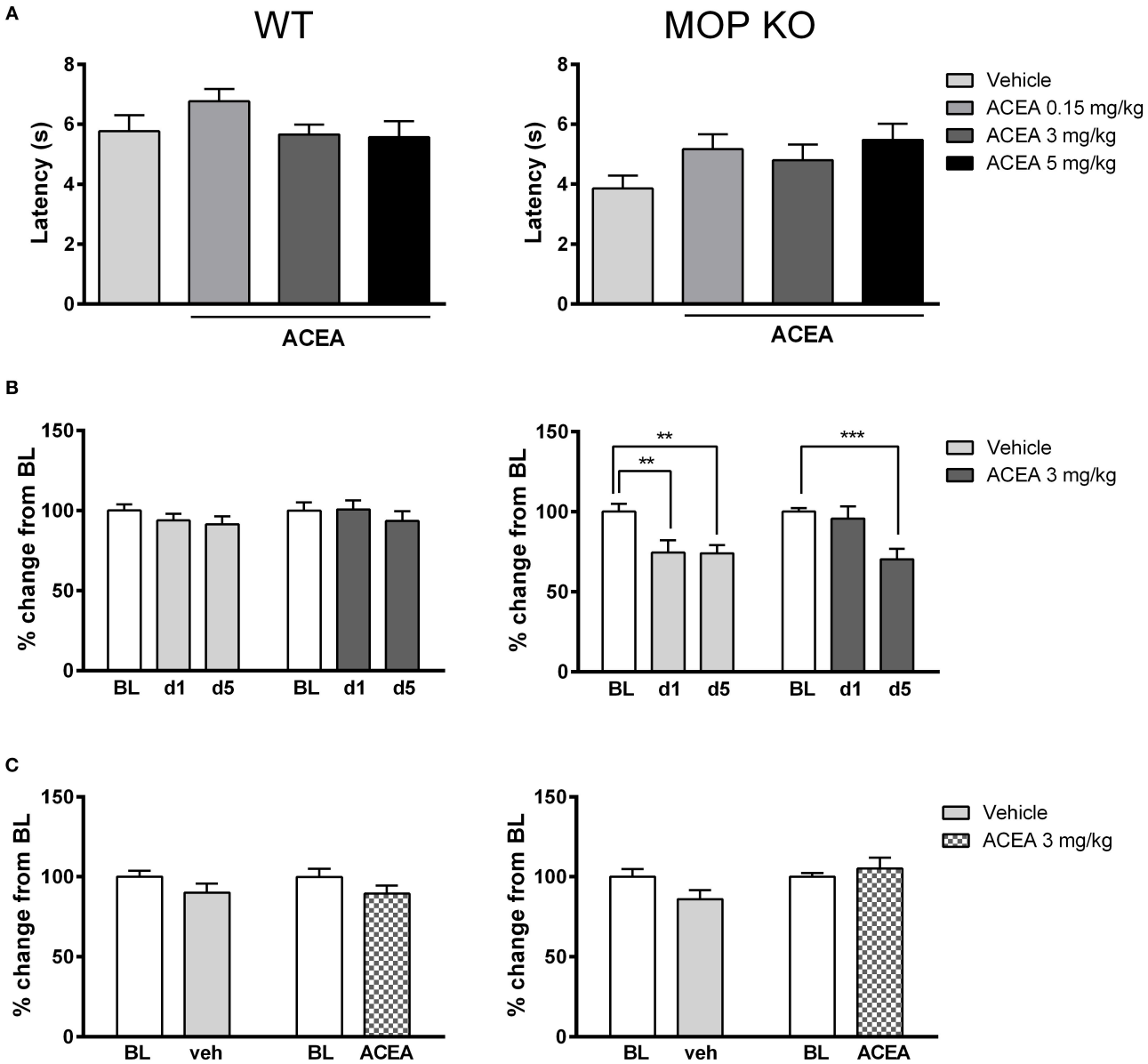

FIGURE 1 | Effects of ACEA on thermal nociceptive thresholds in wildtype (WT) (left) and MOP receptor knockout (KO) (right) mice following acute **(A)** or chronic **(B)** injections and during a post-treatment drug-free test **(C)**. **(A)** Bars represent mean (+SEM) latency (s) to withdraw the tail from a heated bath 45 min after ACEA (0.15, 3, or 5 mg/kg) or vehicle injections. **(B)** Tail withdrawal latencies are expressed as mean (+SEM) % change from baseline (BL) following vehicle or ACEA (3 mg/kg) administration on days 1 and 5 (d1, d5) of a 5-day dosing regime. BL was established one day prior to the first injection. **(C)** Tail withdrawal latencies, shown as mean (+SEM) % change from BL, were assessed 23 h following the final ACEA or vehicle injection. ACEA = arachidonyl-2-chloroethylamide $^{**}P < 0.01$; $^{***}P < 0.001$.

analysis revealed that ACEA treatment increased both somatic symptoms [$F_{(1, 41)} = 24.45$, $P < 0.0001$] and signs of discomfort [$F_{(1\,41)} = 3.80$, $P = 0.058$], although the latter did not reach statistical significance (**Figures 2B,C**). Differences in somatic signs in ACEA- and vehicle-treated mice varied across genotype [$F_{(2, 41)} = 4.50$, $P < 0.05$], with MOP KO showing reduced signs of withdrawal compared to WT and DOP KO mice. Signs of discomfort were relatively low in all animals and did not differ across the three lines [$F_{(2, 41)} = 0.97$, $P = 0.07$].

Light-Dark Test

Figure 3A shows that there were no significant differences in baseline levels of anxiety, measured in the light-dark test, across WT, MOP KO, and DOP KO mice [$F_{(2, 37)} = 1.436$, $P = 0.25$]. Repeated drug injections (ACEA with and without NTI) decreased anxiety-like effects in WT mice (**Figure 3B**) [$F_{(2, 42)} = 7.93$, $P < 0.01$], although there was no overall main effect of drug [$F_{(2, 21)} = 0.02$, $P = 0.97$]. *Post-hoc* tests revealed a significant difference in % time spent in the dark on BL compared to d1 for

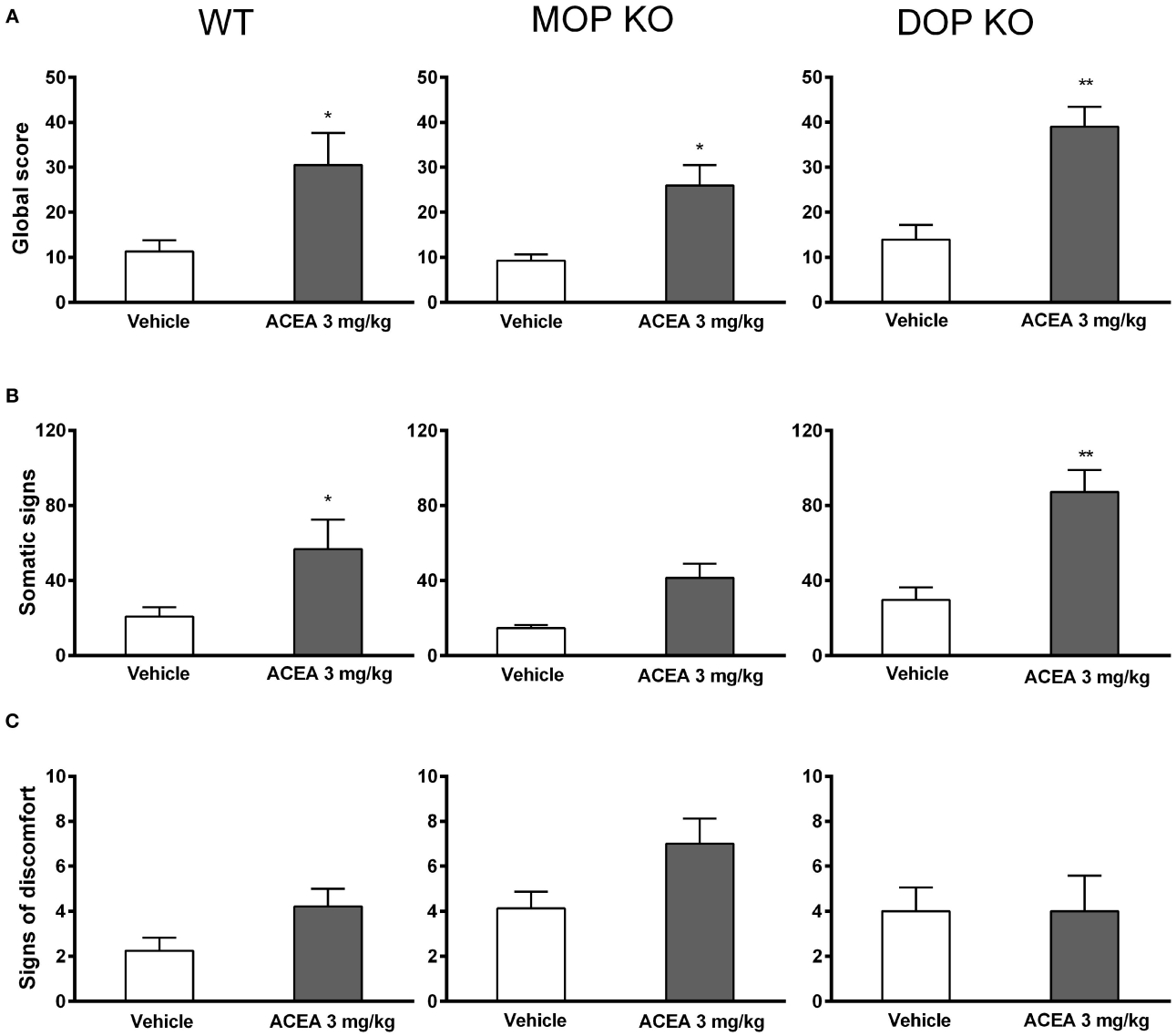

FIGURE 2 | Effects of ACEA treatment on naloxone-precipitated withdrawal in wildtype (WT), MOP knockout (KO), and DOP KO mice. Bars represent mean (+SEM) global withdrawal scores **(A)**, somatic signs **(B)**, and signs of discomfort **(C)** for each genotype over a 20-min observation session. Naloxone (1 mg/kg) was administered 2 h after the last injection of vehicle or ACEA (3 mg/kg per day for 5 days). ACEA = arachidonyl-2-chloroethylamide $^{*}P < 0.05$; $^{**}P < 0.01$.

mice treated with NTI plus ACEA (Bonferroni *post-hoc* $P < 0.05$). Similarly, repeated injections of ACEA increased % time in the dark in both KO lines [$F_{(2, 28)} = 8.57$, $P < 0.01$] (**Figure 3C**), due to significant changes from BL to both d1 and d3 in MOP KO mice (Bonferroni *post-hoc* $P < 0.05$ and $P < 0.01$).

Splash Test

As shown in **Figure 4**, grooming scores of WT controls (**Figure 4A**) were higher than both MOP (**Figure 4C**) and DOP (**Figure 4D**) KO mice following a vehicle injection, although time spent grooming was consistent across genotypes. Statistical analysis revealed that the effect of ACEA on grooming scores in WT and DOP KO mice was modified with repeated testing [WT: $F_{(1, 23)} = 7.15$, $P < 0.05$; DOP KO: $F_{(1, 12)} = 15.50$, $P < 0.01$]. Analysis of time spent grooming revealed a similar drug x time interaction in these groups [WT: $F_{(1, 23)} = 15.25$, $P < 0.0001$; DOP KO: $F_{(1, 12)} = 16.23$, $P < 0.01$]. These effects were due to decreased grooming on day 1 in ACEA- vs. vehicle-treated mice and increased drug-induced grooming on day 3 compared to day 1 (*post-hoc* Ps < 0.05). ACEA increased grooming score [$F_{(1, 29)} = 17.04$, $P < 0.001$], but not time spent grooming [$F_{(1, 29)} = 0.53$, $P = 0.46$], in MOP KO mice. As with WT and DOP KO mice, both measures increased with repeated injections in this group [grooming score: $F_{(1, 29)} = 7.30$, $P < 0.05$; time spent grooming: $F_{(1, 29)} = 5.38$, $P < 0.05$]. In sum, the first ACEA injection decreased grooming in WT and DOP KO mice, while having no

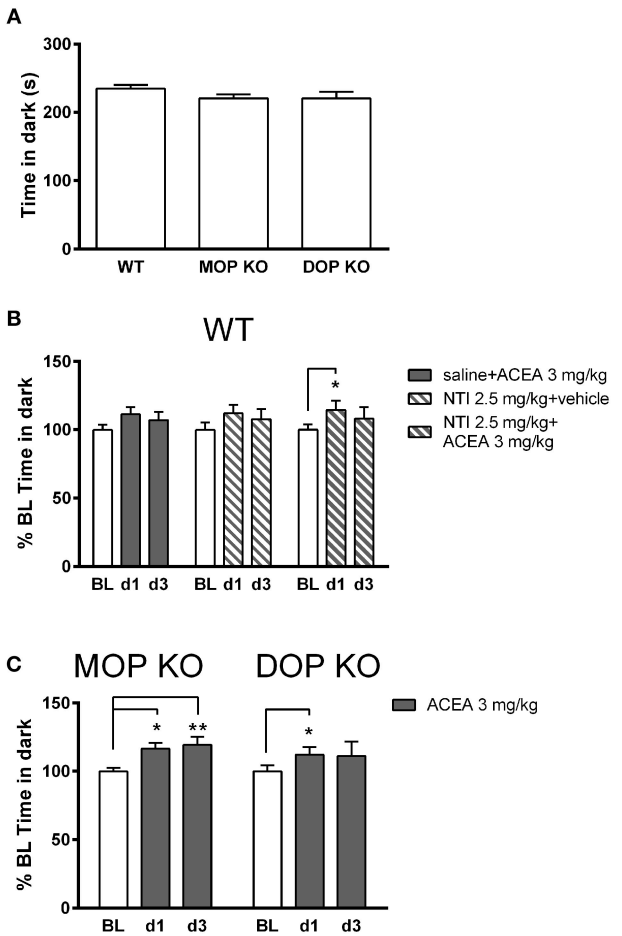

FIGURE 3 | Effects of ACEA treatment on anxiety-like behavior in the light-dark test for wildtype (WT), MOP knockout (KO), and DOP KO mice. **(A)** Bars represent mean (+SEM) time (s) spent in the dark compartment for each genotype over a 5-min drug-free test, which constituted baseline (BL) values. **(B)** Time in the dark compartment is shown as mean (+SEM) % change from BL, assessed on days 1 and 3 (d1, d3) in WT mice, following injections of ACEA plus saline, ACEA combined with naltrindole (NTI), or NTI alone. **(C)** Time in the dark compartment, expressed as mean (+SEM) % change from BL, was assessed in the two KO lines on days 1 and 3 following daily injections of vehicle or ACEA (3 mg/kg). ACEA = arachidonyl-2-chloroethylamide. $^{*}P < 0.05$; $^{**}P < 0.01$.

effect in MOP KO mice. Interestingly, this prodepressive drug effect was altered following 3 days of injections in WT mice: grooming score returned to basal levels and time spent grooming was increased beyond these levels. The pattern was similar in DOP KO mice, showing increases in both measures following the third ACEA injection. In MOP KO mice, grooming score and time spent grooming were not significantly altered in MOP KO mice on day 1; grooming score increased following the 3rd ACEA injection.

We verified this biphasic effect of ACEA on depressive-like behavior in a separate group of WT mice (**Figure 4B**), replicating decreased grooming at day 1 with a return to baseline levels by day 3 [grooming score: $F_{(2, 42)} = 4.41$, $P < 0.05$; time spent grooming: $F_{(2, 42)} = 9.95$, $P < 0.001$]. Pretreatment with NTI had no significant effect on either measure [grooming score: $F_{(2, 21)} = 1.55$, $P = 0.20$; time spent grooming: $F_{(2, 21)} = 1.23$, $P = 0.31$].

DISCUSSION

The goal of this study was to clarify the role of CB1 receptors in addiction-related behaviors and to assess potential interactions with opioid mechanisms, specifically MOP and DOP receptors. Extensive research over the last decades confirms an important contribution of cannabinoid mechanisms to processes such as antinociception, drug dependence, and emotional responses (1, 39, 40), but many of these studies employed THC or other nonselective compounds. Studies using receptor KO mice confirm that both CB1 and CB2 receptors play a role in these processes (1, 41, 42), pointing to the need to examine behavioral effects of pharmacological tools that specifically target each receptor. To this end, we used the selective CB1 agonist, ACEA, as this compound has a Ki of 1.4 nM for CB1, vs. a Ki <2000 nM for CB2, or 1,400 times greater for CB2 compared to CB1 (43). Confirmation of compound selectivity is provided by abolishment of neurotoxicological effects of ACEA in CB1 KO animals (44), although functional selectivity of G protein signaling may be lost with high doses of ACEA (45).

Use of this highly specific compound revealed a distinct pattern of behavioral effects, some of which differed from results using other CB1 agonists. First, we show that ACEA did not alter bodyweight, despite evidence that other CB1 agonists have orexigenic properties in human and rodents, and that CB1 antagonists may facilitate weight loss (46, 47). We also observed no effect of ACEA on antinociceptive thresholds across a range of doses, contradicting previous evidence that CB agonists such as THC, CP55,940, anandamide, or WIN are analgesic (48, 49). We confirmed a lack of ACEA-induced antinociception in a separate group of mice treated with a single dose and revealed no changes in this measure with repeated injections. It is possible that ACEA doses in our study were too low to be effective as higher doses of THC are required to elicit analgesic responses in the tail immersion, compared to the hot plate, test (20). It is also possible that extending the ACEA dosing regime may have revealed behavioral effects that were not apparent following the protocol used in this study. At the same time, high doses of CB agonists, such as THC, may induce hypolocomotion and catalepsy (50), which could interfere with the behavioral expression of pain and confound measures of antinociception. In addition, THC produces aversive effects at higher doses, such as anxiety and weight loss, which may explain contracictory effects of cannabinoid agonists at high and low doses (51–53). Repeated injections of cannabinoid agonists, such as ACEA, could lead to poor health outcomes minimizing the ethological validity of our findings. Importantly, doses of ACEA comparable to those in our study reduce mechanical allodynia in mouse models of osteoarthritic (54) and neuropathic (55) pain. A more plausible explanation for our negative findings, therefore, is that CB1 receptors are not involved in antinociceptive responses in pain naïve states, fitting evidence that ACEA-induced analgesia in

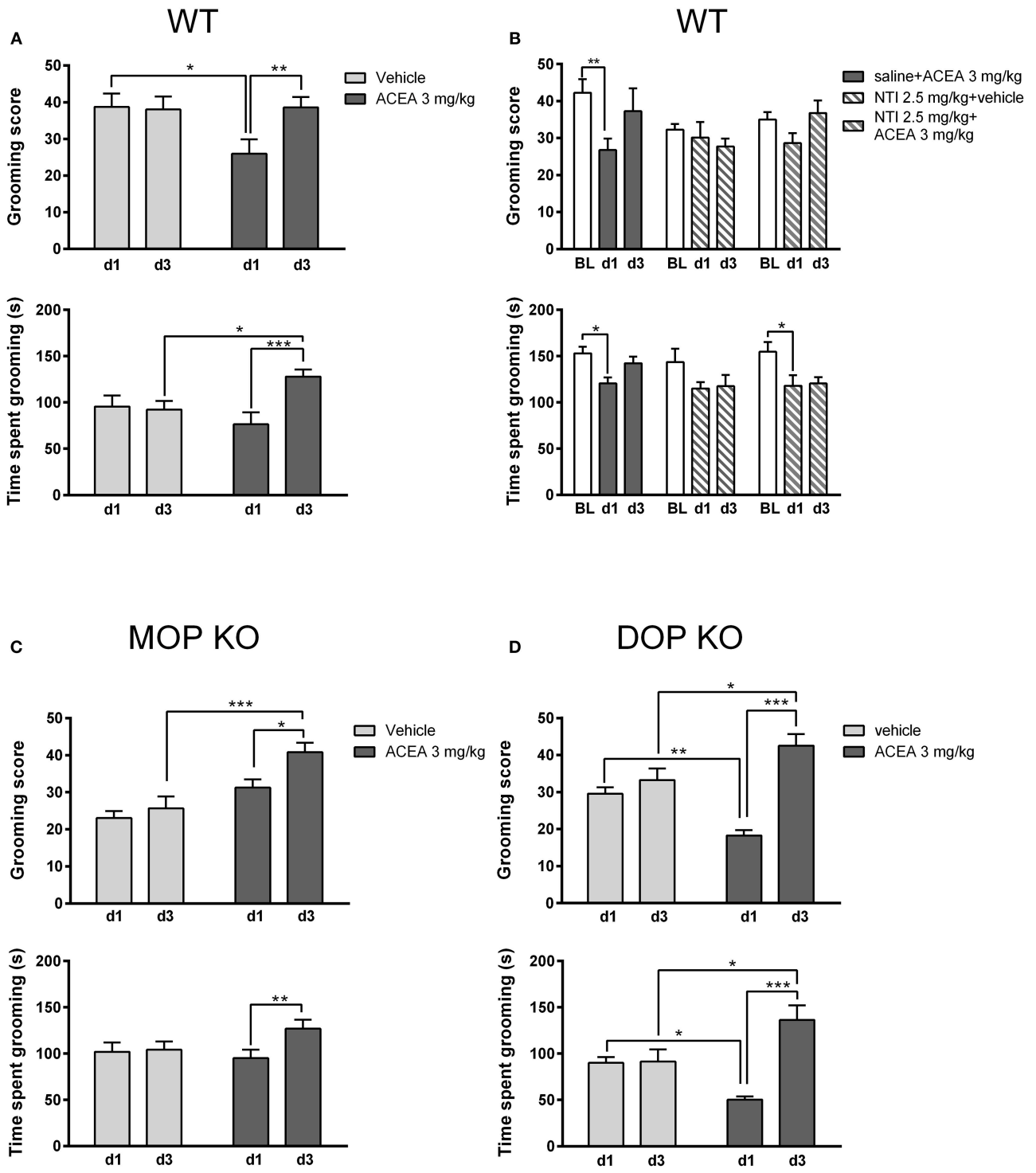

FIGURE 4 | Effects of ACEA treatment on depressive-like behaviors in the splash test for wildtype (WT) **(A)**, MOP knockout (KO) **(C)**, and DOP KO **(D)** mice. Bars represent mean (+SEM) grooming score (left) and time (s) spent grooming (right) in response to vaporization of a 20% sucrose solution on the back fur. Responses were assessed in each genotype over a 5-min period, 45 min after the first and third daily (d1, d3) injections of vehicle or ACEA (3 mg/kg). Grooming responses were assessed in a separate group of WT mice on days 1 and 3 (d1, d3) following injections of ACEA plus saline, ACEA combined with naltrindole (NTI), or NTI alone **(B)**. ACEA = arachidonyl-2-chloroethylamide $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

a neuropathic model does not extend to the paw contralateral to the injury (54). This dissociation in the effectiveness of ACEA treatment may reflect altered endocannabinoid signaling, including changes in CB1 receptor function, that is associated with persistent pain states (56).

Our study also provides the first evidence of naloxone-precipitated withdrawal following chronic ACEA treatment. The effect was consistent across all three genotypes but was substantially lower than symptoms observed following treatment with a classic MOP receptor agonist, morphine (33). This general reduction in withdrawal signs may have obscured any genotypic differences in our study, such as decreased withdrawal in MOP or DOP KO mice. If these do exist, they could be revealed by a more intense treatment regime (i.e., higher dose and/or increased number of injections). Regardless, our finding that naloxone elicited withdrawal symptoms in ACEA-treated mice adds to evidence of opioid-cannabinoid interactions in this behavior. For example, cannabinoid antagonists, such as SR141716A, elicit withdrawal symptoms following chronic morphine injections (17, 19, 57) and opioid antagonists induce withdrawal in rodents previously treated with cannabinoid agonists (17). Studies using genetically modified mice confirm opioid-cannabinoid cross-talk in drug withdrawal: naloxone-precipitated withdrawal is decreased in CB1 receptor knockout mice following morphine treatment (19–21) and MOP KO mice exhibit reductions in SR141716A-induced withdrawal following chronic THC treatment (19). The latter effect is dose dependent and alleviated by morphine injections in wildtype mice, providing further support for cannabinoid-opioid interactions in drug withdrawal.

Given the reciprocal relationship between opioid and cannabinoid systems in drug withdrawal, our observation that ACEA treatment induced withdrawal symptoms in both MOP and DOP KO mice seems counterintuitive. These findings could suggest that the KOP receptor has a critical role in cannabinoid withdrawal. This fits evidence that DOP receptors contribute to negative affective states induced by THC (18, 20, 58), but have no role in the anxiolytic properties of the drug (59). In addition, naloxone is a nonselective antagonist so if a single receptor subtype is deleted (e.g., MOP or DOP), withdrawal symptoms could be elicited through an action at the remaining, intact receptors. This would explain why naloxone-precipitated withdrawal following cannabinoid treatment is not modulated in mice lacking either MOP, DOP, or KOP receptors (18).

A novel finding in our study is that initial administration of a CB1 agonist produces a depressive-like state, which recovers with further drug exposure. This could explain why the rewarding effects of cannabinoid agonists, including THC, are only revealed in self-administration or place conditioning paradigms when animals receive priming injections prior to training (18, 51, 53, 60, 61). Interestingly, the pro-depressive effect of acute ACEA injections (i.e., day 1) was absent in MOP KO mice, mimicking decreased depressive-like symptoms of this genotype in other behavioral tests (29). Despite this initial blunting, repeated ACEA injections reduced depressive-like symptoms in MOP KO animals, matching behavioral effects observed in DOP KO mice. WT mice showed a different profile: repeated ACEA injections restored grooming scores to basal levels but increased time spent grooming above control levels. MOP receptors, therefore, appear to mediate the initial pro-depressive effects of ACEA without affecting the subsequent anti-depressive effects of repeated exposure.

In contrast to depressive-like behaviors, we observed no effect of either acute or chronic ACEA injections on anxiety-like responses in the light/dark box. This appears to contradict previous findings in the elevated plus maze (54), although decreased time spent in open arms was only observed with higher doses of the drug. At least for another CB agonist (THC), anxiolytic properties at low doses (59, 61) are replaced by anxiogenic effects at higher ones (51, 61). As noted previously, hypolocomotion and catalepsy induced by higher dose of THC (50) could modify responses in a number of behavioral tasks including the light-dark test, open field, and elevated plus maze. At the very least, given that we used identical dosing procedures in splash and light/dark tests, our findings provide evidence for a dissociation in the effects of CB1 activation on anxiety- and depressive-like responses in mice.

Given the key role of DOP receptors in anxiety and depression (29), we went on to test whether pharmacological blockade of these receptors in WT mice would alter responses in splash or light/dark tests. The DOP receptor antagonist, NTI, was ineffective on its own, but decreased time spent grooming in the splash test when combined with ACEA. This reduction was not matched by a reduction in grooming score, suggesting that the drug combination may have disrupted a general pattern of naturalistic behavior (i.e., grooming). Overall, our results are consistent with previous studies: although NTI disrupts pro-depressive or anxiogenic effects of DOP agonists (62–64), it is ineffective when administered alone (65, 66). Our findings that pharmacological blockade of DOP receptors has no effect on anxiety- or depressive-like behaviors conflicts with studies using genetically modified mice (67), suggesting that developmental adaptations impact the function of these receptors in emotional expression.

In sum, our results help to clarify how opioid and cannabinoid systems interact in behavioral processes associated with addiction. The use of a selective CB1 agonist revealed no involvement of this receptor in either antinociception or anxiety-like behaviors in mice. In contrast, repeated activation of CB1 receptors induced opioid-dependent withdrawal symptoms and produced a biphasic effect on depressive-like symptoms. In the long-term, this information could facilitate the development of new pain medications that reduce the incidence of affective and substance use disorders that currently characterize long-term opioid use.

AUTHOR CONTRIBUTIONS

L-AR and KB designed and performed the experiments and wrote the manuscript. L-AR collected data and analyzed the results under the guidance of KB. DM provided the animals and consulted on the design of the experiments. MO

contributed to the discussion of the data and writing of the manuscript.

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REFERENCES

1. Befort K. Interactions of the opioid and cannabinoid systems in reward: insights from knockout studies. *Front Pharmacol.* (2015) 6:6. doi: 10.3389/fphar.2015.00006
2. Scavone JL, Sterling RC, Van Bockstaele EJ. Cannabinoid and opioid interactions: implications for opiate dependence and withdrawal. *Neuroscience* (2013) 248:637–54. doi: 10.1016/j.neuroscience.2013.04.034
3. Erbs E, Faget L, Scherrer G, Matifas A, Filliol D, Vonesch J-L, et al. A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* (2015) 220:677–702. doi: 10.1007/s00429-014-0717-9
4. Mansour A, Fox CA, Akil H, Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* (1995) 18:22–9.
5. Mackie K. Cannabinoid receptors: where they are and what they do. *J Neuroendocrinol.* (2008) 20(Suppl 1):10–4. doi: 10.1111/j.1365-2826.2008.01671.x
6. Maldonado R, Berrendero F, Ozaita A, Robledo P. Neurochemical basis of cannabis addiction. *Neuroscience* (2011) 181:1–17. doi: 10.1016/j.neuroscience.2011.02.035
7. Onaivi ES, Ishiguro H, Gong J-P, Patel S, Perchuk A, Meozzi PA, et al. Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Annals N Y Acad Sci.* (2006) 1074:514–36. doi: 10.1196/annals.1369.052
8. Zhang H-Y, Gao M, Liu Q-R, Bi G-H, Li X, Yang H-J, et al. Cannabinoid CB2 receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice. *Proc Natl Acad Sci USA.* (2014) 111:E5007–15. doi: 10.1073/pnas.1413210111
9. Zhang H-Y, Gao M, Shen H, Bi G-H, Yang H-J, Liu Q-R, et al. Expression of functional cannabinoid CB2receptor in VTA dopamine neurons in rats. *Addict Biol.* (2017) 22:752–65. doi: 10.1111/adb.12367
10. Bushlin I, Gupta A, Stockton SD, Miller LK, Devi LA. Dimerization with cannabinoid receptors allosterically modulates delta opioid receptor activity during neuropathic pain. *PLoS ONE* (2012) 7:e49789. doi: 10.1371/journal.pone.0049789
11. Rodriguez JJ, Mackie K, Pickel VM. Ultrastructural localization of the CB1 cannabinoid receptor in mu-opioid receptor patches of the rat Caudate putamen nucleus. *J Neurosci* (2001) 21:823–33.
12. Salio C, Fischer J, Franzoni MF, Mackie K, Kaneko T, Conrath M. CB1-cannabinoid and mu-opioid receptor co-localization on postsynaptic target in the rat dorsal horn. *Neuroreport* (2001) 12:3689–92.
13. Cichewicz DL. Synergistic interactions between cannabinoid and opioid analgesics. *Life Sci.* (2004) 74:1317–24.
14. Fattore L, Deiana S, Spano SM, Cossu G, Fadda P, Scherma M, et al. Endocannabinoid system and opioid addiction: behavioural aspects. *Pharmacol Biochem Behav.* (2005) 81:343–59. doi: 10.1016/j.pbb.2005.01.031
15. Massotte D. *In vivo* opioid receptor heteromerization: where do we stand? *Br J Pharmacol.* (2015) 172:420–34. doi: 10.1111/bph.12702
16. Nielsen S, Sabioni P, Trigo JM, Ware MA, Betz-Stablein BD, Murnion B, et al. Opioid-sparing effect of cannabinoids: a systematic review and meta-analysis. *Neuropsychopharmacology* (2017) 42:1752–65. doi: 10.1038/npp.2017.51
17. Navarro M, Carrera MR, Fratta W, Valverde O, Cossu G, Fattore L, et al. Functional interaction between opioid and cannabinoid receptors in drug self-administration. *J Neurosci.* (2001) 21:5344–50.
18. Ghozland S, Matthes HWD, Simonin F, Filliol D, Kieffer BL, Maldonado R. Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. *J Neurosci.* (2002) 22:1146–54.
19. Lichtman AH, Sheikh SM, Loh HH, Martin BR. Opioid and cannabinoid modulation of precipitated withdrawal in delta(9)-tetrahydrocannabinol and morphine-dependent mice. *J Pharmacol Exp Ther.* (2001) 298:1007–14.
20. Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, et al. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* (1999) 283:401–4.
21. Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O. Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. *Eur J Neurosci.* (2000) 12:4038–46.
22. Pasternak GW. Mu opioid pharmacology: 40 years to the promised land. *Adv Pharmacol.* (2018) 82:261–91. doi: 10.1016/bs.apha.2017.09.006
23. Cichewicz DL, Welch SP. Modulation of oral morphine antinociceptive tolerance and naloxone-precipitated withdrawal signs by oral Delta 9-tetrahydrocannabinol. *J Pharmacol Exp Ther.* (2003) 305:812–7. doi: 10.1124/jpet.102.046870
24. Finn DP, Beckett SRG, Roe CH, Madjd A, Fone KCF, Kendall DA, et al. Effects of coadministration of cannabinoids and morphine on nociceptive behaviour, brain monoamines and HPA axis activity in a rat model of persistent pain. *Eur J Neurosci.* (2004) 19:678–86.
25. Smith PA, Selley DE, Sim-Selley LJ, Welch SP. Low dose combination of morphine and delta9-tetrahydrocannabinol circumvents antinociceptive tolerance and apparent desensitization of receptors. *Eur J Pharmacol.* (2007) 571:129–37. doi: 10.1016/j.ejphar.2007.06.001
26. Valverde O, Noble F, Beslot F, Daugé V, Fournié-Zaluski MC, Roques BP. Delta9-tetrahydrocannabinol releases and facilitates the effects of endogenous enkephalins: reduction in morphine withdrawal syndrome without change in rewarding effect. *Eur J Neurosci.* (2001) 13:1816–24.
27. Dhopeshwarkar A, Mackie K. CB2 Cannabinoid receptors as a therapeutic target-what does the future hold? *Mol Pharmacol.* (2014) 86:430–7. doi: 10.1124/mol.114.094649
28. Pertwee RG. Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Curr Med Chem.* (2010) 17:1360–81.
29. Filliol D, Ghozland S, Chluba J, Martin M, Matthes HWD, Simonin F, et al. Mice deficient for δ- and μ-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet.* (2000) 25:195–200. doi: 10.1038/76061
30. Matthes HWD, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, et al. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. *Nature* (1996) 383:819–23. doi: 10.1038/383819a0
31. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* (1983) 16:109–10. doi: 10.1016/0304-3959(83)90201-4
32. Roeckel LA, Utard V, Reiss D, Mouheiche J, Maurin H, Robé A, et al. Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Sci Rep.* (2017) 7:10406. doi: 10.1038/s41598-017-11120-4
33. Berrendero F, Mendizábal V, Murtra P, Kieffer BL, Maldonado R. Cannabinoid receptor and WIN 55 212-2-stimulated [35S]-GTPgammaS binding in the brain of mu-, delta- and kappa-opioid receptor knockout mice. *Eur J Neurosci.* (2003) 18:2197–202.
34. Goeldner C, Lutz P-E, Darcq E, Halter T, Clesse D, Ouagazzal A-M, et al. Impaired emotional-like behavior and serotonergic function during

protracted abstinence from chronic morphine. *Biol Psychiatry* (2011) 69:236–44. doi: 10.1016/j.biopsych.2010.08.021
35. Takao K, Miyakawa T. Light/dark transition test for mice. *J Visual Exp* (2006) 1:104. doi: 10.3791/104
36. Ducottet C, Belzung C. Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice. *Physiol Behav.* (2004) 81:417–26. doi: 10.1016/j.physbeh.2004.01.013
37. Kalueff AV, Tuohimaa P. Grooming analysis algorithm for neurobehavioural stress research. *Brain Res Brain Res Prot.* (2004) 13:151–8. doi: 10.1016/j.brainresprot.2004.04.002
38. MacHado DG, Cunha MP, Neis VB, Balen GO, Colla A, Grando J, et al. Fluoxetine reverses depressive-like behaviors and increases hippocampal acetylcholinesterase activity induced by olfactory bulbectomy. *Pharmacol Biochem Behav.* (2012) 103:220–9. doi: 10.1016/j.pbb.2012.08.024
39. Lötsch J, Weyer-Menkhoff I, Tegeder I. Current evidence of cannabinoid-based analgesia obtained in preclinical and human experimental settings. *Eur J Pain* (2017) 22:471–84. doi: 10.1002/ejp.1148
40. Tzschentke TM. Measuring reward with the conditioned place preference (CPP) paradigm: Update of the last decade. *Addict Biol.* (2007) 12:227–462. doi: 10.1111/j.1369-1600.2007.00070.x
41. Nadal X, La Porta C, Andreea Bura S, Maldonado R. Involvement of the opioid and cannabinoid systems in pain control: new insights from knockout studies. *Eur J Pharmacol.* (2013) 716:142–57. doi: 10.1016/j.ejphar.2013.01.077
42. Valverde O, Torrens M. CB1 receptor-deficient mice as a model for depression. *Neuroscience* (2012) 204:193–206. doi: 10.1016/j.neuroscience.2011.09.031
43. Hillard CJ, Manna S, Greenberg MJ, DiCamelli R, Ross RA, Stevenson LA, et al. Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J Pharmacol Exp Ther.* (1999) 289:1427–33.
44. Dang DK, Shin EJ, Mai AT, Jang CG, Nah SY, Jeong JH, et al. Genetic or pharmacological depletion of cannabinoid CB1 receptor protects against dopaminergic neurotoxicity induced by methamphetamine in mice. *Free Radical Biol Med.* (2017) 108:204–24. doi: 10.1016/j.freeradbiomed.2017.03.033
45. Diez-Alarcia R, Ibarra-Lecue I, Lopez-CardonaïngelaP, Meana J, Gutierrez-Adïn A, Callado LF, et al. (2016). Biased agonism of three different cannabinoid receptor agonists in mouse brain cortex. *Front Pharmacol.* 7:415. doi: 10.3389/fphar.2016.00415
46. Dore R, Valenza M, Wang X, Rice KC, Sabino V, Cottone P. The inverse agonist of CB1 receptor SR141716 blocks compulsive eating of palatable food. *Addict Biol.* (2014) 19:849–61. doi: 10.1111/adb.12056
47. Vickers SP, Kennett GA. Cannabinoids and the regulation of ingestive behaviour. *Curr Drug Targets* (2005) 6:215–23.
48. Stevens AJ, Higgins MD. A systematic review of the analgesic efficacy of cannabinoid medications in the management of acute pain. *Acta Anaesthesiol Scand.* (2017) 61:268–80. doi: 10.1111/aas.12851
49. Suplita RL, Eisenstein SA, Neely MH, Moise AM, Hohmann AG. Cross-sensitization and cross-tolerance between exogenous cannabinoid antinociception and endocannabinoid-mediated stress-induced analgesia. *Neuropharmacology* (2008) 54:161–71. doi: 10.1016/j.neuropharm.2007.07.006
50. Monory K, Blaudzun H, Massa F, Kaiser N, Lemberger T, Schütz G, et al. Genetic dissection of behavioural and autonomic effects of Delta(9)-tetrahydrocannabinol in mice. *PLoS Biol.* (2007) 5:e269. doi: 10.1371/journal.pbio.0050269
51. Célérier E, Ahdepil T, Wikander H, Berrendero F, Nyberg F, Maldonado R. Influence of the anabolic-androgenic steroid nandrolone on cannabinoid dependence. *Neuropharmacology* (2006) 50:788–806. doi: 10.1016/j.neuropharm.2005.11.017
52. Hutschenson DM, Tzavara ET, Smadja C, Valjent E, Roques BP, Hanoune J, et al. Behavioural and biochemical evidence for signs of abstience in mice chronically treated with delta-9-tetrahyrocannabinol. *Br J Pharmacol.* (1998) 125:1567–77.
53. Soria G, Castañé A, Berrendero F, Ledent C, Parmentier M, Maldonado R, Valverde O. Adenosine A2A receptors are involved in physical dependence and place conditioning induced by THC. *Eur J Neurosci.* (2004) 20:2203–13. doi: 10.1111/j.1460-9568.2004.03682.x
54. La Porta C, Bura SA, Llorente-Onaindia J, Pastor A, Navarrete F, García-Gutiérrez MS, et al. Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain. *Pain* (2015) 156:2001–12. doi: 10.1097/j.pain.0000000000000260
55. Vera G, Cabezos PA, Martín MI, Abalo R. Characterization of cannabinoid-induced relief of neuropathic pain in a rat model of cisplatin-induced neuropathy. *Pharmacol Biochem Behav.* (2013) 105:205–12. doi: 10.1016/j.pbb.2013.02.008
56. Li M-H, Suchland KL, Ingram SL. Compensatory activation of cannabinoid CB2 receptor inhibition of GABA release in the rostral ventromedial medulla in inflammatory pain. *J Neurosci* (2017) 37:626–36. doi: 10.1523/JNEUROSCI.1310-16.2017
57. Navarro M, Chowen J, Rocío A Carrera M, del Arco I, Villanúa MA, Martin Y, et al. CB1 cannabinoid receptor antagonist-induced opiate withdrawal in morphine-dependent rats. *Neuroreport* (1998) 9:3397–402.
58. Zimmer A, Valjent E, Konig M, Zimmer AM, Robledo P, Hahn H, et al. Absence of delta−9-tetrahydrocannabinol dysphoric effects in dynorphin-deficient mice. *J Neurosci.* (2001) 21:9499–505.
59. Berrendero F, Maldonado R. Involvement of the opioid system in the anxiolytic-like effects induced by Delta(9)-tetrahydrocannabinol. *Psychopharmacology* (2002) 163:111–7. doi: 10.1007/s00213-002-1144-9
60. Valjent E, Maldonado R. A behavioural model to reveal place preference to delta 9-tetrahydrocannabinol in mice. *Psychopharmacology* (2000) 147: 436–8.
61. Valjent E, Mitchell JM, Besson M-J, Caboche J, Maldonado R. Behavioural and biochemical evidence for interactions between Δ 9-tetrahydrocannabinol and nicotine. *Br J Pharmacol.* (2002) 135:564–78. doi: 10.1038/sj.bjp.0704479
62. Hudzik TJ, Maciag C, Smith MA, Caccese R, Pietras MR, Bui KH, et al. Preclinical pharmacology of AZD2327: a highly selective agonist of the δ-opioid receptor. *J Pharmacol Exp Ther.* (2011) 338:195–204. doi: 10.1124/jpet.111.179432
63. Jutkiewicz EM. The antidepressant -like effects of delta-opioid receptor agonists. *Mol Intervent.* (2006) 6:162–9. doi: 10.1124/mi.6.3.7
64. Naidu PS, Lichtman AH, Archer CC, May EL, Harris LS, Aceto MD. NIH 11082 produces anti-depressant-like activity in the mouse tail-suspension test through a delta-opioid receptor mechanism of action. *Eur J Pharmacol.* (2007) 566:132–6. doi: 10.1016/j.ejphar.2007.03.031
65. Broom DC, Jutkiewicz EM, Folk JE, Traynor JR, Rice KC, Woods JH. Nonpeptidic delta-opioid receptor agonists reduce immobility in the forced swim assay in rats. *Neuropsychopharmacology* (2002) 26:744–55. doi: 10.1016/S0893-133X(01)00413-4
66. Saitoh A, Kimura Y, Suzuki T, Kawai K, Nagase H, Kamei J. Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents. *J Pharmacol Sci.* (2004) 95:374–80.
67. Lutz P-E, Kieffer BL. Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* (2013) 36:195–206. doi: 10.1016/j.tins.2012.11.002

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The Contribution of the Descending Pain Modulatory Pathway in Opioid Tolerance

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Opioids remain among the most effective pain-relieving therapeutics. However, their long-term use is limited due to the development of tolerance and potential for addiction. For many years, researchers have explored the underlying mechanisms that lead to this decreased effectiveness of opioids after repeated use, and numerous theories have been proposed to explain these changes. The most widely studied theories involve alterations in receptor trafficking and intracellular signaling. Other possible mechanisms include the recruitment of new structural neuronal and microglia networks. While many of these theories have been developed using molecular and cellular techniques, more recent behavioral data also supports these findings. In this review, we focus on the mechanisms that underlie tolerance within the descending pain modulatory pathway, including alterations in intracellular signaling, neural-glial interactions, and neurotransmission following opioid exposure. Developing a better understanding of the relationship between these various mechanisms, within different parts of this pathway, is vital for the identification of more efficacious, novel therapeutics to treat chronic pain.

Keywords: opioid, tolerance, periaqueductal gray (PAG), RVM, dorsal horn

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DESCENDING PAIN PATHWAY IN OPIOID FUNCTIONS

Opioids are widely used pain therapeutics; however, the development of tolerance limits the long-term use of opioids due to the need for dose escalation over time in order to maintain analgesia. While there are four main types of opioid receptors, most pain therapeutics, including morphine, methadone, fentanyl, and oxycodone, target the mu opioid receptor (MOPr). The MOPr is a G-protein coupled receptor that couples to inhibitory heterotrimeric G-proteins ($G_{i/o}$) producing subsequent intracellular signaling and ion conductance (Goode and Raffa, 1997; Gintzler and Chakrabarti, 2004). MOPr expression within the descending pain modulatory pathway, which includes the ventrolateral periaqueductal gray (PAG), rostral ventromedial medulla (RVM), and the dorsal horn (DH) of the spinal cord, contribute to opioid-induced antinociception and the development of opioid tolerance (Fang et al., 1989; Fairbanks and Wilcox, 1997; Tortorici et al., 2001; Morgan et al., 2006; Bobeck et al., 2009).

GABAergic neurons within the PAG are a critical site of action by opioids. Under normal conditions, these neurons have tonic activity (**Figure 1**, naive); however, upon binding of opioids to MOPr, the activity of these neurons is decreased, disinhibiting PAG projections to the RVM (**Figure 1**, Acute Morphine) (Stiller et al., 1996; Vaughan et al., 1997; Bobeck et al., 2014). *In vitro*

electrophysiology studies have shown that opioids reduce the frequency of spontaneous mIPSCs in PAG (Vaughan et al., 1997; Bobeck et al., 2014), which indicates a reduction in the probability of GABA release. This is also supported by *in vivo* studies. Microinjection of bicuculline ($GABA_A$ agonist) into the PAG produces antinociception, which suggests that GABA is being tonically released (Bobeck et al., 2014). Furthermore, microdialysis in the PAG reveals a reduction in extracellular GABA following administration of morphine (Stiller et al., 1996).

Opioids activate different signaling cascades depending on whether the MOPr is expressed at pre- or post- synaptic sites. Opioid binding to postsynaptic MOPr results in the activation of a G-protein inwardly rectifying potassium channels (GIRK) that hyperpolarize GABAergic neurons in the PAG producing an overall decrease in GABAergic neuron activity (**Figure 1**; Acute Moprhine) (North and Williams, 1983; Pan et al., 1990). Alternatively, when MOPr are expressed at presynaptic sites they produce an inhibition of voltage gated calcium channels and voltage gated potassium channels (Kv) resulting in the inhibition of GABA release (**Figure 1**; Acute Morphine) (Wilding et al., 1995; Vaughan et al., 1997; Connor et al., 1999; Williams et al., 2001). Overall, the combined action of MOPr binding by opioids is a decrease in GABAergic neuronal activity, and therefore an increase in output from the PAG to the RVM (**Figure 1**; Moreau and Fields, 1986; Depaulis et al., 1987; Jacquet, 1988; Osborne et al., 1996). Recent studies support the hypothesis that this increase in PAG output to the RVM is a main contributor to the opioid-induced antinociception by demonstrating that selective inhibition of GABAergic neurons or activation of glutamatergic output neurons in the PAG mimics the antinociceptive effects of opioids (Samineni et al., 2017). In summary, these findings support the notion that analgesia is produced by disinhibition of excitatory outputs from the PAG.

The overall effect of MOPr activation in the PAG is an increase in output to the two distinct cell types within the RVM: off-cells and on-cells (Fields et al., 1983; Fields and Heinricher, 1985). The activity of off-cells pauses just prior to the response to a painful stimulus, while the activity of on-cells increases during this response, and both of these activities are blocked during the administration of opioids. There is conflicting evidence regarding the excitatory versus inhibitory nature of the PAG projections to the on- and off-cells in the RVM. Studies in GAD67-GFP mice, a marker for GABAergic neurons, show that retrogradely labeled neurons from the RVM do no co-localize with GAD67 in the PAG (Park et al., 2010), indicating that the PAG to RVM projection is glutamatergic. In contrast, studies in rats demonstrate that PAG to RVM projections are a mix of GABAergic and glutamatergic neurons (Morgan et al., 2008). Furthermore, these studies demonstrate that GABAergic neurons project from PAG and target on-cells and glutamatergic neurons project from the PAG and target off-cells (Morgan et al., 2008). Despite these differences, both studies support the notion that opioids inhibit GABA release from interneurons in the PAG, which disinhibit (i.e., excite) glutamate projections to off-cells. Given that the off-cells in the RVM are GABAergic, they subsequently inhibit pain responses in the DH (Fields et al., 1983; Moreau and Fields, 1986; Morgan et al., 2008). Overall, these studies support the concept that opioid-induced antinociception is mediated by direct excitation of off-cells and subsequent inhibition of pain in the spinal cord.

At each level of this pathway, a myriad of cellular effects drives the physiological changes mentioned above, and are highly correlated with the development of opioid tolerance. One of the most studied mechanisms involves regulation and signaling at the MOPr. Current research demonstrates that while MOPr is a key player in the development of antinociceptive tolerance, mechanisms beyond simple receptor desensitization, including alterations in neurotransmission and β-arrestin dependent signaling, are also critical. Furthermore, MOPr expression in non-neuronal cells, specifically on microglia and astrocytes within the spinal cord, and more recently within the PAG, greatly contributes to the development of opioid tolerance.

OPIOID TOLERANCE AND NEUROTRANSMISSION IN THE DESCENDING PAIN PATHWAY

Evidence suggests that tolerance is due to changes in the properties of GABAergic neurons in the PAG (Morgan et al., 2003). First of all, while microinjection of morphine into the PAG or RVM produces antinociception, repeated microinjection into the PAG and not the RVM results in tolerance (Morgan et al., 2005; Campion et al., 2016). Secondly, inhibition of MOPrs within the PAG blocks tolerance to systemic morphine (Lane et al., 2005). Furthermore, inactivation of RVM by a GABA agonist during direct administration of morphine into the PAG still leads to tolerance development (Lane et al., 2005). Therefore, MOPr within the PAG are necessary and sufficient in the development of opioid tolerance.

However, the development of tolerance within the PAG produces downstream effects along the descending pain pathway. This is evidenced by the fact that direct injection of morphine into the PAG affects RVM signaling, suggesting that their activity is in fact coupled (Tortorici et al., 2001). While acute administration of opioids into the PAG disrupts the activity of on- and off-cells in response to painful stimuli, these cells respond normally following chronic infusion that is associated with tolerance (Lane et al., 2004). Another side effect of chronic morphine treatment is hyperalgesia, or the increased sensitivity to pain following chronic morphine treatment. One theory is that hyperalgesia may manifest as opioid-induced tolerance since increased sensitivity to pain would counteract the pain-relieving effects of opioids. Some studies suggest that increased activation of the descending pain pathway by chronic morphine produces neuroadaptations with in the RVM that result in hyperalgesia (Vanderah et al., 2001). In support of this, one study demonstrated that chronic morphine produced an increase in the number of active on-cells, likely increasing sensitivity to noxious stimuli (Meng and Harasawa, 2007), which may be responsible for morphine-induced hyperalgesia. While RVM plays a role in opioid-induced tolerance, direct injections into the RVM leads to a lesser development of tolerance compared to PAG administration

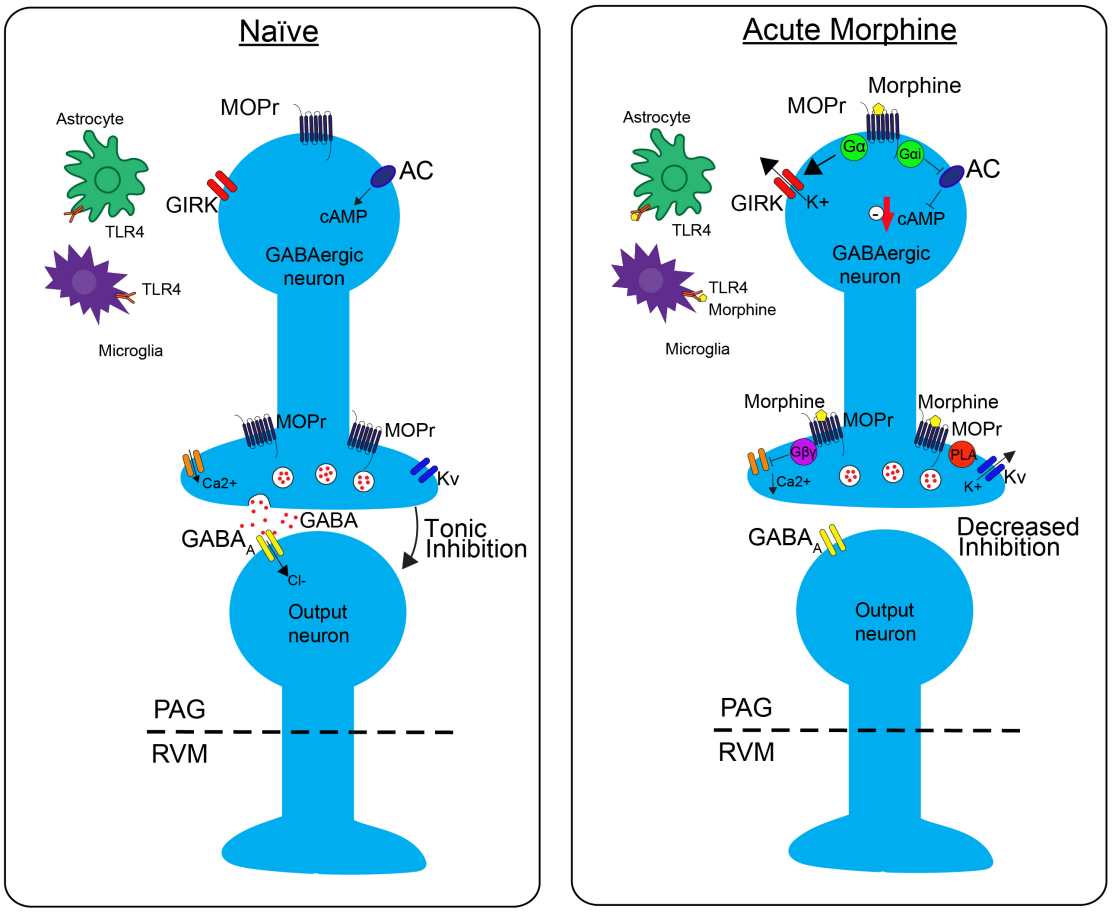

FIGURE 1 | The effects of morphine on neuronal transmission in the descending pain pathway. In the **naïve state**, GABAergic interneurons in the periaqueductal gray (PAG) fire tonically, thereby producing a steady release of GABA and inhibition of PAG output neurons. Upon administration of **acute morphine**, postsynaptic mu opioid receptor (MOPr) activate GIRK channels via Gα proteins resulting in K+ release and hyperpolarization of the neuron. Additionally, MOPr activate $G_{i/o}$ proteins, which result in the inhibition of adenylyl cyclase (AC) and decrease cAMP production. Morphine binding of presynaptic MOPr inhibits voltage dependent calcium (Ca^{2+}) conductances via Gβγ proteins and activated voltage dependent potassium conductances (Kv) via Phospholipase A (PLA). Overall, these two mechanisms block release of the neurotransmitter GABA, therefore suppressing inhibition, increasing output, of the PAG neurons projecting to the rostral ventromedial medulla (RVM). Acute morphine treatment also activates toll-like receptor 4 (TLR4) receptors on astrocytes and microglia in the PAG inducing several signaling cascades.

(Morgan et al., 2005), indicating that activation of the entire descending pain circuit is essential.

The neurophysiological mechanisms of tolerance in the PAG are mediated by MOPr uncoupling from downstream G-protein mediated signaling (**Figure 2**). One key study demonstrated that chronic morphine decreases opioid-mediated GIRK currents in the PAG (Bagley et al., 2005), supporting the notion that morphine tolerance is associated with uncoupling of G-protein mediated signaling. Since GIRK channels regulate neuronal excitability, this mechanism would result in a reduction in the ability of MOPr activation to suppress GABAergic neuron activity. Additionally, morphine tolerance is also associated with decreased efficacy of other MOPr agonists ability to reduce voltage gated calcium currents in the PAG (Bagley et al., 2005). The net effect of the uncoupling of MOPr activation from voltage gated calcium channels would be the attenuation of MOPr mediated inhibition of GABA release. However, the precise mechanisms underlying MOPr uncoupling from voltage gated calcium channels are complex, as cellular tolerance associated with this effect was not observed in β-arrestin two knockout mice (Connor et al., 2015), suggesting that β-arrestin two also plays a role in this interaction.

GABA release is also regulated by signaling through phospholipase A2-mediated activation of voltage gated potassium channels (**Figure 1**; Wimpey and Chavkin, 1991; Vaughan et al., 1997). This signaling pathway is differentially affected by morphine tolerance versus withdrawal. Morphine tolerance is associated with a decrease in opioid-mediated inhibition of GABA release (**Figure 2**) that is not a result of MOPr desensitization (Fyfe et al., 2010). However, during naloxone-precipitated withdrawal following chronic morphine, GABA release is enhanced via an increase in adenylyl cyclase (AC) signaling (Sharma et al., 1975; Ingram et al., 1998; Hack et al., 2003). These two outcomes may be related as studies have

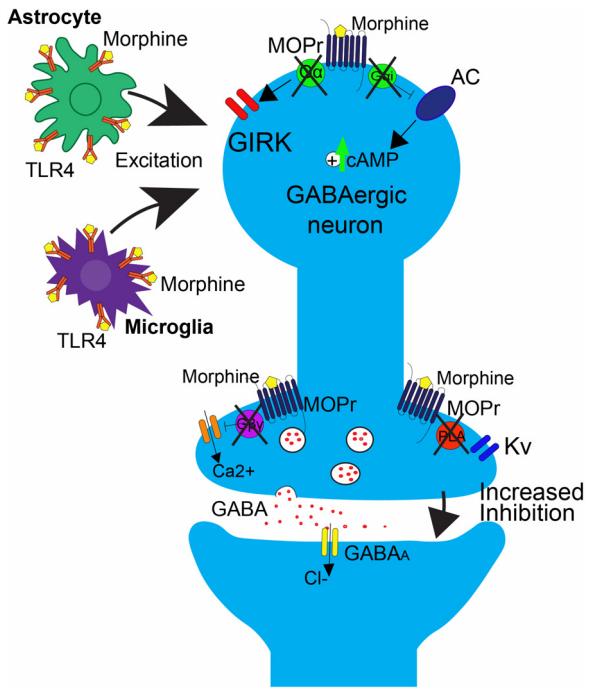

FIGURE 2 | Effects of repeated morphine treatment on glial and neuronal signaling in the PAG. Chronic morphine treatment induces several side effects that block opioid-induced decreases in GABAergic interneuron activity at the neuronal level, postsynaptically. This includes the uncoupling of MOPr from G-protein mediated effects on GIRK channels and AC. This results in an upregulation of cAMP. Uncoupling also occurs in the presynaptic region, blocking the Gβγ mediated inhibition of calcium channels and PLA mediated activation of Kv channels. In this state, binding of opioids to MOPr no longer results in suppression of GABA release. At the level of glial signaling, upon repeated treatment with morphine, there is a rapid upregulation of TLR4 on astrocytes and microglia within the PAG, resulting in an increase in excitatory cytokine release, as well as a switch from $G_{i/o}$ to G_s coupling at MOPr, resulting in an overall increase in excitatory tone that is correlated with opioid tolerance.

demonstrated that inhibition of AC in the PAG prevents the development and expression of morphine tolerance (Bobeck et al., 2014). Moreover, AC activation is sufficient to increase GABA release from PAG neurons (Bobeck et al., 2014).

INTRACELLULAR SIGNALING CHANGES IN THE PAG-RVM-DH PATHWAY IN OPIOID TOLERANCE

Direct activation of the MOPr results in the Gα subunit-mediated inhibition of the AC-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway (**Figure 1**; Sharma et al., 1975; Guitart and Nestler, 1989; Hirst and Lambert, 1995). However, opioid binding activates other signaling proteins, such as protein kinase C (PKC) and extracellular signal-regulated kinase 1/2 (ERK1/2) via β-arrestin pathways, which are independent of G-protein signaling. As mentioned previously, downstream of G-protein mediated signaling, there is an inhibition of calcium channels and activation of potassium channels that leads to hyperpolarization and a reduction in neurotransmitter release in the PAG that produces antinociception (Bourinet et al., 1996; Ippolito et al., 2002; Torrecilla et al., 2002). Chronic morphine produces adaptations that contribute to opioid-tolerance within all these downstream signaling pathways.

Long-term opioid treatment leads to adaptations in many signaling proteins within the PAG-RVM-DH pathway, which have been proposed as mechanisms of opioid tolerance. In contrast to the acute inhibitory effect of opioids on cAMP production, chronic morphine treatment upregulates cAMP (**Figure 2**; Guitart and Nestler, 1989; Gintzler and Chakrabarti, 2004). It has been proposed that this upregulation in cAMP is caused by compensatory changes in intracellular signaling, or an uncoupling of $G_{i/o}$-proteins from the receptor and a switch to coupling with G_s-proteins (Gintzler and Chakrabarti, 2004). Very few *in vivo* studies have evaluated the role of the AC pathway in morphine tolerance, but inhibition of the AC pathway, via either intracerebroventricular (ICV) or intra-PAG injection, during morphine pretreatment has been shown to block the development of morphine tolerance (Smith et al., 2006; Gabra et al., 2008; Bobeck et al., 2014). In the DH, administration of morphine results in no change or even an increase in MOPr expression, but a significant down-regulation of the G-protein activation in the DH, as measured by [^{35}S]-GTPγS (Maher et al., 2001; Ray et al., 2004). The loss of G-protein signaling is likely a switch in MOPr G-protein coupling, from $G_{i/o}$ to G_s coupling (Gintzler and Chakrabarti, 2004). Recently, adrenomedullin, a pronociceptive peptide from the CGRP family, has been implicated in mediating this G-protein switch in the DH. Following chronic morphine, adrenomedullin is significantly upregulated in the DH and dorsal root ganglia, and inhibition of its receptor prevents or reverses morphine tolerance and blocks the MOPr $G_{i/o}$ to G_s switch in coupling (Wang et al., 2016).

Behavioral studies suggest that the mechanisms underlying tolerance are dependent on the specific MOPr agonist being studied. Some agonists, such as morphine, do not readily recruit β-arrestin or internalize the receptor, as compared to high efficacy agonists, such as DAMGO or fentanyl, which readily do both. This difference in signaling suggests differences in tolerance mechanisms, where morphine-mediated tolerance utilizes a G-protein dependent mechanism, and other MOPr agonists, such as DAMGO or fentanyl, use a β-arrestin dependent mechanism (Hull et al., 2010; Melief et al., 2010; Bobeck et al., 2014, 2016; Morgan et al., 2014). For example, inhibition of ERK1/2 within the PAG during the development of tolerance enhances morphine tolerance (Macey et al., 2009), but reduces tolerance to DAMGO and has no effect on fentanyl tolerance (Bobeck et al., 2016). Furthermore, inhibition of the G-protein dependent pathway (i.e., c-Jun N-terminal kinase) blocks development of tolerance to morphine, but not fentanyl. However, inhibition of β-arrestin dependent signaling (i.e., G protein-coupled receptor kinase) blocks expression of fentanyl tolerance (Morgan et al., 2014).

Neuropeptides within the descending pain pathway have also been shown to regulate opioid tolerance. One such neuropeptide, cholecystokinin (CCK), is particularly enriched in supraspinal midbrain regions known to regulate spinal

nociception (King et al., 2005). There is evidence that CCK acting within the PAG-RVM-DH pathway regulates morphine tolerance (Xie et al., 2005; Thomas et al., 2015). A CCK receptor antagonist, directly injected into the PAG, is able to block morphine tolerance (Xie et al., 2005). In the RVM, injection of CCK blocks opioid activation of off-cells that mediate descending antinociception, resulting in a blockade of the analgesic effects of morphine (Xu et al., 2014; Thomas et al., 2015).

N-methyl D-aspartate receptors (NMDArs) have been heavily implicated in the development of both spinal-mediated hyperalgesia and opioid tolerance. NMDAr antagonists or the targeted disruption of the NR2 subunits, NR2a and NR2b, attenuates opioid tolerance (Price et al., 2000; Zhao et al., 2012). Deletion of PSD-93, the anchoring protein for NR2a and NR2b in the synapse, leads to a DH site-specific down-regulation of both subunits from the plasma membrane into the cytosolic compartment, and a reduction in the development of morphine tolerance (Liaw et al., 2008). This is a region-specific effect, as other portions of the descending pain pathway did not see changes in the NR2 subunit localization (Kozela and Popik, 2007). Interestingly, NMDArs in the PAG have not been implicated in tolerance (Morgan et al., 2009).

A few other main signaling targets have been implicated in DH-mediated opioid tolerance, as well. Mammalian target of rapamycin (mTOR) is found to be upregulated following repeated intrathecal morphine administration, and this effect is mediated by activation of PI3K/AKT following MOPr activation (Xu et al., 2015). Administration of mTOR inhibitors is able to both prevent and reverse morphine tolerance (Xu et al., 2014, 2015; Jiang et al., 2016; Chen et al., 2017). Calcium/calmodulin-dependent protein kinase IIα has also been implicated in the development of tolerance (Brüggemann et al., 2000). It has been shown to colocalize with MOPr, in the DH specifically, following opioid administration, possibly resulting in increased MOPr phosphorylation and desensitization (Brüggemann et al., 2000).

IMPACT OF OPIOID-INDUCED NEUROINFLAMMATION ON THE DEVELOPMENT OF TOLERANCE

Over the past few decades, researchers have discovered that opioids are potent activators of immune cells within the CNS, and this inflammation is a strong contributor to the development of opioid tolerance (Giron et al., 2015; Cahill and Taylor, 2017). Specifically, repeated administration of opioids, which leads to activation of glia within the PAG and spinal cord of the descending pain pathway, results in alterations in both intracellular signaling cascades and signaling properties of neurons. Furthermore, microglial inhibitors have been shown to attenuate morphine-induced tolerance (Song and Zhao, 2001; Raghavendra et al., 2002, 2004; Cui et al., 2008; Eidson and Murphy, 2013; Harada et al., 2013). Though the precise mechanisms that underlie these changes are only beginning to be uncovered, a few notable pathways have emerged that are likely significant contributors to the development of opioid tolerance.

One prominent pro-inflammatory signaling cascade that has been implicated in opioid tolerance involves the immune receptor, toll-like receptor 4 (TLR4, **Figure 2**). Upon agonist binding to TRL4, sphingomyelinase produces ceramide, which allows for interaction of the receptor with its co-activators myeloid differentiation factor-2 (MD-2) and CD14, resulting in subsequent activation of 3 parallel pathways: the p38-MAPK pathway, the PI3K/AKT pathway (cell survival/apoptosis), and the NFκB pathway (proinflammatory cytokine release) (Rönnbäck and Hansson, 1988; Watkins et al., 2009; Nakamoto et al., 2012; Eidson and Murphy, 2013). In the spinal cord, TLR4 is primarily expressed on microglial cells and is shown to be upregulated (**Figure 2**) along with its cofactor MD-2 following morphine treatment (Wang et al., 2012), and activation of TLR4 signaling can induce "naïve tolerance" to opioids (Eidson and Murphy, 2013; Grace et al., 2015). Furthermore, inhibition of TLR4, co-activators MD-2 or CD14, or inhibition of ceramide biosynthesis, leads to attenuation of morphine tolerance, as well as decreased microglial activation, suggesting a prominent role for the TLR4 pathway in the development of opioid tolerance, at the level of the spinal cord (Ndengele et al., 2009; Hutchinson et al., 2010, 2011; Muscoli et al., 2010; Thomas et al., 2015).

Interestingly, it is also thought that TLR4 is directly activated by opioids (**Figures 1**, **2**; Hutchinson et al., 2011; Wang et al., 2012; Grace et al., 2015), and, perhaps more importantly, the accessory protein MD-2 is able to non-stereoselectively bind opioids and signal through TRL4 (Grace et al., 2015). Since the classic opioid receptors only bind the (−)-opioid isomer, the (+)-opioid isomer antagonists could be used to block TLR4-mediated microglial activation and pro-inflammatory cytokine production. In fact, studies have demonstrated that (+)-naloxone is able to attenuate morphine-induced analgesia, specifically at the level of the spinal cord (Hutchinson et al., 2010; Lewis et al., 2010). This non-stereoselectivity at the TLR4 receptor complex could potentially be leveraged for the enhancement of the therapeutic efficacy of opioids, including enhancing analgesic effects and reducing tolerance.

How does the activation of glial cells lead to alterations in neuronal signaling? One possibility is through the alteration of neuronal excitability via increased release of glially-derived pro-inflammatory cytokines, including TNF (tumor necrosis factor) and IL-1β, which are known to increase neuronal AMPA and NMDA receptors, as well as down regulate GABA receptors (Viviani et al., 2003; Stellwagen, 2005). Within the PAG, repeated morphine administration results in an upregulation of TLR4, which subsequently leads to an increase in release of TNF and IL-1β (Eidson and Murphy, 2013; Eidson et al., 2017). This upregulation is concurrent with a downregulation of astrocyte glutamate transporters GLT-1 and GLAST, which are responsible for synaptic glutamate uptake. The overall effect is an increase in neuronal excitability, thereby lowering the ability of opioids to hyperpolarize mu-containing GABAergic neurons (**Figure 2**). Within the PAG to RVM circuitry, this results in an inability for morphine to disinhibit output neurons to RVM (Eidson and Murphy, 2013; Eidson et al., 2017).

Another potential point of cross talk is via purinergic receptors, specifically P2X4 and P2X7, which are primarily

expressed on microglia. These receptors are also capable of upregulating pro-inflammatory cytokines, and blocking their activity in the spinal cord attenuates morphine tolerance (Horvath et al., 2010; Zhou et al., 2010; Xiao et al., 2015). P2X4 activates the p38-MAPK pathway, resulting in the release of IL-1β, TNF-α, and BDNF, which, as mentioned above, are known to alter neuronal excitability and contribute to pain hypersensitivity, but no direct connection has been made to opioid tolerance (Ferrini et al., 2013; Grace et al., 2015; Thomas et al., 2015). However, P2X7 mediated release of IL-18 from microglia induces activation of the IL-18 receptor on astrocytes, thereby increasing the release of D-serine, which is able to activate NMDA receptors in neurons. Activation of both receptors is able to alter glial activation and neuronal excitability, suggesting a complicated crosstalk between cell types in the spinal cord that is correlated with morphine tolerance (Chen et al., 2012).

CONCLUSION

The descending pain pathway is a critical modulator of nociception and plays an important role in mediating endogenous and exogenous opioid-induced analgesia. Because of this, it is highly implicated in allostatic cellular and molecular changes following repeated opioid use that lead to the development of tolerance. While this review has touched on a number of those changes at each level of the descending pain pathway, including desensitization of MOPr, altered cellular excitability and signaling, and induction of immune-competent cells, we do not yet have a complete understanding of all the factors that might be contributing to opioid tolerance.

Much of the literature on opioid tolerance has focused the effects of morphine on this system. Future research must expand to include other commonly used opioids, especially in light of the increasing use of oxycodone and fentanyl, as each of these has widely different pharmacokinetic and signaling profiles, and may have differential effects on each level of the PAG-RVM-DH pathway. Indeed, studies looking at cross-tolerance between opioid analgesics suggest that differences in the distribution of the drug within the pain pathway may be differentially contributing to the development of tolerance. Furthermore, the cellular signaling pathways initiated within these spinal and supraspinal regions following administration of different opioids are known to vary.

Finally, the research design of the studies related to opioids and tolerance has varied widely in terms of not only the drugs used, but also routes of administration, length of exposure, and use of biological systems. Also, the majority of studies on opioid tolerance have focused on males and have largely excluded females. Given that males show greater morphine potency, tolerance, and activation of neurons from PAG to RVM following morphine, as compared to females (Loyd et al., 2008), it is imperative to further explore these differences. Overall, these variations in research design have resulted in a myriad of observed cellular changes that correlate with tolerance, but with no definite conclusions or unifying theories of tolerance. While no one specific etiology may exist, future researchers must be careful in designing these studies, in order to make meaningful conclusions regarding the cellular impact of opioids in the development of tolerance.

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REFERENCES

Bagley, E. E., Chieng, B. C. H., Christie, M. J., and Connor, M. (2005). Opioid tolerance in periaqueductal gray neurons isolated from mice chronically treated with morphine. *Br. J. Pharmacol.* 146, 68–76. doi: 10.1038/sj.bjp.0706315

Bobeck, E. N., Chen, Q., Morgan, M. M., and Ingram, S. L. (2014). Contribution of adenylyl cyclase modulation of pre- and postsynaptic GABA neurotransmission to morphine antinociception and tolerance. *Neuropsychopharmacology* 39, 2142–2152. doi: 10.1038/npp.2014.62

Bobeck, E. N., Ingram, S. L., Hermes, S. M., Aicher, S. A., and Morgan, M. M. (2016). Ligand-biased activation of extracellular signal-regulated kinase 1/2 leads to differences in opioid induced antinociception and tolerance. *Behav. Brain Res.* 298, 17–24. doi: 10.1016/j.bbr.2015.10.032

Bobeck, E. N., McNeal, A. L., and Morgan, M. M. (2009). Drug dependent sex-differences in periaqueducatal gray mediated antinociception in the rat. *Pain* 147, 210–216. doi: 10.1016/j.pain.2009.09.008

Bourinet, E., Soong, T. W., Stea, A., and Snutch, T. P. (1996). Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1486–1491. doi: 10.1073/pnas.93.4.1486

Brüggemann, I., Schulz, S., Wiborny, D., and Höllt, V. (2000). Colocalization of the μ-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions. *Mol. Brain Res.* 85, 239–250. doi: 10.1016/S0169-328X(00)00265-5

Cahill, C. M., and Taylor, A. M. (2017). Neuroinflammation—a co-occurring phenomenon linking chronic pain and opioid dependence. *Curr. Opin. Behav. Sci.* 13, 171–177. doi: 10.1016/j.cobeha.2016.12.003

Campion, K. N., Saville, K. A., and Morgan, M. M. (2016). Relative contribution of the dorsal raphe nucleus and ventrolateral periaqueductal gray to morphine antinociception and tolerance in the rat. *Eur. J. Neurosci.* 44, 2667–2672. doi: 10.1111/ejn.13378

Chen, M. L., Cao, H., Chu, Y. X., Cheng, L. Z., Liang, L. L., Zhang, Y. Q., et al. (2012). Role of P2X7 receptor-mediated IL-18/IL-18R signaling in morphine tolerance: Multiple glial-neuronal dialogues in the rat spinal cord. *J. Pain* 13, 945–958. doi: 10.1016/j.jpain.2012.06.007

Chen, S.-P., Zhou, Y.-Q., Liu, D.-Q., Zhang, W., Manyande, A., Guan, X.-H., et al. (2017). PI3K/Akt pathway: a potential therapeutic target for chronic pain. *Curr. Pharm. Des.* 23, 1860–1868. doi: 10.2174/1381612823666170210150147

Connor, M., Bagley, E. E., Chieng, B. C., and Christie, M. J. (2015). β-Arrestin-2 knockout prevents development of cellular μ-opioid receptor tolerance but does not affect opioid-withdrawal-related adaptations in single PAG neurons. *Br. J. Pharmacol.* 172, 492–500. doi: 10.1111/bph.12673

Connor, M., Schuller, A., Pintar, J. E., and Christie, M. J. (1999). μ-opioid receptor modulation of calcium channel current in periaqueductal grey neurons from

C57B16/J mice and mutant mice lacking MOR-1. *Br. J. Pharmacol.* 126, 1553–1558. doi: 10.1038/sj.bjp.0702457

Cui, Y., Liao, X. X., Liu, W., Guo, R. X., Wu, Z. Z., Zhao, C. M., et al. (2008). A novel role of minocycline: attenuating morphine antinociceptive tolerance by inhibition of p38 MAPK in the activated spinal microglia. *Brain. Behav. Immun.* 22, 114–123. doi: 10.1016/j.bbi.2007.07.014

Depaulis, A., Morgan, M. M., and Liebeskind, J. C. (1987). GABAergic modulation of the analgesic effects of morphine microinjected in the ventral periaqueductal gray matter of the rat. *Brain Res.* 436, 223–228. doi: 10.1016/0006-8993(87)91665-9

Eidson, L. N., Inoue, K., Young, L. J., Tansey, M. G., and Murphy, A. Z. (2017). Toll-like receptor 4 mediates morphine-induced neuroinflammation and tolerance via soluble tumor necrosis factor signaling. *Neuropsychopharmacology* 42, 661–670. doi: 10.1038/npp.2016.131

Eidson, L. N., and Murphy, A. Z. (2013). Blockade of toll-like receptor 4 attenuates morphine tolerance and facilitates the pain relieving properties of morphine. *J. Neurosci.* 33, 15952–15963. doi: 10.1523/JNEUROSCI.1609-13.2013

Fairbanks, C. A., and Wilcox, G. L. (1997). Acute tolerance to spinally administered morphine compares mechanistically with chronically induced morphine tolerance. *J. Pharmacol. Exp. Ther.* 282, 1408–1417.

Fang, F. G., Haws, C. M., Drasner, K., Williamson, A., and Fields, H. L. (1989). Opioid peptides (DAGO-enkephalin, dynorphin A(1-13), BAM 22P) microinjected into the rat brainstem: comparison of their antinociceptive effect and their effect on neuronal firing in the rostral ventromedial medulla. *Brain Res.* 501, 116–128. doi: 10.1016/0006-8993(89)91033-0

Ferrini, F., Trang, T., Mattioli, T. A. M., Laffray, S., Del'Guidice, T., Lorenzo, L. E., et al. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl-homeostasis. *Nat. Neurosci.* 16, 183–192. doi: 10.1038/nn.3295

Fields, H. L., Bry, J., Hentall, I., and Zorman, G. (1983). The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *J. Neurosci.* 3, 2545–2552. doi: 10.1523/JNEUROSCI.03-12-02545.1983

Fields, H. L., and Heinricher, M. M. (1985). Anatomy and physiology of a nociceptive modulatory system. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 308, 361–374. doi: 10.1098/rstb.1985.0037

Fyfe, L. W., Cleary, D. R., Macey, T. A., Morgan, M. M., and Ingram, S. L. (2010). Tolerance to the antinociceptive effect of morphine in the absence of short-term presynaptic desensitization in rat periaqueductal gray neurons. *J. Pharmacol. Exp. Ther.* 335, 674–680. doi: 10.1124/jpet.110.172643

Gabra, B. H., Bailey, C. P., Kelly, E., Smith, F. L., Henderson, G., and Dewey, W. L. (2008). Pre-treatment with a PKC or PKA inhibitor prevents the development of morphine tolerance but not physical dependence in mice. *Brain Res.* 1217, 70–77. doi: 10.1016/j.brainres.2008.04.036

Gintzler, A. R., and Chakrabarti, S. (2004). Chronic morphine-induced plasticity among signalling molecules. *Novartis Found. Symp.* 261, 191–193. doi: 10.1002/0470869127.ch13

Giron, S. E., Griffis, C. A., and Burkard, J. F. (2015). Chronic pain and decreased opioid efficacy: an inflammatory link. *Pain Manag. Nurs.* 16, 819–831. doi: 10.1016/j.pmn.2015.04.001

Goode, T. L., and Raffa, R. B. (1997). An examination of the relationship between mu-opioid antinociceptive efficacy and G-protein coupling using pertussis and cholera toxins. *Life Sci.* 60, l107–l113. doi: 10.1016/S0024-3205(96)00684-4

Grace, P. M., Maier, S. F., and Watkins, L. R. (2015). Opioid-Induced central immune signaling: implications for opioid analgesia. *Headache* 55, 475–489. doi: 10.1111/head.12552

Guitart, X., and Nestler, E. J. (1989). Identification of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the locus coeruleus and other regions of rat brain: regulation by acute and chronic morphine. *J. Neurosci.* 9, 4371–4387. doi: 10.1523/JNEUROSCI.09-12-04371.1989

Hack, S. P., Vaughan, C. W., and Christie, M. J. (2003). Modulation of GABA release during morphine withdrawal in midbrain neurons in vitro. *Neuropharmacology* 45, 575–584. doi: 10.1016/S0028-3908(03)00205-3

Harada, S., Nakamoto, K., and Tokuyama, S. (2013). The involvement of midbrain astrocyte in the development of morphine tolerance. *Life Sci.* 93, 573–578. doi: 10.1016/j.lfs.2013.08.009

Hirst, R. A., and Lambert, D. G. (1995). Adenylyl cyclase in SH-SY5Y human neuroblastoma cells is regulated by intra- and extracellular calcium. *Biochem. Pharmacol.* 49, 1633–1640. doi: 10.1016/0006-2952(95)00075-B

Horvath, R. J., Romero-Sandoval, E. A., and Leo, J. A. D. (2010). Inhibition of microglial P2X4receptors attenuates morphine tolerance, Iba1, GFAP and μ opioid receptor protein expression while enhancing perivascular microglial ED2. *Pain* 150, 401–413. doi: 10.1016/j.pain.2010.02.042

Hull, L. C., Llorente, J., Gabra, B. H., Smith, F. L., Kelly, E., Bailey, C., et al. (2010). The effect of protein kinase C and G protein-coupled receptor kinase inhibition on tolerance induced by mu-opioid agonists of different efficacy. *J. Pharmacol. Exp. Ther.* 332, 1127–1135. doi: 10.1124/jpet.109.161455

Hutchinson, M. R., Shavit, Y., Grace, P. M., Rice, K. C., Maier, S. F., and Watkins, L. R. (2011). Exploring the neuroimmunopharmacology of opioids: an integrative review of mechanisms of central immune signaling and their implications for opioid analgesia. *Pharmacol. Rev.* 63, 772–810. doi: 10.1124/pr.110.004135

Hutchinson, M. R., Zhang, Y., Shridhar, M., Evans, J. H., Buchman, M. M., Zhao, T. X., et al. (2010). Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav. Immun.* 24, 83–95. doi: 10.1016/j.bbi.2009.08.004

Ingram, S. L., Vaughan, C. W., Bagley, E. E., Connor, M., and Christie, M. J. (1998). Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway. *J. Neurosci.* 18, 10269–10276. doi: 10.1523/JNEUROSCI.18-24-10269.1998

Ippolito, D. L., Temkin, P. A., Rogalski, S. L., and Chavkin, C. (2002). N-terminal tyrosine residues within the potassium channel Kir3 modulate GTPase activity of Galphai. *J. Biol. Chem.* 277, 32692–32696. doi: 10.1074/jbc.M204407200

Jacquet, Y. F. (1988). The NMDA receptor: central role in pain inhibition in rat periaqueductal gray. *Eur. J. Pharmacol.* 154, 271–276. doi: 10.1016/0014-2999(88)90201-4

Jiang, Z., Wu, S., Wu, X., Zhong, J., Lv, A., Jiao, J., et al. (2016). Blocking mammalian target of rapamycin alleviates bone cancer pain and morphine tolerance via μ-opioid receptor. *Int. J. Cancer* 138, 2013–2020. doi: 10.1002/ijc.29927

King, T., Ossipov, M. H., Vanderah, T. W., Porreca, F., and Lai, J. (2005). Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance? *Neurosignals* 14, 194–205. doi: 10.1159/000087658

Kozela, E., and Popik, P. (2007). A complete analysis of NMDA receptor subunits in periaqueductal grey and ventromedial medulla of morphine tolerant mice. *Drug Alcohol Depend.* 86, 290–293. doi: 10.1016/j.drugalcdep.2006.06.018

Lane, D. A., Patel, P. A., and Morgan, M. M. (2005). Evidence for an intrinsic mechanism of antinociceptive tolerance within the ventrolateral periaqueductal gray of rats. *Neuroscience* 135, 227–234. doi: 10.1016/J.NEUROSCIENCE.2005.06.014

Lane, D. A., Tortorici, V., and Morgan, M. M. (2004). Behavioral and electrophysiological evidence for tolerance to continuous morphine administration into the ventrolateral periaqueductal gray. *Neuroscience* 125, 63–69. doi: 10.1016/j.neuroscience.2004.01.023

Lewis, S. S., Hutchinson, M. R., Rezvani, N., Loram, L. C., Zhang, Y., Maier, S. F., et al. (2010). Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1β. *Neuroscience* 165, 569–583. doi: 10.1016/j.neuroscience.2009.10.011

Liaw, W. J., Zhu, X. G., Yaster, M., Johns, R. A., Gauda, E. B., and Tao, Y. X. (2008). Distinct expression of synaptic NR2A and NR2B in the central nervous system and impaired morphine tolerance and physical dependence in mice deficient in postsynaptic density-93 protein. *Mol. Pain* 4:45. doi: 10.1186/1744-8069-4-45

Loyd, D. R., Morgan, M. M., and Murphy, A. Z. (2008). Sexually dimorphic activation of the periaqueductal gray-rostral ventromedial medullary circuit during the development of tolerance to morphine in the rat. *Eur. J. Neurosci.* 27, 1517–1524. doi: 10.1111/j.1460-9568.2008.06100.x

Macey, T. A., Bobeck, E. N., Hegarty, D. M., Aicher, S. A., Ingram, S. L., and Morgan, M. M. (2009). Extracellular signal-regulated kinase 1/2 activation counteracts morphine tolerance in the periaqueductal gray of the rat. *J. Pharmacol. Exp. Ther.* 331, 412–418. doi: 10.1124/jpet.109.152157

Maher, C. E., Eisenach, J. C., Pan, H. L., Xiao, R., and Childers, S. R. (2001). Chronic intrathecal morphine administration produces homologous mu receptor/G-protein desensitization specifically in spinal cord. *Brain Res.* 895, 1–8. doi: 10.1016/S0006-8993(00)03093-6

Melief, E. J., Miyatake, M., Bruchas, M. R., and Chavkin, C. (2010). Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11608–11613. doi: 10.1073/pnas.1000751107

Meng, I. D., and Harasawa, I. (2007). Chronic morphine exposure increases the proportion of on-cells in the rostral ventromedial medulla in rats. *Life Sci.* 80, 1915–1920. doi: 10.1016/j.lfs.2007.02.022

Moreau, J. L., and Fields, H. L. (1986). Evidence for GABA involvement in midbrain control of medullary neurons that modulate nociceptive transmission. *Brain Res.* 397, 37–46. doi: 10.1016/0006-8993(86)91367-3

Morgan, M. M., Bobeck, E. N., and Ingram, S. L. (2009). Glutamate modulation of antinociception, but not tolerance, produced by morphine microinjection into the periaqueductal gray of the rat. *Brain Res.* 1295, 59–66. doi: 10.1016/j.brainres.2009.07.100

Morgan, M. M., Clayton, C. C., and Boyer-Quick, J. S. (2005). Differential susceptibility of the PAG and RVM to tolerance to the antinociceptive effect of morphine in the rat. *Pain* 113, 91–98. doi: 10.1016/j.pain.2004.09.039

Morgan, M. M., Clayton, C. C., and Lane, D. A. (2003). Behavioral evidence linking opioid-sensitive GABAergic neurons in the ventrolateral periaqueductal gray to morphine tolerance. *Neuroscience* 118, 227–232. doi: 10.1016/S0306-4522(02)00822-9

Morgan, M. M., Fossum, E. N., Levine, C. S., and Ingram, S. L. (2006). Antinociceptive tolerance revealed by cumulative intracranial microinjections of morphine into the periaqueductal gray in the rat. *Pharmacol. Biochem. Behav.* 85, 214–219. doi: 10.1016/j.pbb.2006.08.003

Morgan, M. M., Reid, R. A., and Saville, K. A. (2014). Functionally selective signaling for morphine and fentanyl antinociception and tolerance mediated by the rat periaqueductal gray. *PLoS One* 9:e114269. doi: 10.1371/journal.pone.0114269

Morgan, M. M., Whittier, K. L., Hegarty, D. M., and Aicher, S. A. (2008). Periaqueductal gray neurons project to spinally projecting GABAergic neurons in the rostral ventromedial medulla. *Pain* 140, 376–386. doi: 10.1016/j.pain.2008.09.009

Muscoli, C., Doyle, T., Dagostino, C., Bryant, L., Chen, Z., Watkins, L. R., et al. (2010). Counter-regulation of opioid analgesia by glial-derived bioactive sphingolipids. *J. Neurosci.* 30, 15400–15408. doi: 10.1523/JNEUROSCI.2391-10.2010

Nakamoto, K., Kawasaki, S., Kobori, T., Fujita-Hamabe, W., Mizoguchi, H., Yamada, K., et al. (2012). Involvement of matrix metalloproteinase-9 in the development of morphine tolerance. *Eur. J. Pharmacol.* 683, 86–92. doi: 10.1016/j.ejphar.2012.03.006

Ndengele, M. M., Cuzzocrea, S., Masini, E., Vinci, M. C., Esposito, E., Muscoli, C., et al. (2009). Spinal ceramide modulates the development of morphine antinociceptive tolerance via peroxynitrite-mediated nitroxidative stress and neuroimmune activation. *J. Pharmacol. Exp. Ther.* 329, 64–75. doi: 10.1124/jpet.108.146290

North, R. A., and Williams, J. T. (1983). Opiate activation of potassium conductance inhibits calcium action potentials in rat locus coeruleus neurones. *Br. J. Pharmacol.* 80, 225–228. doi: 10.1111/j.1476-5381.1983.tb10023.x

Osborne, P. B., Vaughan, C. W., Wilson, H. I., and Christie, M. J. (1996). Opioid inhibition of rat periaqueductal grey neurones with identified projections to rostral ventromedial medulla in vitro. *J. Physiol.* 490, 383–389. doi: 10.1113/jphysiol.1996.sp021152

Pan, Z. Z., Williams, J. T., and Osborne, P. B. (1990). Opioid actions on single nucleus raphe magnus neurons from rat and guinea? pig in vitro. *J. Physiol.* 427, 519–532. doi: 10.1113/jphysiol.1990.sp018185

Park, C., Kim, J.-H., Yoon, B.-E., Choi, E.-J., Lee, C. J., and Shin, H.-S. (2010). T-type channels control the opioidergic descending analgesia at the low threshold-spiking GABAergic neurons in the periaqueductal gray. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14857–14862. doi: 10.1073/pnas.1009532107

Price, D. D., Mayer, D. J., Mao, J., and Caruso, F. S. (2000). NMDA-receptor antagonists and opioid receptor interactions as related to analgesia and tolerance. *J. Pain Symptom Manage.* 19(1 Suppl.), S7–S11. doi: 10.1016/S0885-3924(99)00121-9

Raghavendra, V., Rutkowski, M. D., and DeLeo, J. A. (2002). The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J. Neurosci.* 22, 9980–9989. doi: 10.1523/jneurosci.1850-04.2004

Raghavendra, V., Tanga, F. Y., and DeLeo, J. A. (2004). Attenuation of morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammtory immune responses by propentofylline in rats. *Neuropsychopharmacology* 29, 327–334. doi: 10.1038/sj.npp.1300315

Ray, S. B., Gupta, H., and Gupta, Y. K. (2004). Up-regulation of mu-opioid receptors in the spinal cord of morphine-tolerant rats. *J. Biosci.* 29, 51–56. doi: 10.1007/BF02702561

Rönnbäck, L., and Hansson, E. (1988). Are astroglial cells involved in morphine tolerance? *Neurochem. Res.* 13, 87–103.

Samineni, V. K., Grajales-Reyes, J. G., Copits, B. A., O'Brien, D. E., Trigg, S. L., Gomez, A. M., et al. (2017). Divergent modulation of nociception by glutamatergic and gabaergic neuronal subpopulations in the periaqueductal gray. *Eneuro* 4:ENEURO.0129-16.2017. doi: 10.1523/ENEURO.0129-16.2017

Sharma, S. K., Klee, W. A., and Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 72, 3092–3096. doi: 10.1073/pnas.72.8.3092

Smith, F. L., Javed, R. R., Smith, P. A., Dewey, W. L., and Gabra, B. H. (2006). PKC and PKA inhibitors reinstate morphine-induced behaviors in morphine tolerant mice. *Pharmacol. Res.* 54, 474–480. doi: 10.1016/j.phrs.2006.09.007

Song, P., and Zhao, Z.-Q. (2001). The involvement of glial cells in the development of morphine tolerance. *Neurosci. Res.* 39, 281–286. doi: 10.1016/S0168-0102(00)00226-1

Stellwagen, D. (2005). Differential Regulation of AMPA Receptor and GABA receptor trafficking by tumor necrosis factor-. *J. Neurosci.* 25, 3219–3228. doi: 10.1523/JNEUROSCI.4486-04.2005

Stiller, C. O., Bergquist, J., Beck, O., Ekman, R., and Brodin, E. (1996). Local administration of morphine decreases the extracellular level of GABA in the periaqueductal gray matter of freely moving rats. *Neurosci. Lett.* 209, 165–168. doi: 10.1016/0304-3940(96)12638-0

Thomas, J., Mustafa, S., Johnson, J., Nicotra, L., and Hutchinson, M. (2015). The relationship between opioids and immune signaling in the spinal cord. *Handb. Exp. Pharmacol.* 227, 207–238. doi: 10.1007/978-3-662-46450-2_11

Torrecilla, M., Marker, C. L., Cintora, S. C., Stoffel, M., Williams, J. T., and Wickman, K. (2002). G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons. *J. Neurosci.* 22, 4328–4334. doi: 10.1523/JNEUROSCI.22-11-04328.2002

Tortorici, V., Morgan, M. M., and Vanegas, H. (2001). Tolerance to repeated microinjection of morphine into the periaqueductal gray is associated with changes in the behavior of off- and on-cells in the rostral ventromedial medulla of rats. *Pain* 89, 237–244. doi: 10.1016/S0304-3959(00)00367-5

Vanderah, T. W., Suenaga, N. M., Ossipov, M. H., Malan, T. P., Lai, J., and Porreca, F. (2001). Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. *J. Neurosci.* 21, 279–286. doi: 10.1523/JNEUROSCI.21-01-00279.2001

Vaughan, C. W., Ingram, S. L., Connor, M. A., and Christie, M. J. (1997). How opioids inhibit GABA-mediated neurotransmission. *Nature* 390, 611–614. doi: 10.1038/37610

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., et al. (2003). Interleukin-1β enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J. Neurosci.* 23, 8692–8700. doi: 10.1523/JNEUROSCI.23-25-08692.2003

Wang, D., Zeng, J., Li, Q., Huang, J., Couture, R., and Hong, Y. (2016). Contribution of adrenomedullin to the switch of G protein-coupled μ-opioid receptors from Gi to Gs in the spinal dorsal horn following chronic morphine exposure in rats. *Br. J. Pharmacol.* 173, 1196–1207. doi: 10.1111/bph.13419

Wang, X., Loram, L. C., Ramos, K., de Jesus, A. J., Thomas, J., Cheng, K., et al. (2012). Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6325–6330. doi: 10.1073/pnas.1200130109

Watkins, L. R., Hutchinson, M. R., Rice, K. C., and Maier, S. F. (2009). The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. *Trends Pharmacol. Sci.* 30, 581–591. doi: 10.1016/j.tips.2009.08.002

Wilding, T. J., Womack, M. D., and McCleskey, E. W. (1995). Fast, local signal transduction between the mu opioid receptor and Ca2+ channels. *J. Neurosci.* 15(5 Pt 2), 4124–4132. doi: 10.1523/JNEUROSCI.15-05-04124.1995

Williams, J. T., Christie, M. J., and Manzoni, O. (2001). Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev.* 81, 299–343. doi: 10.1152/physrev.2001.81.1.299

Wimpey, T. L., and Chavkin, C. (1991). Opioids activate both an inward rectifier and a novel voltage-gated potassium conductance in the hippocampal formation. *Neuron* 6, 281–289. doi: 10.1016/0896-6273(91)90363-5

Xiao, Z., Li, Y. Y., and Sun, M. J. (2015). Activation of P2X7 receptors in the midbrain periaqueductal gray of rats facilitates morphine tolerance. *Pharmacol. Biochem. Behav.* 135, 145–153. doi: 10.1016/j.pbb.2015.06.002

Xie, J. Y., Herman, D. S., Stiller, C.-O., Gardell, L. R., Ossipov, M. H., Lai, J., et al. (2005). Cholecystokinin in the rostral ventromedial medulla mediates opioid-induced hyperalgesia and antinociceptive tolerance. *J. Neurosci.* 25, 409–416. doi: 10.1523/JNEUROSCI.4054-04.2005

Xu, J., Sun, L., Lutz, B., Bekker, A. T., and Tao, Y. (2015). Intrathecal rapamycin attenuates morphine-induced analgesic tolerance and hyperalgesia in rats with neuropathic pain. *Transl. Perioper. Pain Med.* 2, 27–34.

Xu, J.-T. T., Yaster, M., Tao, Y.-X. X., Zhao, J.-Y. Y., Zhao, X., Ligons, D., et al. (2014). Opioid receptor-triggered spinal mTORC1 activation contributes to morphine tolerance and hyperalgesia. *J. Clin. Invest* 124, 592–603. doi: 10.1172/JCI70236

Zhao, Y. L., Chen, S. R., Chen, H., and Pan, H. L. (2012). Chronic opioid potentiates presynaptic but impairs postsynaptic N-methyl-D-aspartic acid receptor activity in spinal cords: implications for opioid hyperalgesia and tolerance. *J. Biol. Chem.* 287, 25073–25085. doi: 10.1074/jbc.M112.378737

Zhou, D., Chen, M.-L., Zhang, Y.-Q., and Zhao, Z.-Q. (2010). Involvement of Spinal microglial P2X7 receptor in generation of tolerance to morphine analgesia in rats. *J. Neurosci.* 30, 8042–8047. doi: 10.1523/JNEUROSCI.5377-09.2010

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Drug Addiction: From Neuroscience to Ethics

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In the present paper, we suggest a potential new ethical analysis of addiction focusing on the relationship between aware and unaware processing in the brain. We take the case of the opioids epidemics to argue that a consideration of both aware and unaware processing provides a more comprehensive ethical framework to discuss the ethical issues raised by addiction. Finally, our hypothesis is that in addition to identified Central Nervous System's neuronal/neurochemical factors contributing to addictive dynamics, the socio-economic status plays a causal role through epigenetic processes, originating the need for additional reward in the brain. This provides a strong base for a socio-political form of responsibility for preventing and managing addiction crisis.

Keywords: addiction, ethics of addiction, unaware processing, opioids epidemics, drugs addiction

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INTRODUCTION

Even if, as recognized, among others, by the World Health Organization and by the American Psychiatric Association, addiction can be described as a chronically relapsing brain disorder which shares the same brain pathways of reward systems, there is a growing discussion about whether addiction should be understood as a brain disease/disorder or as resulting from a non-pathological brain dynamics/development (1–8). The point being whether addiction results from a pathological neurobiological disorder or rather from a brain dynamics eventually manifesting in addicted behavior. In both scenarios addiction is correlated with some changes in brain systems, particularly in networks mediating experience and anticipation of reward, perception and memory, and cognitive control (7), but the point at stake is whether such changes should be regarded as pathological or rather as brain developments caused by particular biological, psychological and environmental factors. These two alternative views result in different interventions: if addiction is a neurobiological pathology medication is the only way to treat it; if addiction is a non-pathological brain development, then changing the factors causing it would restore a non-addicted brain state. We acknowledge the controversy around the definition of addiction as a disease, as well as, its potential impacts on different levels (from diagnosis to prognosis, from ethics to policy definition). Yet, as we will see in details in the following, we think that new scientific perspectives on brain development and on consciousness/non-conscious processing relationship offer the possibility of conceptualizing addiction beyond a dualistic interpretation of disease and dynamical models.

Beyond the abovementioned scientific controversy, addiction has emerged as one of the most compelling emergencies of contemporary society. Many factors contribute to making addiction a complex and multifaceted issue, the analysis of which requires the contribution of different fields.

Several scientific (e.g., from neuroscience to medicine) and social analyses of addiction have been produced in recent years (4, 9). However, the ethical discussion seems to be more limited and it is mainly focused on normative and practical issues (10, 11), i.e., on the regulatory and practical questions related to the off-label abuse of opioid medication. An ethical analysis of the

factors leading to addictive behaviors and, specifically, of the responsibility for such behaviors seems lacking. This paper aims to contribute to this kind of ethical analysis on the basis of neuroscientific data about information processing in the brain. In particular, we focus on the role of external influences on unaware processing (also referred to as unconscious, preconscious, or non-conscious processing) and its driving role in addictive behavior.

We will argue that in addition to the central nervous system's neuronal/neurochemical bases of addiction, particularly to opiate crisis, socio-economic status modulates through non-conscious processing what can be described as the person's subjective "global well-being," raising the need for additional rewards in the brain. In the light of the impact of external factors, we argue that some people are particularly vulnerable to a sort of alienation by the political-socio-economical capitalistic system, and that this stressful condition, which has both aware and unaware components, is one of the main causes of addiction.

In fact, different factors contribute to define the complexity of addictive behavior, which includes both physiological/pharmacological and psychological/social components. As a result, the subject acts as conditioned by both aware and unaware drives. Aware and unaware levels, their respective interaction, and the impact of external factors should be taken into account when attempting to provide a more adequate ethical analysis of addiction.

The case of opioid epidemics is highly illustrative in this respect. It clearly shows how addiction is affected by both internal and external factors, e.g., physiological, psychological, and environmental mostly social.

A CONTEMPORARY EPIDEMICS

Opioids Abuse Data

Data shows that opioids abuse has increased significantly in the last decades just in the US (9, 12): the rate of opioid addiction affected about 2.5 million adults in 2014, while in 2016 91.8 million of US civilian used prescription opioids, and 11.5 million misused them (13).

Media coverage of the opioids addiction has been growing in recent years. In the last few years, *Time* magazine devoted two covers to the abuse of opioids painkillers [June 2015 (14) and March 2018 (15)]. Yet many other communication media reserve significant attention to the phenomenon almost every day: just to give some recent examples, *CNN* website recently published the data of the fast increase of opioid crisis (16); *The Washington Post* published a tough story of life devastated by opioids (17); and *The New York Times* described the stories of the children of addicted mothers (18).

Increases in opioids abuse are related to increases in therapeutic opioids prescription (12). The main claimed reason for those prescriptions is chronic pain, whose prevalence among adult Americans is between 30 and 40% (19). Opioid medication is now the most prescribed medication in the US (20).

As recently outlined by Volkow and McLellan, two facts seem indubitable: first, opioid analgesics are widely distributed and improperly used, resulting in a high number of overdose deaths and addictions; second, the main source of opioids distribution is physicians' prescriptions (9, 21, 22).

These two facts raise important ethical issues that should be addressed. The main issue is the responsibility, i.e., the original cause, for such a widespread improper use of opioids that many people define as an epidemic. What are the real causes leading to an improper use of opioids?

The Legal Use of Opioids as Pain Killers

Different causes can be related to the actual massive use of opioid analgesics. One of the main factors is the perception of pain as a negative experience to be cured and eventually eliminated. More specifically, the Joint Commission on the Accreditation of Healthcare Organizations (JCAHO), incorrectly assuming that clinical use of opioids rarely generates addiction, reported that effective narcotic analgesics were wrongly *not* used in US because of an irrational fear of addiction (23). A strong focus on pain management by opioids ensued, accompanied by pharmaceutical companies marketing.

The pro-opioids for pain management movement gained strength from this report and had a large impact on the public, contributing to subtly change the perception of the medical use of opioids. As a consequence, the prescription of opioid analgesics increased considerably (24). The problem is that the number of non-prescribed, diverted use of opioids seems to be proportionally related to the number of prescriptions (12); the transition from medication to addiction being something subtle and not consciously perceived by the affected subject (25). Addiction starts as an unaware process: only in subsequent stages does the subject become aware of her/his addiction and takes the drug knowing it is a drug.

An important reason for the continued use of opioids is that they are prescribed by physicians, hence perceived as either less or not dangerous at all (26). This fact points not just to the physicians' responsibility (both as causal role and accountability) but also shows the influence of implicit biases on the resulting addiction behaviors. In general, the doctor is implicitly seen as an ethically normative actor, someone who clearly makes the difference between licit and illicit behaviors (27). Moreover, because opioids are legally prescribed as painkillers there is a tendency to regard them as safer drugs (28). The impact of these implicit biases on the final addiction behavior raises the issue of socio-political responsibility: legally allowing the prescription of opioids medication is not neutral with regard to the perception of its risks. In fact, we think that, in the light of both aware and unaware influences that can be exercised on the final users, legally allowing the prescription and subsequent use of opioids risks to be a way to endorse them.

Among the personal reasons to continue opioids use, the most important seems to be their role as a response to life stressors or as a means of self-medicating psychological issues, effects of trauma, or emotional pain. Other, minor reasons include normalization (e.g., to not feel uncomfortable), increased energy, boredom, enhanced sexual intimacy, self-blame/addictive personality (12). Co-morbidity of opioids addiction with underlying psychiatric disorders is quite high in prescribed opioid addicted subjects. The risk of addiction has also genetic and developmental roots:

in particular adolescents are more prone to develop addiction (29, 30).

The above suggests that addiction has a strong basis in the brain on different levels of consciousness, aware, as well as, unaware. For this reason we think that an ethical analysis of addiction purporting to highlight the reasons behind it must include a specific focus also on the lower levels of consciousness, not connected to awareness.

THE PHARMACOLOGICAL IMPACT OF ADDICTION ON AWARENESS

While different theoretical models of conscious processing have been elaborated on the basis of cognitive neuroscience [e.g., the Global Neuronal Workspace (31, 32)], little attention has been dedicated to the pharmacology of conscious experience (33). There is evidence that self-awareness, an important component of conscious experience, is determined by a paralimbic circuitry of γ synchrony regulated by GABAergic interneurons under the control of acetylcholine and dopamine. Accordingly, specific chemical agents and their respective balance modulate awareness.

Specifically, self-awareness has been shown to be linked to hemodynamic activity in a medial paralimbic circuitry involving medial prefrontal/anterior cingulate, medial parietal/posterior cingulate, and subcortical regions, associated with the lateral parietal cortex, typically the angular gyri, and insula (33). On the basis of experiments with transcranial magnetic stimulation (TMS), Changeux and Lou conclude that the paralimbic circuitry plays a causal role with regard to self-awareness. Interestingly γ synchronization in the paralimbic circuitry regions increases proportionally with self-processing. The conclusion by Changeux and Lou is that the paralimbic synchronization enables unity of consciousness through coherence of serial conscious experiences (e.g., self-control) by acting as their common neural path (33). In other words, γ synchronization of paralimbic regions plays a crucial role in self-awareness and self-control. γ synchronization is regulated by GABAergic interneurons, which are affected in particular by two neurotransmitters: acetylcholine and dopamine.

Behaviorally, addiction may be described as the result of the loss of or the serious impairment of self-control, decision-making, and emotion processing by the subject, where an initially voluntary substance use or behavior gradually becomes compulsive (33, 34). More specifically, three stages of addiction have been identified (2): preoccupation/anticipation; binge/intoxication; withdrawal/negative effects. Importantly, these three stages feed into each other reinforcing an addiction cycle.

At the neurophysiological and neurobiological levels, addiction causes the impairment of the paralimbic circuitry that we have seen to be critical for self-awareness and self-control. Consequently, addiction results in a pharmacological disorder or chemical impairment of conscious self-control and self-regulation through the impairment of paralimbic medial circuitry normal function (33, 35). The abovementioned three stages are correlated with changes occurring in three brain systems related to particular functions: preoccupation/anticipation is correlated with changes in the prefrontal cortex, which underlies executive function; binge/intoxication is correlated with changes in the basal ganglia, which underlies incentive salience; and withdrawal/negative effect is correlated with extended amygdala, which underlies negative effect withdrawal (2).

In short, in cases of addiction there are important changes in the brain reward and stress mechanisms, underlying the passage from impulsive to compulsive behavior and from positive to negative reinforcement. Importantly, addiction affects, in particular, the dopaminergic system, which we have seen plays an important role in modulating self-awareness and self-control. In the end, addiction causes a pharmacological disconnection from top-down GNW processing, i.e., a moving from self-awareness to unchecked goal-directed actions (36).

In other words, addiction causes the disruption of the chemical balance critical for self-awareness and self-control, causing a vicious circle for which the dependence from the substance constantly increases (33).

ETHICS OF ADDICTION

In addition to the neurochemical bases of consciousness and of the addiction's impact on it described above, socio-economic and ecological contexts play an important role in addiction insofar as they have a significant impact on the brain aware and unaware processes. The connection between the brain and its living contexts gives us new tools for detecting ethical responsibility for addiction.

An important scientific theory for exploring the connection between brain and external world is the epigenetic theory of neuronal development, which promises to help us illustrating addiction dynamics as well.

Synaptic Epigenesis and the Internalization of the Socio-Cultural Environment During Development

As recently summarized elsewhere (37), recent advances in neuronal epigenesis studies reveal a deep relationship between the brain and its environment, including social and cultural contexts (38). There is evidence that because of this interaction, an active epigenetic selection of neuronal networks results in the internalization of the cultural and ethical rules prevalent in the social community to which the child and her/his family belongs (39). Arguably, this internalization is mostly implemented at the unaware level and importantly contributes to shaping the brain's architecture on all levels of conscious processing.

The epigenetic theory of neuronal development together with other studies about the intrinsic predisposition of the brain to interact with the world (40) suggest a reciprocal causality between the brain and its external environments, and a mutual epistemic relevance in understanding the two realms (biological vs. socio-cultural) (37). Specifically, understanding the brain requires reference to the experiences and social structures that shape it, and knowledge of the brain is also relevant to understanding the development of those social structures (41, 42).

From the above we can infer that the brain is not a closed, self-referential information device or a simple input-output machine, but rather a plastic and interacting organ shaped by a panoply of factors, including biological, experiential, and social causes.

The Quality of Life and General Welfare

One of the reasons for addictive opioids consumption is likely the lack of well-being understood in its widest sense, that is including both psychological and physical components. As is characteristic of any addictive behavior, an initial need for treating an undesirable condition (e.g., physical pain) is subsequently replaced by the urge to "feel high" and then the need to oppose the withdrawal symptoms. Significantly, the transition from using opioids as painkillers to using them addictively takes place without the subject being aware of it: it seems like the user loses control of what s/he intakes and of the true reasons why she/he intakes it (25).

The critical component in this addiction dynamics is how "being well" is conceived and consequently exposed to external influences. As seen above, recent evidence from neuroscience depicts the brain as a cognitive and emotional, spontaneously active organ, which is shaped and modulated by the interaction with the environment (43). Its cognitive and emotional actions are not limited to the aware level, nor do they result from internal factors only: environmental influences on the development of the cognitive and emotional brain, at both aware and unaware levels, are massive and even critical.

This suggests the view of "being well" as a multilevel and multidimensional condition: well-being can generally be perceived at both unaware and aware levels, and it results from different factors, both internal and external to the subject (e.g., bodily components and environmental influences). Among the factors impacting on brain development are the influences on subjective well-being coming from the socio-cultural environment, including political, cultural, and educational contexts: the information coming from these sources are internalized by the subject and contribute to shaping his personal aware and unaware well-being. The relevance of external factors in shaping individual actions raises the issue of social responsibility, if not in ethical terms, at least in terms of public policy.

It is significant that the opioids addiction described above primarily if not exclusively affects advanced industrial countries, and in particular the US. Opioids consumption is not new in society: for instance, they were abundantly used in ancient societies. Anyway, even if always questionable, the use of drugs usually had a different socio-political meaning and function, and they were almost systematically used in social and religious rituals under a very stringent control, for instance by shamans. Today, the reason, why to use drugs is different: coping with life stressors and looking for well-being seems to be among the main reasons leading to addictive opioids consumption. Such addiction is prevalently affecting advanced industrial countries, so that it is reasonable to infer that these societies may be affected by a general feeling of dissatisfaction, which emerges as a psychological, social, political, and ethical issue. Even further, such dissatisfaction might be at the root of the search for enhancement, including brain enhancement, which has been one of the priorities of US neuroscience research, e.g., in military research (see https://www.darpa.mil/program/targeted-neuroplasticity-training).

This is comparable with the general tendency lately spread in Europe and also the US to think about the need to go over present humans, like in the transhumanist philosophy, which aims at purifying humankind from its poor present state (44), finally emerging as a sort of secular eschatology. A main issue with important ethical implications raised by this view is the definition of the quality of life standards from which we can infer whether our status is good or bad. The internalization of such standards, which result from several external sources and subsequently affect how the subject consciously thinks and acts, is arguably happening mainly below the level of awareness.

Finally, we think that the abovementioned feeling of dissatisfaction has its roots in the value system of Western societies, dominated by a capitalist worldview according to which personal success is measured by economic and financial success. The Western value system grounded on competition vs. cooperation is arguably one of the causes of the life condition leading to addictive consumption of drugs. It is a remarkable phenomenon that the recent dramatic increase of opiates overdose casualties closely follows that of income inequality in the US (45). Again, from an ethical point of view, an alternative to this value system is possible, for instance in stressing the priority of cooperation over competition, and community and esthetic pleasure as a social value (46).

The Aware Feeling of Pain

The original reason why opioids medication was massively introduced in the healthcare system was the need to manage and treat pain, seen as something to be eliminated. This raises the question of the definition of pain. This is a scientific issue with important ethical implications. In fact, we can generally describe pain as an evolutionary warning system, a sort of safety device making subjects consciously aware of a danger without necessarily being aware of the causes of the pain. Accordingly, pain acts as a homeostatic behavioral regulator: it is both an emotion (i.e., interoceptive knowledge of physiological condition) and a behavioral motivation originating from the need to maintain homeostasis (47–49). Homeostasis can be described as a dynamic and ongoing process maintaining an optimal balance in the physiological condition of the body for its survival (50).

If so, from an evolutionary point of view pain is not a negative but rather a necessary phenomenon. It is reasonable to think that without pain the chances of survival of humankind, and of any animal species, would be much lower. The inherited condition known as congenital insensitivity to pain confirms the necessity of pain for surviving: people affected by such disorder frequently die prematurely due to complications of trauma and injuries (51).

The issue to address, then, is whether and to what extent it is worth to manage pain simply by silencing or abolishing it. Completely suppressing pain would mean eliminating a system of physiological feedback regulation between the subject and the outside world. In other words, the capacity for experiencing

pain is necessary for the survival of living organisms. The individual fitness would be consequently affected if pain was simply removed. The reason is that both positive and negative rewards are necessary for an appropriate evaluation of the external world.

This is not to say that all pain is "valuable," or that not being able tolerating pain is unjustifiable or in any way worthy of stigmatization. There may be conditions of pain that the subject cannot—and should not be requested to—endure [which they are is open to controversy, e.g., particularly debilitating chronic pain, or end-of-life conditions. See for instance the Final Report by the President's Commission on Combating Drug Addiction and the Opioid Drugs (52)]. In these cases, the use of painkillers, including exceptionally opioids, would indeed be both medically and ethically justified. Our argument directs itself exclusively against the use of opioids, where this is not—or not demonstrably—the case, recognizing that the drawing of this limit can be a difficult challenge. Moreover, we stress also the ethical need to enhance the search for alternative, non-addictive painkillers, including non-addictive opioids like recently suggested by Severino et al. (53).

The Responsibility of Drug Companies and Medical Doctors

The opioids epidemics was partly initiated by the pharmaceutical companies which developed a very potent opiate analgesic without warning against—or at worst even denying—the risk of addiction. A pro-opioids campaign was initiated on the basis of the erroneous assumption that the use of this medication was free of any risk of addiction (23). These companies put great social pressure on medical doctors and this massively affected both public opinion and professional standards. The influence that pharmaceutical companies have on society and how they impact public opinion and professional choices is ethically problematic and requires a specific analysis.

First, the reasons behind such influence should be scrutinized: what is the aim of drug companies' battle in favor of opioids medication? Is it patients' interest or rather their own (economic) interest, or a combination of both?

The behavior of medical doctors should also be critically assessed. If even when they know about the risk of addiction physicians still choose to prescribe opioids, such choice may be ethically problematic. It is true that it is not easy for the doctor to choose how to best help the patient and maybe the sole cost-benefit analysis is insufficient *per se* to solve the dilemma. The risk of addiction may be outweighed by the benefit of pain-relief, but this is a difficult medical and psychological evaluation that must be done with great care, not least since there may be quite large differences between different individuals concerning the best possible treatment. Moreover, if the mere fact that a medical doctor prescribes a medication makes lay people believe that such medication is ethically unproblematic, this means that what the doctor says is not neutral but has important consequences both in terms of opinions and in terms of action, mainly influencing them at the unaware level. This fact is ethically significant.

An ethical warning informed by the scientific data about aware and unaware brain processes should be part of both drug companies' policies and medical doctors' professional skills, and relevant tools should be implemented to increase understanding of these topics.

A Look Forward

The discussion above suggests that a number of considerations should be taken into account in the search for a feasible and effective strategy to manage addiction.

In the first place, any attempt to cope with addiction should start from the relevant scientific knowledge, particularly from the neuroscience of the involved aware and unaware processes. In fact, the dynamics of addiction includes both aware and unaware components: as illustrated by the case of opioids, at the beginning the subject consciously chooses to take the drug to alleviate a negative experience (e.g., pain). This (apparently) fully aware decision is partly affected by unaware dynamics that are beyond direct subjective control. Whether undergoing pain is taken to be a negative experience and what amount of pain can be tolerated depend in part on external information (e.g., from professional organizations, educational actors, social media) that eventually becomes interiorized and affects subjective behavior at the unaware level. Thus, when the subject asks the doctor for an opioid prescription, and she/he consciously starts to take the medication, her/his behavior is already subtly conditioned and eventually guided by both aware and unaware drives until an addictive use of the substance is established. As emerges from first person accounts (12), initially addicted subject has no knowledge of being addicted, she/he is not aware. The realization of addiction comes only at a later stage, when she/he continues to take the drug knowing that it is an addiction and unable to stop using it because of withdrawal and other negative symptoms.

In the end, it is necessary to be aware of this continued oscillation between aware and unaware drives, which denote different psychological, neurological, and pharmacological processes in the brain. Since neuroscience is providing increasing knowledge of these processes, management strategies should consider both the aware and unaware brain. Of course, such strategies can be implemented in different ways, e.g., through a direct pharmacological approach or through an indirect approach aiming at influencing the brain by altering external environmental conditions, including cultural and social institutions. In particular, considering that brain development is particularly sensible to external inputs for about 20 years after birth (43), the experiences during this period of time, especially familiar and educational conditions, play a crucial role in exposing the subject to the risk of addiction.

CONCLUSION

An ethical framework for a balanced analysis of addiction should take into account emerging neuroscientific data about aware and unaware processes involved. In order to clarify the ethical responsibility of the final user, of the medical doctors, and of the pharmaceutical companies, and to suggest a strategy for an ethically sound management of addiction it is necessary to include different levels of conscious processing in the brain, not only awareness, and to outline the critical role they play

in addiction behavior, the extent to which external influences shape it, and the possibility to take care of it. Thus, we suggest that an ethics of addiction (i.e., an ethically sound treatment of addiction) importantly requires taking due care of the brain also below the levels of awareness.

In short, our argument rests on the following:

1 Medical: Addiction causes the disruption of the chemical balance critical for self-awareness and self-control, resulting in a pharmacological impairment of awareness and causing a vicious circle for which the dependence from the substance constantly increases.
2 Scientific: Non-conscious brain processes are massively influenced by socio-economic and ecological factors.
3 Psychological: Addiction is mainly dependent from non-conscious brain processes, i.e., from loss of conscious control.
4 Ethical: Given the scientific and psychological factors mentioned, the socio-economic and ecological contexts are highly relevant to addictive dynamics, especially through the influence they have on unaware brain processes.

Finally, our hypothesis is that in addition to identified Central Nervous System's neuronal/neurochemical factors contributing to addictive dynamics, the socio-economic status plays a causal role through epigenetic processes, originating the need for additional reward in the brain. For this reason, we consider addiction to be, *in addition* to a medical and mental disorder, *also* a social disorder.

This provides a strong base for a socio-political form of responsibility for preventing and managing addiction crisis.

AUTHOR CONTRIBUTIONS

MF wrote the manuscript and was responsible for general ideas. J-PC contributed to revising and developing ideas. J-PC and KE commented on previous versions of the manuscript and helped in developing lines of argument.

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REFERENCES

1. Thibaut F, Hoehe M. Addictive behaviors: where do we stand, and where are we going? *Dialogues Clin Neurosci*. (2017) 19:215.
2. George O, Koob GF. Individual differences in the neuropsychopathology of addiction. *Dialogues Clin Neurosci*. (2017) 19:217–29.
3. Leshner AI. Addiction is a brain disease, and it matters. *Science* (1997) 278:45–7.
4. Volkow ND, Koob GF, McLellan AT. Neurobiologic advances from the brain disease model of addiction. *N Engl J Med*. (2016) 374:363–71. doi: 10.1056/NEJMra1511480
5. Lewis MD. *The Biology of Desire: Why Addiction Is Not a Disease*. 1st ed. New York, NY: PublicAffairs (2015). p. 238.
6. Hall W, Carter A, Forlini C. The brain disease model of addiction: is it supported by the evidence and has it delivered on its promises? *Lancet Psychiatry* (2015) 2:105–10. doi: 10.1016/S2215-0366(14)00126-6
7. Lewis M. Addiction and the brain: development, not disease. *Neuroethics* (2017) 10:7–18. doi: 10.1007/s12152-016-9293-4
8. Satel S, Lilienfeld SO. Addiction and the brain-disease fallacy. *Front Psychiatry* (2013) 4:141. doi: 10.3389/fpsyt.2013.00141
9. Volkow ND, McLellan AT. Opioid abuse in chronic pain–misconceptions and mitigation strategies. *N Engl J Med*. (2016) 374:1253–63. doi: 10.1056/NEJMra1507771
10. Carter A, Hall W, Illes J. *Addiction Neuroethics : The Ethics of Addiction Neuroscience Research and Treatment*. 1st ed. London: Academic Press (2012). p. 320.
11. Carter A, Hall WD. What is addiction neuroethics? In: Clausen J, Levy N, editors. *Handbook of Neuroethics*. Dordrecht: Springer Science+Business Media (2015). p. 995–8.
12. Cicero TJ, Ellis MS. The prescription opioid epidemic: a review of qualitative studies on the progression from initial use to abuse. *Dialogues Clin Neurosci*. (2017) 19:259–69.
13. Substance, Administration AaMHS. *Results From the 2016 National Survey on Drug Use and Health: Detailed Tables* (2017) Available online at: https://www.samhsa.gov/data/sites/default/files/NSDUH-DetTabs-2016/NSDUH-DetTabs-2016.pdf
14. Calabresi M. Why America Can't Kick Its Painkiller Problem. *Time*. (2015, 5 June).
15. The Opioid Diaries. *Time* (2018, 5 March).
16. CNN. *Opioid Crisis Fast Facts* (2018). Available online at: https://edition.cnn.com/2017/09/18/health/opioid-crisis-fast-facts/index.html
17. McCoy T. *Unintended Consequences. Inside the Fallout of the America's Crackdown on Opioids* (2018). Available online at: https://www.washingtonpost.com/graphics/2018/local/impact-of-americas-opioid-crackdown/?utm_term=0.818701c0d42b
18. Egan J. *Children of the Opioid Epidemic* (2018). Available online at: https://www.nytimes.com/2018/05/09/magazine/children-of-the-opioid-epidemic.html
19. Johannes CB, Le TK, Zhou X, Johnston JA, Dworkin RH. The prevalence of chronic pain in United States adults: results of an Internet-based survey. *J Pain* (2010) 11:1230–9. doi: 10.1016/j.jpain.2010.07.002
20. Prevention CfDCa. *FastStats. Therapeutic Drug Use* (2014). Available online at: http://www.cdc.gov/nchs/fastats/drug-use-therapeutic.htm
21. Compton WM, Boyle M, Wargo E. Prescription opioid abuse: problems and responses. *Prev Med*. (2015) 80:5–9. doi: 10.1016/j.ypmed.2015.04.003
22. Shei A, Rice JB, Kirson NY, Bodnar K, Birnbaum HG, Holly P, et al. Sources of prescription opioids among diagnosed opioid abusers. *Curr Med Res Opin*. (2015) 31:779–84. doi: 10.1185/03007995.2015.1016607
23. Phillips DM. JCAHO pain management standards are unveiled. Joint Commission on Accreditation of Healthcare Organizations. *JAMA* (2000) 284:428–9. doi: 10.1001/jama.284.4.423b
24. Control CfD. Vital signs: overdoses of prescription opioid pain relievers—United States, 1999–2008. *MMWR Morb Mortal Wkly Rep*. (2011) 60:1487–92.
25. Back SE, Lawson KM, Singleton LM, Brady KT. Characteristics and correlates of men and women with prescription opioid dependence. *Addict Behav*. (2011) 36:829–34. doi: 10.1016/j.addbeh.2011.03.013
26. Daniulaityte R, Falck R, Carlson RG. "I'm not afraid of those ones just 'cause they've been prescribed": perceptions of risk among illicit users of pharmaceutical opioids. *Int J Drug Policy* (2012) 23:374–84. doi: 10.1016/j.drugpo.2012.01.012

27. Rigg KK, Murphy JW. Understanding the etiology of prescription opioid abuse: implications for prevention and treatment. *Qual Health Res*. (2013) 23:963–75. doi: 10.1177/1049732313488837
28. Inciardi JA, Surratt HL, Cicero TJ, Beard RA. Prescription opioid abuse and diversion in an urban community: the results of an ultrarapid assessment. *Pain Med*. (2009) 10:537–48. doi: 10.1111/j.1526-4637.2009.00603.x
29. Chambers RA, Taylor JR, Potenza MN. Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am J Psychiatry* (2003) 160:1041–52. doi: 10.1176/appi.ajp.160.6.1041
30. Mistry CJ, Bawor M, Desai D, Marsh DC, Samaan Z. Genetics of opioid dependence: a review of the genetic contribution to opioid dependence. *Curr Psychiatry Rev*. (2014) 10:156–67. doi: 10.2174/1573400510666140320000928
31. Dehaene S, Sergent C, Changeux JP. A neuronal network model linking subjective reports and objective physiological data during conscious perception. *Proc Natl Acad Sci USA*. (2003) 100:8520–5. doi: 10.1073/pnas.1332574100
32. Dehaene S, Changeux JP. Experimental and theoretical approaches to conscious processing. *Neuron* (2011) 70:200–27. doi: 10.1016/j.neuron.2011.03.018
33. Changeux JP, Lou HC. Emergent pharmacology of conscious experience: new perspectives in substance addiction. *FASEB J*. (2011) 25:2098–108. doi: 10.1096/fj.11-0702ufm
34. Verdejo-Garcia A, Bechara A, Recknor EC, Perez-Garcia M. Negative emotion-driven impulsivity predicts substance dependence problems. *Drug Alcohol Depend*. (2007) 91:213–9. doi: 10.1016/j.drugalcdep.2007.05.025
35. Romer Thomsen K, Joensson M, Lou HC, Moller A, Gross J, Kringelbach ML, et al. Altered paralimbic interaction in behavioral addiction. *Proc Natl Acad Sci USA*. (2013) 110:4744–9. doi: 10.1073/pnas.1302374110
36. Changeux JP. Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. *Nat Rev Neurosci*. (2010) 11:389–401. doi: 10.1038/nrn2849
37. Farisco M, Salles A, Evers K. Neuroethics: a Conceptual Approach. *Camb Q Healthc Ethics* (2018) 27:717–27. doi: 10.1017/S0963180118000208.
38. Changeux JP, Courrège P, Danchin A. A theory of the epigenesis of neuronal networks by selective stabilization of synapses. *Proc Natl Acad Sci USA*. (1973) 70:2974–8.
39. Evers K. Can we be epigenetically proactive? In: Metzinger TWJ, editor. *Open Mind: Philosophy and the Mind Sciences in the 21st Century*. Cambridge, MA: MIT Press (2015), 497-518.
40. Changeux JP. *The Physiology of Truth*. Cambridge, MA: Belknap Press (2004).
41. Salles A, Evers, K. Social neuroscience and neuroethics: a fruitful synergy. In: Ibanez A, Sedeno L, Garcia A, editors. *Social Neuroscience and Social Science: The Missing Link*. Cham: Springer International Publishing (2017). p. 531–46.
42. Evers K, Changeux JP. Proactive epigenesis and ethical innovation: a neuronal hypothesis for the genesis of ethical rules. *EMBO Rep*. (2016) 17:1361–4. doi: 10.15252/embr.201642783
43. Changeux J-P. *The Physiology of Truth : Neuroscience and Human Knowledg*e. Cambridge, MA: Belknap Press of Harvard University Press (2004) p. 324.
44. More M, Vita-More N. *The Transhumanist Reader : Classical and Contemporary Essays on the Science, Technology, and Philosophy of the Human Future*. Chichester: Wiley-Blackwell (2013). p. 460.
45. Piketty T, Saez E, Zucman G. Distributional national accounts: methods and estimates for the United States. *Q J Econ*. (2018) 133:553–609. doi: 10.1093/qje/qjx043
46. O'Brien JE. *Critique of Rationality. Judgement and Creativity from Benjamin to Merleau-Ponty*. Leiden; Boston, MA: Brill (2016).
47. Craig AD. A new view of pain as a homeostatic emotion. *Trends Neurosci*. (2003) 26:303–7. doi: 10.1016/S0166-2236(03)00123-1
48. Craig AD. How do you feel? Interoception: the sense of the physiological condition of the body. *Nat Rev Neurosci*. (2002) 3:655–66. doi: 10.1038/nrn894
49. Bonavita V, De Simone R. Pain as an evolutionary necessity. *Neurol Sci*. (2011) 32(Suppl. 1):S61–6. doi: 10.1007/s10072-011-0539-y
50. Cannon WB. *The Wisdom of the Body*. New York, NY: W.W. Norton & Company (1939). p. 19–333.
51. Nagasako EM, Oaklander AL, Dworkin RH. Congenital insensitivity to pain: an update. *Pain* (2003) 101:213–9. doi: 10.1016/S0304-3959(02)00482-7
52. President's, Drugs CoCDAatO. *Final Report* (2017). Available online at: https://www.whitehouse.gov/sites/whitehouse.gov/files/images/Final_Report_Draft_11-1-2017.pdf
53. Severino AL, Shadfar A, Hakimian JK, Crane O, Singh G, Heinzerling K, et al. Pain Therapy guided by purpose and perspective in light of the opioid epidemic. *Front Psychiatry* (2018) 9:119. doi: 10.3389/fpsyt.2018.00119

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Heteromerization Modulates mu Opioid Receptor Functional Properties *in vivo*

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Mu opioid receptors modulate a large number of physiological functions. They are in particular involved in the control of pain perception and reward properties. They are also the primary molecular target of opioid drugs and mediate their beneficial analgesic effects, euphoric properties as well as negative side effects such as tolerance and physical dependence. Importantly, mu opioid receptors can physically associate with another receptor to form a novel entity called heteromer that exhibits specific ligand binding, signaling, and trafficking properties. As reviewed here, *in vivo* physical proximity has now been evidenced for several receptor pairs, subsequent impact of heteromerization on native mu opioid receptor signaling and trafficking identified and a link to behavioral changes established. Selective targeting of heteromers as a tool to modulate mu opioid receptor activity is therefore attracting growing interest and raises hopes for innovative therapeutic strategies.

Keywords: mu opioid receptor, heteromer, GPCR, delta opioid receptor, morphine, hypertension, addiction, pain

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INTRODUCTION

The mu opioid (mu) receptor is a G protein-coupled receptor (GPCR) that neuromodulates several physiological functions, in particular nociception (Kieffer and Evans, 2009). This receptor also mediates the reinforcing properties of natural stimuli. In addition, mu receptors are the primary molecular target of opioid drugs used in the clinic (e.g., morphine, codeine, oxycodone, fentanyl, tramadol), and are responsible for their analgesic properties but also for the side effects associated with their acute (e.g., respiratory depression, nausea, dizziness, sedation, constipation) (Kieffer, 1999) and chronic use (tolerance, hyperalgesia, and physical dependence) (Matthes et al., 1996; Williams et al., 2013). Moreover, mu receptors mediate opioid rewarding and euphoric properties that underlie their abuse potential (Matthes et al., 1996). The latter is at the root of the epidemic that has developed in North America upon misuse and/or abuse of prescription opioid drugs after an initial therapeutic use or in patients that self-medicate (Vowles et al., 2015). It underscores the need for designing effective opioid analgesics devoid of side effects and has prompted considerable efforts to better understand the molecular and cellular mechanisms underlying mu receptor activity. In this context, functional consequences elicited by physical association of the mu receptor with another GPCR attracted attention. Here, we review evidence of molecular, cellular, and behavioral modulation induced by mu receptor heteromerization in native tissue.

MU RECEPTOR HETEROMERS IN NATIVE TISSUE

Heteromers involving the mu receptor have been extensively studied in heterologous systems (Fujita et al., 2014a). Receptor physical proximity has now been established in native tissue for several receptor pairs using receptor co-immunoprecipitation, co-localization by electron microscopy or *in situ* proximity ligation assay (PLA), and/or disruption of physical contact by an interfering peptide. The use of interfering peptide and/or mice deficient for one receptor also significantly contributed to demonstrate the specificity of the functional changes associated with heteromer formation and to establish a causal link with behavioral outputs. In native tissue, the mu receptor heteromerizes with the delta opioid (delta) or kappa opioid (kappa) receptors or with the non-opioid receptors ORL1, cannabinoid CB_1, galanin Gal1, adrenergic α_{2a}, somatostatin sst_2, dopamine D_1, chemokine CCR5, and vasopressin V_{1b}. Association between the mu receptor splice variant MOR_{1D} and the gastrin-releasing peptide receptor (GRPR) has also been reported as well as mu physical proximity with the ion channel NMDA (**Table 1**). Heteromerization with a $G\alpha_{i/o}$ coupled GPCR is thus the most frequently reported to date but association with the $G\alpha_q$ coupled GRPR and vasopressin V_{1B} receptors or the $G\alpha_s$ coupled dopamine D_1 receptor indicates no specific requirement. Additional heteromers likely exist *in vivo* since mu receptor heteromerizes with serotonin $5HT_{1a}$ (Cussac et al., 2012), neuropeptide FF $NPFF_2$ (Roumy et al., 2007), melanocortin MC3 (Rediger et al., 2009), neurokinin NK1 (Pfeiffer et al., 2003), and possibly, dopamine D4 (Qian et al., 2018) receptors in co-transfected cells, and neuronal co-localization with chemokine CXCR4 (Patel et al., 2006; Heinisch et al., 2011), metabotropic glutamate mGluR5 (Schröder et al., 2009) and serotonin $5HT_{2a}$ (Lopez-Gimenez et al., 2008), and dopamine D_4 (Rivera et al., 2017) receptors has been reported (see also http://www.gpcr-hetnet.com for further information on the GPCR interaction network, and interacting or non-interacting receptor pairs; Borroto-Escuela et al., 2014).

Expression of native heteromers is dynamic. Chronic morphine treatment enhances mu-delta heteromer density in brain regions associated with the reward pathway (Gupta et al., 2010). Concomitant increase in delta receptor localization at the cell surface is observed and is mu receptor dependent (Gendron et al., 2015; Ong et al., 2015; Erbs et al., 2016). Heteromers form intracellularly in native tissue. In the mouse dorsal root ganglia (DRG), mu and delta opioid receptors associate in the endoplasmic reticulum (ER), which requires phosphorylation of the delta receptor at threonine 161 by the cdk5 kinase (Walwyn et al., 2009; Xie et al., 2009). Mu-delta density could also be affected in other pathological conditions enhancing delta receptor presence at the cell surface such as inflammatory pain conditions (Cahill et al., 2003) or voluntary alcohol consumption (van Rijn et al., 2012). In addition, expression of mu-α_{2a} heteromers in the nucleus of the solitary tract (NTS) is dynamically regulated and increased in hypertensive rats (Sun et al., 2015). In human peripheral blood mononuclear cells (PBMC), the mu agonist DAMGO induced CCR5 receptor synthesis through a TGFβ1 dependent mechanism (Happel et al., 2008), suggesting a role for mu-CCR5 heteromers in HIV1 entry in opiate abusers.

MODULATION OF G PROTEIN SIGNALING IN NATIVE MU HETEROMERS

In SK-N-SH neuroblastoma cells co-expressing mu and delta receptors, occupancy of the binding site of one receptor by a non-signaling concentration of ligand increased binding and Gα signaling of the other receptor (Gomes et al., 2000, 2004, 2011). The nature of the first ligand did not seem important since agonist, antagonist or inverse agonist induced similar effects. Data therefore suggest that mu-delta heteromerization induces cross-allosteric modulation with a positive cooperativity promoted upon binding of the first ligand (**Figure 1**). Accordingly, co-application of the delta antagonist TIPPψ and mu agonist DAMGO or co-application of the mu antagonist CTAP and delta agonists deltorphin II or DPDPE increased hyperpolarization in a subset of neurons in the ventral tegmental area (VTA) (Margolis et al., 2017). Similarly, co-injection of subthreshold doses of the mu agonist DAMGO and the delta agonist deltorphin II in the rostral ventromedial medulla (RVM) of rats chronically treated with morphine increased γ aminobutyric acid (GABA)ergic inhibition through synergistic activation of the phospholipase A2 and cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) dependent pathways (Zhang and Pan, 2010). Moreover, mu-delta preferential coupling to the pertussis toxin insensitive Gαz subunit would not be desensitized by chronic morphine administration in the rat striatum and hippocampus (George et al., 2000; Kabli et al., 2014). Altogether, mu-delta positive crosstalk reinforces the inhibition of neuronal activity.

In contrast, heteromers formed with a non-opioid receptor appear to negatively modulate mu receptor G protein dependent signaling (**Figure 1**). In the VTA, co-activation of mu-Gal1 heteromers by galanin and endomorphin 1 decreased extracellular signal-regulated kinase ERK1/2, protein kinase B (AKT), and cyclic AMP response element binding protein (CREB) phosphorylation (Moreno et al., 2017). Accordingly, galanin could not prevent dopamine release promoted by local infusion of endomorphin 1 in the presence of an interfering peptide that disrupt mu-Gal1 physical interaction (Moreno et al., 2017). In addition, acute morphine administration enhanced ERK1/2 activation in the nucleus accumbens (Nacc) and amygdala of galanin knock-out mice compared to wild type mice (Hawes et al., 2008). These data suggest a negative crosstalk mediated by mu-Gal1 heteromers by which galanin dampens mu receptor signaling. In addition, the mu antagonist CTOP counteracted galanin induced ERK1/2, AKT and CREB phosphorylation indicative of a cross-antagonism on Gal1 receptor signaling (Moreno et al., 2017).

In BE(2)-C neuroblastoma cells co-expressing mu and ORL1 receptors, pretreatment with nociceptin decreased DAMGO potency and efficacy to inhibit adenylate cyclase (Mandyam et al., 2002). This effect was abolished in HEK293 cells co-transfected with receptor pairs unable to physically associate, which supports heteromer specificity (Wang et al., 2005).

TABLE 1 | Identification, properties, and functional outcome of native heteromers involving the mu opioid receptor.

Receptor pair	*In vivo* physical proximity			Specific properties of native heteromers			
	Tissue	Technique	References	Ligand binding, receptor signaling and trafficking	Tissue	Functional outcome	References
MU HETEROMERS INVOLVING ASSOCIATION WITH A GI/O COUPLED RECEPTOR							
Mu-delta	Mouse brain, SC, DRG	Co-IP Disruptive peptide	Xie et al., 2009; Kabli et al., 2013; Erbs et al., 2015	Reciprocal positive crosstalk upon co-activation with an agonist, inverse agonist or antagonist for the other receptor (positive binding cooperativity, increased Gα signaling)	SKNSH, SC VTA		Gomes et al., 2000, 2004, 2011; Margolis et al., 2017
	Increased by morphine in Selected brain areas	Specific mu-delta antibody	Gupta et al., 2010				
				Synergy upon co-activation in chronic morphine treated rats	RVM	Increased analgesia	Zhang and Pan, 2010
				Synergy upon co-activation in chronic inflammatory condition	RVM	Increased analgesia	Sykes et al., 2007
				Mu-delta surface expression	DRG, SC	Increased analgesia	Walwyn et al., 2009; Xie et al., 2009
				Disruption mu-delta heteromer	SC, DRG	Increased Morphine tolerance	Xie et al., 2009; He et al., 2011
				Mu-delta co-internalization (UFP-512, CYM51010)	Striatum, hippocampus	Anxiolytic, anti-depressive, analgesic, decreased morphine tolerance and dependence	Gomes et al., 2013; Kabli et al., 2013; Derouiche et al., 2018
				Increased β-arrestin signaling	SKNSH cells		Rozenfeld and Devi, 2007
				No uncoupling from Gz after chronic morphine	Striatum, hippocampus		Kabli et al., 2014
				DAMGO induced delta recycling to plasma membrane after chronic morphine	DRG		Ong et al., 2015
Mu-kappa	Rat SC proestrous females	Co-IP	Chakrabarti et al., 2010	Co-activation morphine/ dyn1-17 induced synergy		Increases morphine analgesia females	Chakrabarti et al., 2010; Liu N. J. et al., 2011
Mu-ORL1	DRG	Co-IP	Evans et al., 2010	Co-activation induced negative crosstalk on ORL1 signaling	Neuroblastoma	Nociception	Mandyam et al., 2002
Mu-CB_1	Rat striatum	Electron microscopy	Rodriguez et al., 2001	Co-activation induced bidirectional negative crosstalk, decreased mu agonist binding Bidirectional cross antagonism (Nacc)	SKNSH, striatum Mu KO mice CB_1 KO mice CB_1 antagonist	Neuritogenesis Social play	Vaysse et al., 1987; Rios et al., 2006 Manduca et al., 2016
Mu-Gal1	Mouse VTA	Disruptive peptide	Moreno et al., 2017	Co-activation induced negative crosstalk Cross-antagonism on Gal1 signaling	VTA	Opioid drug reward	Moreno et al., 2017
Mu-α_{2a} adrenergic	Rat NTS Increased expression in hypertensive rats	Co-IP PLA	Sun et al., 2015	Opiate induced increased co-expression Co-activation induced negative crosstalk receptor co-internalization	RVM Primary SC neurons DRG	Hypertension	Sun et al., 2015 Jordan et al., 2003 Tan et al., 2009

(Continued)

TABLE 1 | Continued

Receptor pair	*In vivo* physical proximity			Specific properties of native heteromers			
	Tissue	Technique	References	Ligand binding, receptor signaling and trafficking	Tissue	Functional outcome	References
Mu-sst2	Human pancreatic cancer cells	Co-IP FCS	Jorand et al., 2016	Co-activation increased β-arrestin signaling, decreased EMT	Pancreatic cancer cell line	Increased cancer metastatis	Jorand et al., 2016
Mu-CCR5	Human and monkey PBMC	Co-IP	Suzuki et al., 2002	Negative crosstalk Cross-antagonism	CCR5 KO mice CCR5 antagonist	Decreased nociception HIV infection	Lee et al., 2013 Szabo et al., 2002
MU HETEROMERS INVOLVING ASSOCIATION WITH A Gs COUPLED RECEPTOR							
Mu-D_1	Mouse striatum mPFC	Co-IP Co-localization	Tao et al., 2017	Cross-antagonism	D_1 KO mice D_1 antagonist	Opiate locomotor sensitization	Tao et al., 2017
MU HETEROMERS INVOLVING ASSOCIATION WITH A Gq COUPLED RECEPTOR							
Mu-V_{1b}	Mouse RVM	ISH Truncated V_{1b} receptor	Koshimizu et al., 2018	Increased β-arrestin signaling	RVM	Enhanced morphine tolerance	Koshimizu et al., 2018
MOR1D-GRPR	Mouse SC	Co-IP Disruptive peptide	Liu X. Y. et al., 2011	Positive crosstalk on GRPR signaling	SC	Morphine induced itch	Liu X. Y. et al., 2011
MU HETEROMERS INVOLVING ASSOCIATION WITH AN ION CHANNEL							
Mu-NMDA	Mouse PAG	Co-IP	Rodríguez-Muñoz et al., 2012	Positive crosstalk on mu receptor and negative crosstalk on NMDA CAMKII pathway	PAG	Decreased morphine analgesia and increase morphine tolerance	Rodríguez-Muñoz et al., 2012

Co-IP, Co-immunoprecipitation; DRG, Dorsal Root Ganglia; PAG, Periaqueductal Gray; PBMC, peripheral blood mononuclear cells; PLA, Proximity Ligation Assay; RVM, Rostral Ventral Medulla; SC, Spinal Cord; VTA, Ventral Tegmental Area.

Additional examples of negative crosstalk on mu receptor signaling can be linked to heteromerization. Co-activation of mu and cannabinoid CB_1 receptors by the mu agonist morphine and a non-signaling dose of the CB_1 agonist WIN 55,212-2 decreased [^{35}S]guanosine 5′-[γ-thio]triphosphate (GTPγS) binding and ERK1/2 signaling in SK-N-SH neuroblastoma cells (Rios et al., 2006) and Δ^9-tetrahydrocannabinol (THC) allosterically decreased dihydromorphine binding at the mu receptor in rat striatal membranes (Vaysse et al., 1987). Similarly, co-activation of mu and adrenergic α_{2a} receptors decreased ERK1/2 phosphorylation in primary spinal cord neurons (Jordan et al., 2003). Also, the chemokine CCL5 induced phosphorylation of the mu receptor in human PBMC indicating cross-desensitization (Szabo et al., 2002). Finally, the dopamine D_1 antagonist SCH233390 decreased G protein activation and ERK1/2 phosphorylation induced by the mu agonist DAMGO in mouse striatal membrane from wild type but not mice deficient for the D_1 receptor (Tao et al., 2017). Activation of mu-NMDA heteromers by N-methyl-D-aspartate (NMDA) in the periaqueductal gray (PAG) also negatively regulated mu receptor activity by promoting PKA-dependent dissociation of the heteromer and subsequent mu receptor phosphorylation. This in turn promoted G protein uncoupling and receptor desensitization (Rodríguez-Muñoz et al., 2012).

Interestingly, constitutive activity has been reported for delta opioid (Costa and Herz, 1989), kappa opioid (Sirohi and Walker, 2015), ORL1 (Beedle et al., 2004), cannabinoid CB_1 (Fioravanti et al., 2008), adrenergic α_{2a} (Pauwels et al., 2000), and mu opioid receptors (Wang et al., 2004). Since heteromers involving the mu receptor form in the ER in a ligand independent manner, receptor constitutive activity could represent an important determinant of the allosteric modulation and could contribute to the basal homeostasis of the cell in the absence of receptor stimulation. The release of endogenous peptides would however further modulate their functional impact because these receptors can still be activated by agonists (Canals and Milligan, 2008).

ACTIVATION OF β-ARRESTIN SIGNALING IN NATIVE MU HETEROMERS

In SK-N-SH neuroblastoma cells co-expressing mu and delta receptors, activation by the mu agonist DAMGO changed the spatio-temporal profile of ERK1/2 phosphorylation (Rozenfeld and Devi, 2007) (**Figure 1**). This was abolished in the presence of a β-arrestin 2 small interfering ribonucleic acid (siRNA) or in the presence of the delta selective antagonist TIPPψ (Rozenfeld and Devi, 2007) suggesting that activation of heteromers involving the mu receptor can promote β-arrestin dependent signaling. Mice deficient for β-arrestin 2 developed less tolerance to morphine (Bohn et al., 2002), data thus suggest that the recruitment of the β-arrestin pathway by mu heteromers contributes to morphine tolerance. This hypothesis is also supported by the observation that tolerance to morphine develops more slowly in mice deficient for the vasopressin V_{1B} receptor or in the presence of a V_{1B} selective antagonist

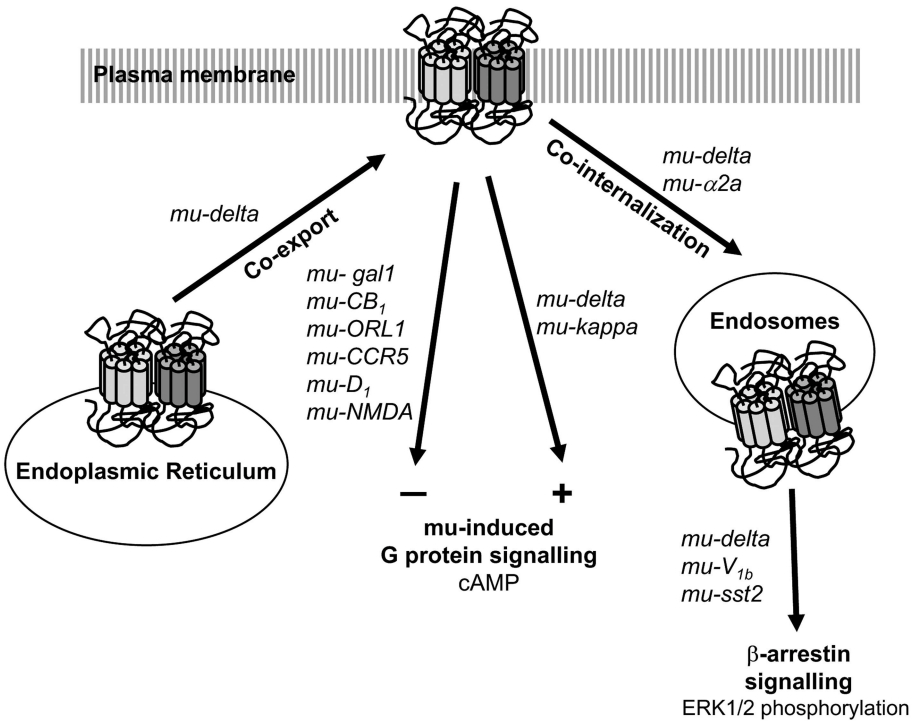

FIGURE 1 | Impact of heteromerization on mu opioid receptor signaling and trafficking. Heteromerization can take place in the endoplasmic reticulum. Association with another opioid receptor positively modulates mu opioid receptor G protein-dependent signaling whereas association with a non-opioid receptor negatively regulates it. Heteromerization also favors the recruitment the β-arrrestin dependent pathway upon internalization in the endosomal compartments. Native receptor pairs for which information is available are indicated.

(Koshimizu et al., 2018). In the mouse RVM, vasopressin V_{1B} receptors constitutively associate with β-arrestin 2 through a leucine rich motif present in the V_{1B} C-terminus (Koshimizu et al., 2018). This suggests that physical association with the V_{1B} receptor facilitates the recruitment of the β-arrestin pathway by the mu receptor, and contributes to the development of morphine tolerance. Accordingly, removal by genome editing with the clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) system of the leucine rich motif responsible for the receptor V_{1B}-β-arrestin interaction increased morphine-induced analgesia and reduced adenylate cyclase supersensitization and morphine-induced tolerance and physical dependence (Koshimizu et al., 2018).

Of note, co-activation of mu and somatostatin sst2 receptors by dermorphin and L-054,264 in pancreatic cancer lines similarly altered the spatio-temporal profile of ERK1/2 phosphorylation, potentiating the epithelial to mesenchymal transition (Jorand et al., 2016).

RECEPTOR CO-INTERNALIZATION IN NATIVE MU HETEROMERS

Co-internalization of endogenous heteromers is less documented largely due to the lack of appropriate tools (**Figure 1**). Receptor internalization contributes to desensitize G protein dependent signaling and favors β-arrestin dependent signaling (Calebiro et al., 2010). Accordingly, co-internalization of mu and adrenergic α_{2a} receptors was dependent on β-arrestin 2 recruitment and mitogen-activated protein kinase (MAPK) p38 activation in the mouse DRGs (Tan et al., 2009).

Mu-delta co-internalization was observed following activation by the mu-delta biased agonist CYM51010 in primary hippocampal neurons from fluorescent double knock-in mice (Derouiche et al., 2018) but could not be detected in the spinal cord following SNC80 application (Wang et al., 2018). Since SNC80 promoted mu-delta co-internalization in co-transfected HEK293 cells (He et al., 2011), this observation highlights the influence of the cellular environment.

MU HETEROMERS MODULATE NOCICEPTION, MORPHINE ANALGESIA AND TOLERANCE

Several observations support the implication of mu heteromers in the control of the nociceptive threshold. The lower response to inflammatory or chemical stimuli in CCR5 knock-out mice or upon injection of a CCR5 antagonist indicate that mu-CCR5 heteromers contribute to dampen the basal nociceptive threshold by exerting a negative crosstalk on mu receptor signaling (Lee et al., 2013). Mapping mu and delta receptors

in the central and peripheral nervous systems using double fluorescent knock-in mice revealed mu-delta co-expression in discrete neuronal populations located in networks involved in the perception and processing of nociceptive stimuli (Erbs et al., 2015). Accordingly, disrupting mu-delta physical interaction with an interfering peptide in naïve mice increased morphine-induced thermal analgesia (He et al., 2011). In rats chronically treated with morphine or with persistent inflammatory pain, co-administration of low doses of mu and delta agonists in the RVM enhanced mechanical and thermal analgesia (Sykes et al., 2007; Zhang and Pan, 2010). Since delta receptor expression is increased in both pathological conditions (Cahill et al., 2003; Gendron et al., 2015; Ong et al., 2015; Erbs et al., 2016), this synergistic effect can be explained by the positive crosstalk at mu-delta heteromer elicited by receptor co-activation (Gomes et al., 2000, 2004, 2011). Similarly, co-activation by morphine and the subsequently released dynorphin 1–17 acted synergistically at mu-kappa heteromers to increase spinal morphine analgesia (Chakrabarti et al., 2010). However, this effect is sex-dependent and more pronounced in proestrous female mice where mu-kappa heteromers are most abundant (Chakrabarti et al., 2010; Liu N. J. et al., 2011).

Activation of mu-delta heteromers by the mu agonist DAMGO (Rozenfeld and Devi, 2007) or co-activation of mu-V_{1b} heteromers by vasopressin and morphine (Koshimizu et al., 2018) increased β-arrestin 2 recruitment and signaling. Importantly, this pathway participates to the development of morphine tolerance (Bohn et al., 2000), which suggests a contribution from mu heteromers. Accordingly, disruption of the physical contact between the mu and delta opioid receptors (Xie et al., 2009; He et al., 2011) or between the mu and vasopressin V_{1b} receptors (Koshimizu et al., 2018) decreased morphine tolerance. In addition, activation of mu-NMDA heteromers in the PAG reduces morphine efficacy through a dual mechanism. Indeed, stimulation by NMDA decreases the analgesic effect of morphine by exerting a negative crosstalk on mu signaling whereas morphine binding to the mu receptor potentiates the NMDA-Ca^{2+}/calmodulin-dependent protein kinase (CAMKII) pathway and contributes to morphine tolerance (Rodríguez-Muñoz et al., 2012).

Other roles for mu heteromers include morphine-induced itch generated by cross-activation of the GRPR signaling in MORD1-GRPR heteromers (Liu X. Y. et al., 2011). Moreover, mu-α_{2a}, mu-CB_1, or mu-ORL1 heteromers very likely represent additional key players since all four receptors modulate nociception but, to date, a direct link to heteromerization with the mu receptor is still lacking.

MU HETEROMERS MODULATE REWARD PROCESSING AND ADDICTION TO OPIOID DRUGS

Modulation of mu receptor signaling by heteromer formation in the mesocorticolimbic pathway is bound to have a profound impact on the rewarding properties of opioid drugs and natural stimuli. Accordingly, galanin-dependent dampening of opiate reinforcing and rewarding properties was abolished upon disruption of mu-Gal1 heteromers in the VTA (Moreno et al., 2017) or in galanin knock-out mice (Hawes et al., 2008). Also, chronic morphine treatment increased mu-delta heteromer expression in several brain regions including the VTA and Nacc (Gupta et al., 2010). Therefore, the positive cross talk at mu-delta heteromers observed in a subset of VTA neurons could contribute to increased dopamine release in the Nacc and opiate reinforcing properties (Margolis et al., 2017).

Also, systemic injection of the endocannabinoid 2-arachidonoyl (2-AG) hydrolysis inhibitor JZL184 increased the concentration of the endogenous ligand and enhanced social play behavior in adolescent rodents (Manduca et al., 2016). This effect was blocked by infusing the mu antagonist CTAP in the Nacc and was absent in mu receptor knock-out mice (Manduca et al., 2016). Reciprocally, systemic injection of the mu agonist morphine increased social play and was abolished by the CB_1 antagonist SR1417-16 or in CB_1 receptor knock-out mice (Manduca et al., 2016). This bidirectional cross-antagonism suggests that mu-CB_1 heteromers in the Nacc modulate the strong rewarding value of social play.

Mu receptors are also involved in other aspects of opiate addiction such as locomotor sensitization and could achieve their modulatory control through heteromerization with dopamine D_1 receptors. Indeed, opiate hyperlocomotion and locomotor sensitization were abolished in dopamine D_1 receptor in knock-out mice or following local injection of the D_1 antagonist SCH23390 in the Nacc (Tao et al., 2017).

MU HETEROMERS MODULATE ANXIETY AND DEPRESSION

Pharmacological and knock-out based studies linked an anxiogenic and depressant phenotype to mu receptor activation and, on the opposite, associated an anxiolytic and antidepressant phenotype with delta receptor activation (Lutz et al., 2014).

Systemic administration or local micro-infusion in the Nacc of the delta agonist UFP512 promoted anxiolytic- and anti-depressant-like activity (Vergura et al., 2008; Kabli et al., 2013). These effects were abolished by pretreatment with the mu antagonist CTOP or the delta antagonist naltrindole or following disruption of mu-delta physical contact in the Nacc (Kabli et al., 2013). These data therefore suggest that accumbal mu-delta heteromers participate to the modulation of anxio-depressive states.

MU HETEROMERS MODULATE METABOLIC DISORDERS

Mu receptors are known to control autonomous functions. Higher levels of mu-α_{2a} heteromers in the NTS were correlated with increased blood pressure in hypertensive rats (Sun et al., 2015). In normotensive rats, mu-α_{2a} heteromerization induced by the mu agonist DAMGO was paralleled by increased blood pressure. Treatment with the mu antagonist CTAP antagonized DAMGO changes in normotensive rats

and reduced mu-α_{2a} heteromerization and blood pressure in hypertensive rats (Sun et al., 2015). Thus, activation of the mu receptor by endogenous opioid peptides dampens the activity of the α_{2a} adrenergic receptors thereby potentiating hypertension.

Interactions between mu and somatostatin receptors have been postulated to influence tumor cell growth (Hatzoglou et al., 2005). Recently, mu-sst2 heteromers were identified in pancreatic cancer lines and in tissue from patients with pancreatic ductal adenocarcinoma. Co-activation of the receptors initiated the epithelial to mesenchymal transition, which is associated with increased metastatic potential (Jorand et al., 2016).

MU HETEROMERS AS A NOVEL THERAPEUTIC TARGET

The bivalent ligand MDAN-21 composed of the mu agonist oxymorphone and the delta antagonist naltrindole tethered by a 21 amino acid long linker was developed to selectively target mu-delta heteromers (Daniels et al., 2005). The length of the linker was designed to enable simultaneous binding of the two ligand moieties to the orthosteric binding pockets of two GPCRs in physical contact. MDAN-21 induced analgesia with low tolerance, low physical dependence and no reinforcing properties (Daniels et al., 2005; Lenard et al., 2007; Aceto et al., 2012) providing a proof of concept that selective targeting of mu-delta heteromers may represent a valid therapeutic strategy, in particular for patients on opiate maintenance treatment.

More recently, the bivalent ligand MCC22 linking the mu agonist oxymorphone to the CCR5 antagonist TAK220 has been proposed to inhibit inflammatory and neuropathic pain by targeting mu-CCR5 heteromers (Akgün et al., 2015). This is in line with the enhanced nociception observed in CCR5 receptor knock-out mice or in the presence of a CCR5 antagonist (Lee et al., 2013).

A major limitation to the therapeutic use of bivalent ligands is their poor capacity to cross the blood brain barrier (Le Naour et al., 2013; Jörg et al., 2015). Therefore, monovalent bifunctional ligands that would selectively target mu heteromers have been developed (Schiller, 2010; Günther et al., 2018). Eluxadoline is a mixed mu agonist delta antagonist recently been approved by the FDA for the treatment of the irritable bowel syndrome (FDA application N°206940). Arguments in favor of binding to mu-delta heteromers include lower efficacy in mice deficient for the delta receptor and reduced signaling in the presence of mu-delta selective antibodies (Fujita et al., 2014b). Eluxadoline thus represents the first drug on the market designed to target heteromers. In preclinical models, other ligands further support preferential activation of mu heteromers as a valuable therapeutic approach. The mu-delta biased agonist CYM51010 induced potent thermal analgesia comparable to morphine but less tolerance and physical dependence (Gomes et al., 2013) and the mu-kappa agonist NNTA produced strong analgesia devoid of tolerance, physical dependence, or reinforcing properties upon intrathecal injection in mice (Yekkirala et al., 2011).

CONCLUSION

Our current appreciation of the role of mu heteromer is still in its infancy and their contribution to mu receptor-dependent behavior likely underestimated. So far, physical proximity has only been validated for a limited number of receptor pairs *in vivo* and their functional interactions addressed in a handful of tissue or brain areas. Moreover, heteromer expression is dynamically regulated depending on physiopathological conditions. No doubt that both novel functions and receptor pairs will be uncovered in the future, which further emphasizes their potential as innovative therapeutic targets.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Aceto, M. D., Harris, L. S., Negus, S. S., Banks, M. L., Hughes, L. D., Akgun, E., et al. (2012). MDAN-21: a bivalent opioid ligand containing mu-agonist and delta-antagonist pharmacophores and its effects in rhesus monkeys. *Int. J. Med. Chem.* 2012:327257. doi: 10.1155/2012/327257

Akgün, E., Javed, M. I., Lunzer, M. M., Powers, M. D., Sham, Y. Y., Watanabe, Y., et al. (2015). Inhibition of inflammatory and neuropathic pain by targeting a Mu Opioid Receptor/Chemokine Receptor5 Heteromer (MOR-CCR5). *J. Med. Chem.* 58, 8647–8657. doi: 10.1021/acs.jmedchem.5b01245

Beedle, A. M., McRory, J. E., Poirot, O., Doering, C. J., Altier, C., Barrere, C., et al. (2004). Agonist-independent modulation of N-type calcium channels by ORL1 receptors. *Nat. Neurosci.* 7, 118–125. doi: 10.1038/nn1180

Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J., and Caron, M. G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408, 720–723. doi: 10.1038/35047086

Bohn, L. M., Lefkowitz, R. J., and Caron, M. G. (2002). Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. *J. Neurosci.* 22, 10494–10500. doi: 10.1523/JNEUROSCI.22-23-10494.2002

Borroto-Escuela, D. O., Brito, I., Romero-Fernandez, W., Di Palma, M., Oflijan, J., Skieterska, K., et al. (2014). The G protein-coupled receptor heterodimer network (GPCR-HetNet) and its hub components. *Int. J. Mol. Sci.* 15, 8570–8590. doi: 10.3390/ijms15058570

Cahill, C. M., Morinville, A., Hoffert, C., O'donnell, D., and Beaudet, A. (2003). Up-regulation and trafficking of delta opioid receptor in a model of chronic inflammation: implications for pain control. *Pain* 101, 199–208. doi: 10.1016/S0304-3959(02)00333-0

Calebiro, D., Nikolaev, V. O., Persani, L., and Lohse, M. J. (2010). Signaling by internalized G-protein-coupled receptors. *Trends Pharmacol. Sci.* 31, 221–228. doi: 10.1016/j.tips.2010.02.002

Canals, M., and Milligan, G. (2008). Constitutive activity of the cannabinoid CB1 receptor regulates the function of co-expressed Mu opioid receptors. *J. Biol. Chem.* 283, 11424–11434. doi: 10.1074/jbc.M710300200

Chakrabarti, S., Liu, N. J., and Gintzler, A. R. (2010). Formation of mu-/kappa-opioid receptor heterodimer is sex-dependent and mediates female-specific opioid analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20115–20119. doi: 10.1073/pnas.1009923107

Costa, T., and Herz, A. (1989). Antagonists with negative intrinsic activity at d opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7321–7325. doi: 10.1073/pnas.86.19.7321

Cussac, D., Rauly-Lestienne, I., Heusler, P., Finana, F., Cathala, C., Bernois, S., et al. (2012). mu-Opioid and 5-HT1A receptors heterodimerize and show signalling crosstalk via G protein and MAP-kinase pathways. *Cell. Signal.* 24, 1648–1657. doi: 10.1016/j.cellsig.2012.04.010

Daniels, D. J., Lenard, N. R., Etienne, C. L., Law, P. Y., Roerig, S. C., and Portoghese, P. S. (2005). Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc. Natl. Acad. Sci. U.S.A.* 102, 19208–19213. doi: 10.1073/pnas.0506627102

Derouiche, L., Ory, S., and Massotte, D. (2018). "Double fluorescent knock-in mice to investigate endogenous mu-delta opioid heteromer subscellular distribution," in *Receptor-Receptor Interactions in the Central Nervous System, Neuromethods,* eds K. Fuxe and D. Borroto-Escuela (New York, NY: The Humana Press; Springer Nature), 149–162.

Erbs, E., Faget, L., Ceredig, R. A., Matifas, A., Vonesch, J. L., Kieffer, B. L., et al. (2016). Impact of chronic morphine on delta opioid receptor-expressing neurons in the mouse hippocampus. *Neuroscience* 313, 46–56. doi: 10.1016/j.neuroscience.2015.10.022

Erbs, E., Faget, L., Scherrer, G., Matifas, A., Filliol, D., Vonesch, J. L., et al. (2015). A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* 220, 677–702. doi: 10.1007/s00429-014-0717-9

Evans, R. M., You, H., Hameed, S., Altier, C., Mezghrani, A., Bourinet, E., et al. (2010). Heterodimerization of ORL1 and opioid receptors and its consequences for N-type calcium channel regulation. *J. Biol. Chem.* 285, 1032–1040. doi: 10.1074/jbc.M109.040634

Fioravanti, B., De Felice, M., Stucky, C. L., Medler, K. A., Luo, M. C., Gardell, L. R., et al. (2008). Constitutive activity at the cannabinoid CB1 receptor is required for behavioral response to noxious chemical stimulation of TRPV1: antinociceptive actions of CB1 inverse agonists. *J. Neurosci.* 28, 11593–11602. doi: 10.1523/JNEUROSCI.3322-08.2008

Fujita, W., Gomes, I., and Devi, L. A. (2014a). Revolution in GPCR Signaling: Opioid receptor heteromers as novel therapeutic targets. *Br. J. Pharmacol.* 171, 4155–4176. doi: 10.1111/bph.12798

Fujita, W., Gomes, I., Dove, L. S., Prohaska, D., Mcintyre, G., and Devi, L. A. (2014b). Molecular characterization of eluxadoline as a potential ligand targeting mu-delta opioid receptor heteromers. *Biochem. Pharmacol.* 92, 448–456. doi: 10.1016/j.bcp.2014.09.015

Gendron, L., Mittal, N., Beaudry, H., and Walwyn, W. (2015). Recent advances on the delta opioid receptor: from trafficking to function. *Br. J. Pharmacol.* 172, 403–419. doi: 10.1111/bph.12706

George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., et al. (2000). Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J. Biol. Chem.* 275, 26128–26135. doi: 10.1074/jbc.M000345200

Gomes, I., Fujita, W., Gupta, A., Saldanha, S. A., Negri, A., Pinello, C. E., et al. (2013). Identification of a mu-delta opioid receptor heteromer-biased agonist with antinociceptive activity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12072–12077. doi: 10.1073/pnas.1222044110

Gomes, I., Gupta, A., Filipovska, J., Szeto, H. H., Pintar, J. E., and Devi, L. A. (2004). A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 101, 5135–5139. doi: 10.1073/pnas.0307601101

Gomes, I., Ijzerman, A. P., Ye, K., Maillet, E. L., and Devi, L. A. (2011). G protein-coupled receptor heteromerization: a role in allosteric modulation of ligand binding. *Mol. Pharmacol.* 79, 1044–1052. doi: 10.1124/mol.110.070847

Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, N., Nagy, V., and Devi, L. A. (2000). Heterodimerization of mu and delta opioid receptors: a role in opiate synergy. *J. Neurosci.* 20, 1–5. doi: 10.1523/JNEUROSCI.20-22-j0007.2000

Günther, T., Dasgupta, P., Mann, A., Miess, E., Kliewer, A., Fritzwanker, S., et al. (2018). Targeting multiple opioid receptors - improved analgesics with reduced side effects? *Br. J. Pharmacol.* 175, 2857–2868. doi: 10.1111/bph.13809

Gupta, A., Mulder, J., Gomes, I., Rozenfeld, R., Bushlin, I., Ong, E., et al. (2010). Increased abundance of opioid receptor heteromers after chronic morphine administration. *Sci. Signal.* 3:ra54. doi: 10.1126/scisignal.2000807

Happel, C., Steele, A. D., Finley, M. J., Kutzler, M. A., and Rogers, T. J. (2008). DAMGO-induced expression of chemokines and chemokine receptors: the role of TGF-beta1. *J. Leukoc. Biol.* 83, 956–963. doi: 10.1189/jlb.1007685

Hatzoglou, A., Kampa, M., and Castanas, E. (2005). Opioid-somatostatin interactions in regulating cancer cell growth. *Front. Biosci.* 10, 244–256. doi: 10.2741/1524

Hawes, J. J., Brunzell, D. H., Narasimhaiah, R., Langel, U., Wynick, D., and Picciotto, M. R. (2008). Galanin protects against behavioral and neurochemical correlates of opiate reward. *Neuropsychopharmacology* 33, 1864–1873. doi: 10.1038/sj.npp.1301579

He, S. Q., Zhang, Z. N., Guan, J. S., Liu, H. R., Zhao, B., Wang, H. B., et al. (2011). Facilitation of mu-opioid receptor activity by preventing delta-opioid receptor-mediated codegradation. *Neuron* 69, 120–131. doi: 10.1016/j.neuron.2010.12.001

Heinisch, S., Palma, J., and Kirby, L. G. (2011). Interactions between chemokine and mu-opioid receptors: anatomical findings and electrophysiological studies in the rat periaqueductal grey. *Brain Behav. Immun.* 25, 360–372. doi: 10.1016/j.bbi.2010.10.020

Jorand, R., Biswas, S., Wakefield, D. L., Tobin, S. J., Golfetto, O., Hilton, K., et al. (2016). Molecular signatures of mu opioid receptor and somatostatin receptor 2 in pancreatic cancer. *Mol. Biol. Cell* 27, 3659–3672. doi: 10.1091/mbc.e16-06-0427

Jordan, B. A., Gomes, I., Rios, C., Filipovska, J., and Devi, L. A. (2003). Functional interactions between mu opioid and alpha 2A-adrenergic receptors. *Mol. Pharmacol.* 64, 1317–1324. doi: 10.1124/mol.64.6.1317

Jörg, M., May, L. T., Mak, F. S., Lee, K. C., Miller, N. D., Scammells, P. J., et al. (2015). Synthesis and pharmacological evaluation of dual acting ligands targeting the adenosine A2A and dopamine D2 receptors for the potential treatment of Parkinson's disease. *J. Med. Chem.* 58, 718–738. doi: 10.1021/jm501254d

Kabli, N., Fan, T., O'dowd, B. F., and George, S. R. (2014). mu-delta opioid receptor heteromer-specific signaling in the striatum and hippocampus. *Biochem. Biophys. Res. Commun.* 450, 906–911. doi: 10.1016/j.bbrc.2014.06.099

Kabli, N., Nguyen, T., Balboni, G., O'dowd, B. F., and George, S. R. (2013). Antidepressant-like and anxiolytic-like effects following activation of the mu-delta opioid receptor heteromer in the nucleus accumbens. *Mol. Psychiatry* 19, 968–994. doi: 10.1038/mp.2013.115

Kieffer, B. L. (1999). Opioids: first lessons from knockout mice. *Trends Pharmacol.* 20, 19–26. doi: 10.1016/S0165-6147(98)01279-6

Kieffer, B. L., and Evans, C. J. (2009). Opioid receptors: from binding sites to visible molecules *in vivo*. *Neuropharmacology* 56(Suppl. 1), 205–212. doi: 10.1016/j.neuropharm.2008.07.033

Koshimizu, T. A., Honda, K., Nagaoka-Uozumi, S., Ichimura, A., Kimura, I., Nakaya, M., et al. (2018). Complex formation between the vasopressin 1b receptor, beta-arrestin-2, and the mu-opioid receptor underlies morphine tolerance. *Nat. Neurosci.* 21, 820–833. doi: 10.1038/s41593-018-0144-y

Le Naour, M., Akgun, E., Yekkirala, A., Lunzer, M. M., Powers, M. D., Kalyuzhny, A. E., et al. (2013). Bivalent ligands that target mu opioid (MOP) and cannabinoid1 (CB1) receptors are potent analgesics devoid of tolerance. *J. Med. Chem.* 56, 5505–5513. doi: 10.1021/jm4005219

Lee, Y. K., Choi, D. Y., Jung, Y. Y., Yun, Y. W., Lee, B. J., Han, S. B., et al. (2013). Decreased pain responses of C-C chemokine receptor 5 knockout mice to chemical or inflammatory stimuli. *Neuropharmacology* 67, 57–65. doi: 10.1016/j.neuropharm.2012.10.030

Lenard, N. R., Daniels, D. J., Portoghese, P. S., and Roerig, S. C. (2007). Absence of conditioned place preference or reinstatement with bivalent ligands containing mu-opioid receptor agonist and delta-opioid

receptor antagonist pharmacophores. *Eur. J. Pharmacol.* 566, 75–82. doi: 10.1016/j.ejphar.2007.02.040

Liu, N. J., Chakrabarti, S., Schnell, S., Wessendorf, M., and Gintzler, A. R. (2011). Spinal synthesis of estrogen and concomitant signaling by membrane estrogen receptors regulate spinal kappa- and mu-opioid receptor heterodimerization and female-specific spinal morphine antinociception. *J. Neurosci.* 31, 11836–11845. doi: 10.1523/JNEUROSCI.1901-11.2011

Liu, X. Y., Liu, Z. C., Sun, Y. G., Ross, M., Kim, S., Tsai, F. F., et al. (2011). Unidirectional cross-activation of GRPR by MOR1D uncouples itch and analgesia induced by opioids. *Cell* 147, 447–458. doi: 10.1016/j.cell.2011.08.043

Lopez-Gimenez, J. F., Vilaro, M. T., and Milligan, G. (2008). Morphine desensitization, internalization, and down-regulation of the mu opioid receptor is facilitated by serotonin 5-hydroxytryptamine2A receptor coactivation. *Mol. Pharmacol.* 74, 1278–1291. doi: 10.1124/mol.108.048272

Lutz, P. E., Ayranci, G., Chu-Sin-Chung, P., Matifas, A., Koebel, P., Filliol, D., et al. (2014). Distinct mu, delta, and kappa opioid receptor mechanisms underlie low sociability and depressive-like behaviors during heroin abstinence. *Neuropsychopharmacology* 39, 2694–2705. doi: 10.1038/npp.2014.126

Manduca, A., Lassalle, O., Sepers, M., Campolongo, P., Cuomo, V., Marsicano, G., et al. (2016). Interacting cannabinoid and opioid receptors in the nucleus accumbens core control adolescent social play. *Front. Behav. Neurosci.* 10:211. doi: 10.3389/fnbeh.2016.00211

Mandyam, C. D., Thakker, D. R., Christensen, J. L., and Standifer, K. M. (2002). Orphanin FQ/nociceptin-mediated desensitization of opioid receptor-like 1 receptor and mu opioid receptors involves protein kinase C: a molecular mechanism for heterologous cross-talk. *J. Pharmacol. Exp. Ther.* 302, 502–509. doi: 10.1124/jpet.102.033159

Margolis, E. B., Fujita, W., Devi, L. A., and Fields, H. L. (2017). Two delta opioid receptor subtypes are functional in single ventral tegmental area neurons, and can interact with the mu opioid receptor. *Neuropharmacology* 123, 420–432. doi: 10.1016/j.neuropharm.2017.06.019

Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383, 819–823. doi: 10.1038/383819a0

Moreno, E., Quiroz, C., Rea, W., Cai, N. S., Mallol, J., Cortes, A., et al. (2017). Functional mu-opioid-galanin receptor heteromers in the ventral tegmental area. *J. Neurosci.* 37, 1176–1186. doi: 10.1523/JNEUROSCI.2442-16.2016

Ong, E. W., Xue, L., Olmstead, M. C., and Cahill, C. M. (2015). Prolonged morphine treatment alters delta opioid receptor post-internalization trafficking. *Br. J. Pharmacol.* 172, 615–629. doi: 10.1111/bph.12761

Patel, J. P., Sengupta, R., Bardi, G., Khan, M. Z., Mullen-Przeworski, A., and Meucci, O. (2006). Modulation of neuronal CXCR4 by the micro-opioid agonist DAMGO. *J. Neurovirol.* 12, 492–500. doi: 10.1080/13550280601064798

Pauwels, P. J., Tardif, S., Wurch, T., and Colpaert, F. C. (2000). Facilitation of constitutive alpha(2A)-adrenoceptor activity by both single amino acid mutation (Thr(373)Lys) and g(alphao) protein coexpression: evidence for inverse agonism. *J. Pharmacol. Exp. Ther.* 292, 654–663.

Pfeiffer, M., Kirscht, S., Stumm, R., Koch, T., Wu, D., Laugsch, M., et al. (2003). Heterodimerization of substance P and mu-opioid receptors regulates receptor trafficking and resensitization. *J. Biol. Chem.* 278, 51630–51637. doi: 10.1074/jbc.M307095200

Qian, M., Vasudevan, L., Huysentruyt, J., Risseeuw, M. D. P., Stove, C., Vanderheyden, P. M. L., et al. (2018). Design, synthesis, and biological evaluation of bivalent ligands targeting dopamine D2 -like receptors and the mu-opioid receptor. *ChemMedChem* 13, 944–956. doi: 10.1002/cmdc.201700787

Rediger, A., Tarnow, P., Bickenbach, A., Schaefer, M., Krude, H., Gruters, A., et al. (2009). Heterodimerization of hypothalamic G-protein-coupled receptors involved in weight regulation. *Obes. Facts* 2, 80–86. doi: 10.1159/000209862

Rios, C., Gomes, I., and Devi, L. A. (2006). mu opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. *Br. J. Pharmacol.* 148, 387–395. doi: 10.1038/sj.bjp.0706757

Rivera, A., Gago, B., Suarez-Boomgaard, D., Yoshitake, T., Roales-Bujan, R., Valderrama-Carvajal, A., et al. (2017). Dopamine D4 receptor stimulation prevents nigrostriatal dopamine pathway activation by morphine: relevance for drug addiction. *Addict. Biol.* 22, 1232–1245. doi: 10.1111/adb.12407

Rodriguez, J. J., Mackie, K., and Pickel, V. M. (2001). Ultrastructural localization of the CB1 cannabinoid receptor in μ-opioid receptor patches of the rat Caudate putamen nucleus. *J. Neurosci.* 21, 823–833. doi: 10.1523/JNEUROSCI.21-03-00823.2001

Rodríguez-Muñoz, M., Sanchez-Blazquez, P., Vicente-Sanchez, A., Berrocoso, E., and Garzon, J. (2012). The mu-opioid receptor and the NMDA receptor associate in PAG neurons: implications in pain control. *Neuropsychopharmacology* 37, 338–349. doi: 10.1038/npp.2011.155

Roumy, M., Lorenzo, C., Mazeres, S., Bouchet, S., Zajac, J. M., and Mollereau, C. (2007). Physical association between neuropeptide FF and micro-opioid receptors as a possible molecular basis for anti-opioid activity. *J. Biol. Chem.* 282, 8332–8342. doi: 10.1074/jbc.M606946200

Rozenfeld, R., and Devi, L. A. (2007). Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. *FASEB J.* 21, 2455–2465. doi: 10.1096/fj.06-7793com

Schiller, P. W. (2010). Bi- or multifunctional opioid peptide drugs. *Life Sci.* 86, 598–603. doi: 10.1016/j.lfs.2009.02.025

Schröder, H., Wu, D. F., Seifert, A., Rankovic, M., Schulz, S., Hollt, V., et al. (2009). Allosteric modulation of metabotropic glutamate receptor 5 affects phosphorylation, internalization, and desensitization of the micro-opioid receptor. *Neuropharmacology* 56, 768–778. doi: 10.1016/j.neuropharm.2008.12.010

Sirohi, S., and Walker, B. M. (2015). Maturational alterations in constitutive activity of medial prefrontal cortex kappa-opioid receptors in Wistar rats. *J. Neurochem.* 135, 659–665. doi: 10.1111/jnc.13279

Sun, G. C., Ho, W. Y., Chen, B. R., Cheng, P. W., Cheng, W. H., Hsu, M. C., et al. (2015). GPCR dimerization in brainstem nuclei contributes to the development of hypertension. *Br. J. Pharmacol.* 172, 2507–2518. doi: 10.1111/bph.13074

Suzuki, S., Chuang, L. F., Yau, P., Doi, R. H., and Chuang, R. Y. (2002). Interactions of opioid and chemokine receptors: oligomerization of mu, kappa, and delta with CCR5 on immune cells. *Exp. Cell Res.* 280, 192–200. doi: 10.1006/excr.2002.5638

Sykes, K. T., White, S. R., Hurley, R. W., Mizoguchi, H., Tseng, L. F., and Hammond, D. L. (2007). Mechanisms responsible for the enhanced antinociceptive effects of micro-opioid receptor agonists in the rostral ventromedial medulla of male rats with persistent inflammatory pain. *J. Pharmacol. Exp. Ther.* 322, 813–821. doi: 10.1124/jpet.107.121954

Szabo, I., Chen, X. H., Xin, L., Adler, M. W., Howard, O. M., Oppenheim, J. J., et al. (2002). Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10276–10281. doi: 10.1073/pnas.102327699

Tan, M., Walwyn, W. M., Evans, C. J., and Xie, C. W. (2009). p38 MAPK and beta-arrestin 2 mediate functional interactions between endogenous micro-opioid and alpha2A-adrenergic receptors in neurons. *J. Biol. Chem.* 284, 6270–6281. doi: 10.1074/jbc.M806742200

Tao, Y. M., Yu, C., Wang, W. S., Hou, Y. Y., Xu, X. J., Chi, Z. Q., et al. (2017). Heteromers of mu opioid and dopamine D1 receptors modulate opioid-induced locomotor sensitization in a dopamine-independent manner. *Br. J. Pharmacol.* 174, 2842–2861. doi: 10.1111/bph.13908

van Rijn, R. M., Brissett, D. I., and Whistler, J. L. (2012). Distinctive modulation of ethanol place preference by delta opioid receptor-selective agonists. *Drug Alcohol Depend.* 122, 156–159. doi: 10.1016/j.drugalcdep.2011.09.024

Vaysse, P. J., Gardner, E. L., and Zukin, R. S. (1987). Modulation of rat brain opioid receptors by cannabinoids. *J. Pharmacol. Exp. Ther.* 241, 534–539.

Vergura, R., Balboni, G., Spagnolo, B., Gavioli, E., Lambert, D. G., Mcdonald, J., et al. (2008). Anxiolytic- and antidepressant-like activities of H-Dmt-Tic-NH-CH(CH2-COOH)-Bid (UFP-512), a novel selective delta opioid receptor agonist. *Peptides* 29, 93–103. doi: 10.1016/j.peptides.2007.10.012

Vowles, K. E., Mcentee, M. L., Julnes, P. S., Frohe, T., Ney, J. P., and Van Der Goes, D. N. (2015). Rates of opioid misuse, abuse, and addiction in chronic pain: a systematic review and data synthesis. *Pain* 156, 569–576. doi: 10.1097/01.j.pain.0000460357.01998.f1

Walwyn, W., John, S., Maga, M., Evans, C. J., and Hales, T. G. (2009). Delta receptors are required for full inhibitory coupling of mu-receptors to voltage-dependent Ca(2+) channels in dorsal root ganglion neurons. *Mol. Pharmacol.* 76, 134–143. doi: 10.1124/mol.109.055913

Wang, D., Raehal, K. M., Lin, E. T., Lowery, J. J., Kieffer, B. L., Bilsky, E. J., et al. (2004). Basal signaling activity of mu opioid receptor in mouse brain: role in narcotic dependence. *J. Pharmacol. Exp. Ther.* 308, 512–520. doi: 10.1124/jpet.103.054049

Wang, D., Tawfik, V. L., Corder, G., Low, S. A., Francois, A., Basbaum, A. I., et al. (2018). Functional divergence of delta and mu opioid receptor organization in CNS pain circuits. *Neuron* 98, 90-108.e105. doi: 10.1016/j.neuron.2018.03.002

Wang, H. L., Hsu, C. Y., Huang, P. C., Kuo, Y. L., Li, A. H., Yeh, T. H., et al. (2005). Heterodimerization of opioid receptor-like 1 and mu-opioid receptors impairs the potency of micro receptor agonist. *J. Neurochem.* 92, 1285–1294. doi: 10.1111/j.1471-4159.2004.02921.x

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., Von Zastrow, M., Schulz, S., et al. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254. doi: 10.1124/pr.112.005942

Xie, W. Y., He, Y., Yang, Y. R., Li, Y. F., Kang, K., Xing, B. M., et al. (2009). Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the delta-opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. *J. Neurosci.* 29, 3551–3564. doi: 10.1523/JNEUROSCI.0415-09.2009

Yekkirala, A. S., Lunzer, M. M., Mccurdy, C. R., Powers, M. D., Kalyuzhny, A. E., Roerig, S. C., et al. (2011). N-naphthoyl-beta-naltrexamine (NNTA), a highly selective and potent activator of mu/kappa-opioid heteromers. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5098–5103. doi: 10.1073/pnas.1016277108

Zhang, Z., and Pan, Z. Z. (2010). Synaptic mechanism for functional synergism between delta- and mu-opioid receptors. *J. Neurosci.* 30, 4735–4745. doi: 10.1523/JNEUROSCI.5968-09.2010

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The Clinical Concept of Opioid Addiction Since 1877: Still Wanting After All These Years

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In 1877, the psychiatrist Edward Levinstein authored the first monograph on opioid addiction. The prevalence of opioid addiction prior to his publication had risen in several countries including England, France and Germany. He was the first to call it an illness, but doubted that it was a mental illness because the impairment of volition appeared to be restricted to opioid use: it was not pervasive, since it did not extend to other aspects of the individuals' life. While there has been huge progress in understanding the underlying neurobiological mechanisms, there has been little progress in the clinical psychopathology of addiction and in understanding how it relates to these neurobiological mechanisms. A focus on cravings has limited the exploration of other important aspects such as anosognosia and addiction-related behaviors like smuggling opioids into treatment and supporting the continued provision of co-patients. These behaviors are usually considered secondary reactions, but in clinical practice they appear to be central to addiction, indicating that an improved understanding of the complexity of the disorder is needed. We propose to consider an approach that takes into account the embodied, situated, dynamic, and phenomenological aspects of mental processes. Addiction in this context can be conceptualized as a habit, understood as a distributed network of mental, behavioral, and social processes, which not only shapes the addict's perceptions and actions, but also has a tendency to self-maintain. Such an approach may help to develop and integrate psychopathological and neurobiological research and practice of addictions.

Keywords: addiction, opioid use disorders, psychopathology, habits, 4E cognition, enactivism, anosognosia

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INTRODUCTION

The last decades have seen a great deal of progress in our understanding of the underlying neurobiological mechanisms of opioid and other substance use disorders and on the perception of addiction as a public health issue. However, we believe that the clinical psychopathology of addiction has undergone scant development. In our view addiction is a mental disorder. However, many, including psychiatrists, often seem to struggle to support this statement. We contend that this resistance is attributable to an inadequate scientific theory of the psychopathology of addiction, and especially a restricted conception of the addicted mind as core problems in the current discussion of addiction, including opioid addiction.

One hundred and forty years ago, Edward Levinstein, Director of the *Maison de Santé* in Berlin, published a monograph entitled *Die Morphiumsucht* (1). This was the first monograph identifying opioid addiction as a disorder. One year later it was translated into English (2) and French (3). Around 1853, injectable morphine had become available in Germany, and 25 years later, Germany, like other countries, was experiencing a wave of problems with non-prescribed injection of opiates (4).

Levinstein's definition of addiction as an "uncontrollable desire to use" has held up in the last 140 years. He argued that morphine addiction is a disease, but not a mental disease, because the *will* of the addicted individual was not *pervasively* damaged. Individuals could use their will successfully in the context of work and family, but not with opiates (2). Conversely, current versions of the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases (ICD) include opioid use disorders as *mental* disorders. Nevertheless, a great deal of ambivalence remains toward the psychiatric diagnosis of substance use as a mental disorder, not only in the general population, but also among health professionals in general, including psychiatrists (5–8). In our view, this ambivalence reflects the lack of a comprehensive theory of addiction that takes into account the full complexity of the phenomenon in its neurobiological, psychological, and sociocultural aspects.

In this perspective paper, we undertake a critical appraisal of the current status of the psychopathology of opioid use disorder from a clinical point of view. We further suggest that recent developments in cognitive science, in particular enactivism (9, 10), serve as a suitable framework to overcome some of the shortcomings of the current approach by providing a more comprehensive model of addiction that integrates life and social sciences, dynamical and complex systems theory, and philosophical-phenomenological approaches.

LIMITATIONS OF THE CURRENT PSYCHOPATHOLOGY OF ADDICTION

Most physicians and psychiatrists would find it difficult to respond if asked about the nature of addiction. We propose that this difficulty can be attributed to a lack of a mature theory regarding the clinical psychopathology of addiction. Searches for articles using the keywords *psychopathology* and *addiction* or *substance use disorder* will mainly retrieve articles on psychopathology of other mental disorders and concurrent addiction, but not about the psychopathology of addiction as such. We think that a glance at the origin and development of psychopathology and the concept of mental disorders may help to understand this deficit.

The original development of the concepts of psychopathology and mental disorders has been attributed to Karl Jaspers. Jaspers based his concept of psychopathology on Edmund Husserl's phenomenology, focusing on conscious experiences and excluding non-conscious aspects (11). He felt uncomfortable with Sigmund Freud's speculations of the impact of non-conscious aspects of the mind on salience, motivation and decision-making (12). Jasper's philosophical anchoring in phenomenology as a disciplined investigation of conscious experience seems to have been lost (13, 14). Instead, current clinical psychopathology (signs and symptoms) continues to be based on the naïve common sense concepts of the mind derived from folk psychology, with all its limitations and scientifically unsustainable assumptions (15). But even Jaspers in his original formulations made no attempt to systematically probe the phenomenology of actual lived experience of addiction as reported by addicted individuals.

In a recent attempt to develop such a phenomenology of addiction, Owen Flanagan talked about shame and normative failures, not pathological craving (16). Patients appear to experience cravings as intuitive drives, not as uncontrollable or *foreign* urges. This is different from other disorders, such as obsessive-compulsive disorder (OCD), where urges can be experienced as intrusive, overwhelming and dysfunctional. It appears that individuals with an addiction do not tend to spontaneously report a feeling of a "loss of control." If individuals suffering from addiction step back and evaluate their lives, they can articulate the negative impact of their substance use, but this is distinct from a feeling of losing control due to craving. While this is a clinical observation not unfamiliar to treatment providers, data and studies regarding this phenomenon are lacking and current psychopathology of addiction has remained silent to it. Although clinical experience, including clinical psychopathology, cannot replace scientific evidence, clinical psychopathology is important to understand the expression of the disorder in a patient's life and to relate the neurobiological mechanisms to the relevant aspects of this clinical disorder.

Sense of control and *will* are central concepts in commonsense psychology, but they are surprisingly poorly conceptualized or investigated in current psychopathology. If pressed, professionals will express contradictory views: they will argue either that the will of an addict is in principle intact, as conceptualized by Levinstein (2); or that it is impaired (lack of willpower), as argued by Jaspers, Kraepelin and others (17, 18). This contradiction might arise because commonsense psychology endows an individual with a consistent, single will that is either "healthy" or "sick" in addiction. Imposing a dichotomy does not do justice to the complexity of agency. Discussion of the role of volition, will and agency is closely related to questions around free will. Free will is a conceptual cornerstone of the prevalent Western folk intuitions of individuals as responsible human beings (19–21). This is a deeply engrained perspective, and also may be a reason why addiction is associated with such a high level of stigma. The image of a hijacked brain, endorsed by the National Institute on Drug Abuse (NIDA) as a metaphor aimed at lay audiences, circumvents this stigma by describing the brain as seized by an unnamed outside agent (e.g., drugs or addiction processes) that forces it to follow a new trajectory.

It is undeniable that the brain undergoes neuroplastic changes in response to substance abuse. However, neuroplasticity does

not imply that the brain has been "hijacked" (22). Furthermore, this metaphor may undermine the patients' possibility of taking at least partial responsibility for their actions and does little to support their capacity for change (23). Accepting to be completely controlled by drugs might contribute to a low self-efficacy in addiction. We think that this last point is relevant for recovery, since self-efficacy (24) has been found to be a significant determinant of behavior change and relapse prevention in studies on smoking cessation and alcoholism treatments (25–27). Pickard's framework of "responsibility without blame" (28), which proposes to change attitudes toward addiction by decoupling responsibility from morality, might be useful in clinical practice for avoiding stigma and blame without removing the patients' sense of agency. Additionally, a more nuanced theory of agency, which can defend the sense of being an autonomous individual, while acknowledging the constraints of biological embodiment also appears to be advantageous or even necessary, as it might help to identify healthy aspects of agency supporting a restructuring of an individual's life. In the next section, we will argue that enactivism can provide such a theory.

Another feature of addiction, which we feel needs more attention, is that it involves a host of characteristic behaviors beyond use itself. Levinstein already provided a broad range of examples of the effect of morphine addiction on the patients' responses and behavior, e.g., when the treatment provider has to expect that, independently of the "respectability" of the patients, they will try to smuggle morphine into treatment. He also pointed out that "hardly any person suffering from morbid craving for morphia[1] is able truthfully to state the daily quantity of morphia used, and the hour when he last injected morphia" (2). Furthermore it seems to require considerable effort to switch from supporting substance use of others to supporting recovery and abstinence of others, even in the context of a joint recovery. These behaviors and social phenomena are familiar to anybody treating patients suffering from substance use disorders, and yet remain rarely discussed as an integrated part of the disorder. We believe that they once again point toward the need for a more elaborate and far-reaching theory of addiction.

Neuroscience will play an essential role in developing a more comprehensive conceptualization of addiction. For instance, some of the aforementioned aspects have been subsumed under the description of "denial" (29). Denial can be considered a refusal to accept reality or facts, acting as if an uncomfortable event, thought, or feeling does not exist (30). Recently, Nora Volkow and other authors (31–33) have touched upon denial in addiction by discussing *anosognosia*, conceptualizing it as a "dysfunction of brain networks subserving insight and self-awareness" (31). Another example is the theory of *allostasis*, developed by Sterling and Eyer (34) to explain the relationship between stress and diseases. George Koob and other researchers (35–38) have incorporated it into the field of addiction to explain the neurobiological mechanisms underlying vulnerability to drug addiction and relapse. According to this theory, a pathological equilibrium related to sustained changes in the stress response system or *allostatic load* (39) leads to a self-reinforcing drug use pattern. This theory explains compulsion and relapse as behaviors aimed at reestablishing hedonic homeostasis by relieving the allostatic load, which manifests itself as a spiraling affective tension resulting from withdrawal, repeated failures in self-regulation, and other daily stressors. Similar to the anosognosia concept, this framework captures aspects of addiction familiar to clinicians, but currently not covered by clinical psychopathology. One key feature about this theory is that it emphasizes the integral causality between the whole body and the environment, making it clear that the brain does not work in isolation, but only as a part of a complex system. This fact has also been acknowledged by Thomas Fuchs, who regards the brain as "an organ of mediation" between the organism as a whole and its environment (23).

In general, it can be said that acknowledgement of neurobiological aspects has had a very limited impact in the psychopathology of addiction. This may be partly because clinical psychopathology appears to be increasingly disconnected from biological psychiatry. In fact, from the perspective of the latter, psychopathology is sometimes considered a barrier for progress (40, 41). Psychiatrists conducting genomic and neuroscientific research have tried to circumvent it, creating new biological concepts such as endophenotypes (42) and the Research Domain Criteria (RDoC) (41). Behavioral neuroscience certainly is an essential source of progress for research on and treatment of addictions, but it does not replace clinical psychopathology. We see a need for both neuroscience and clinical psychopathology to more effectively inform each other to obtain a more comprehensive understanding of opioid use disorders and other addictions. In the forthcoming section, we suggest that one promising avenue for collaboration might come from an enactive approach to cognitive sciences.

TOWARD A NEW UNDERSTANDING OF OPIOID AND OTHER DRUG ADDICTIONS

Recent developments in embodied, embedded, extended, and enactive (4E) cognitive science have done much to highlight how embodied interactions, tool-use, affectivity, language, material environment, and socio-cultural practices shape lived experience and the functioning of the mind. A theory of addictions based on 4E theory seems to be an attractive option to move the field forward.

Walter (43) recently described the 4E approach to cognition as the potential base for a third wave in biological psychiatry. By treating the mind/brain as embodied, embedded, extended, and enactive, processes external to the brain are considered to be constitutive of mental processes and thus also constitutive of disordered and pathological mental processes. We agree and see much promise of applying these insights to addiction. In particular, we propose that an *enactive* approach may do the

[1]The term "morphia" used in the English edition is equivalent to the term "morphine" that is employed nowadays.

clinical phenomena of addiction more justice, while also being consistent with biological findings.

Enactivism emerged as an alternative to current mainstream cognitive science, emphasizing the dynamical, self-organized, embodied, affective, intersubjective, and situated nature of cognition, as well as its phenomenological dimension (10). The enactive approach emphasizes the centrality of agency for understanding mind and behavior. An agent is understood as "a self-constructed unity that engages the world by actively regulating its exchanges with it for adaptive purposes that are meant to serve its continued viability" (44). This means that agents generate an *identity* through their activity in the world, and strive to preserve it in the face of external perturbations and in spite of its intrinsic precariousness and entropic trends. In order to do that, agents need to be *adaptive,* i.e., they need to regulate themselves to stay within the limits of their viability (44).

On a biological level, agents seek to preserve a metabolic identity in order to survive. However, in the case of humans, they also strive to maintain habitual identities (45). In this regard, according to enactivism, the preservation of habits constitutes a central source of normativity that guides an agent's perception, thought and behavior: agents will tend to avoid situations and actions that may threaten their habitual identities and to look for favorable ones (46). Accordingly, agents create meaningful relations with the world in the sense that everything that contributes to the conservation of their biological and habitual identity is seen as intrinsically *good* and *attractive,* while everything that challenges its subsistence as intrinsically *bad* and *aversive* (47).

This framework also suggests "bundles of habits" (48) constituting a complex network of *regional identities* that involve bodily and neural processes, as well as interactions with the material, social, and cultural environment. These identities mutually enable and restrain each other (49), giving rise through their interaction to a global identity i.e., a loosely assembled *self*.

Under this perspective, addiction is considered one of the many habitual identities that constitute an addict's form of life and that is so deeply ingrained into the agent's physiology that it alters her metabolic autonomy and escalates to dependence. In this sense, addiction can be regarded as a *bad or pathological* habit because it endangers or constrains some of the addict's other identities, such as the biological or social ones. In dynamical systems terms, it can be said that addicts are stuck in a suboptimal attractor, which creates a tension that may manifest as frustration or anxiety for not being able to develop other regional identities. This view thus places addiction within the self, and not as a compulsion or an alien force. Additionally, it acknowledges addicts as autonomous agents that strive to preserve an identity that they have forged through a long history of interactions with their material, social, and cultural environment.

This perspective helps to explain the puzzling but common behaviors of individuals initiating treatment, but smuggling drugs into it and failing to disclose the full extent of usage: these behaviors can be seen as ways of maintaining the addict's form of life, which is being threatened by treatment. Furthermore, addictions may be so difficult to override not only because of their self-sustaining character, but also because their dynamics influence the formation and maintenance of other related habits, including social ones, thus making it necessary to change many other regional identities and, eventually to perform more extensive reshaping of the addict's entire self and its narratives. In order to do this, the enactive approach emphasizes the need to take into account the embodied, affective, situated, intersubjective, and extended aspects of addiction, as well as its phenomenological and dynamical dimensions to achieve a broader understanding and an impact on treatment. We propose that these factors should be a prominent focus of future research on addiction.

While we argue for this approach within the context of a very "underdeveloped" clinical psychopathology, its value will only be realized if it can better integrate diverse aspects of the disorder, including psychopathology and neurobiological findings; if it can predict patients' trajectories; and if it facilitates the development of new effective treatments. One future line of research can come from relating this enactive perspective with the theory of allostasis. In this regard, for example, the enactive notion of *adaptivity*, understood as "the capacity of an organism to regulate itself with respect to the boundaries of its own viability" (44) can be conceptually linked to that of allostasis, which refers to the principle that "to maintain stability an organism must *vary* all the parameters of its internal milieu and match them appropriately to environmental demands." (34). Additionally, both frameworks regard the brain as an interacting dynamical system embedded within larger ecological systems. In fact, the notion of allostasis has started to be incorporated within the enactive approach in relation to autonomy and self-regulation (50). We believe that this exchange will be mutually beneficial, for it can provide enactivism with a more solid physiological and empirical grounding and connect allostasis theory with science informed cutting-edge philosophy of mind.

CONCLUSIONS

Levinstein's monograph ends with case histories. The last case is about Darius, who dies during treatment. The author suggests that he died because he relapsed and overdosed (2). This may be taken as a reminder of the high human and societal cost induced by addiction. A more mature theory of the "pathologies" of the mind, as well as their relationship to individuals' experiences, actions, and brain mechanisms seems to be urgently required. This need may be most pronounced in the field of substance use disorders, and it appears to be time to move beyond the traditional framework of "folk psychology" and brain mechanisms. The need to incorporate science-based and philosophically informed developments in understanding the mind, such as those suggested by the 4E approaches to cognition, appears to

be more than a mere academic exercise; it might actually be considered a necessary step to successfully integrate and further develop preclinical neuroscience and clinical psychopathology.

AUTHOR CONTRIBUTIONS

CS wrote the initial draft of the manuscript. SR-V expanded the section on new approaches to cognitive science. All authors worked on shaping the manuscript into its final form.

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REFERENCES

1. Levinstein E. *Die Morphiumsucht: eine Monographie*. Berlin: Hirschwald (1877).
2. Levinstein E. *Morbid Craving for Morphia: A Monograph Founded on Personal Observations*. London: Smith, Elder (1878).
3. Levinstein E. *La Morphiomanie, Monographie Basée sur des Observations Personnelles*. Paris: Masson (1878). P. 156.
4. Schadewaldt H. Einführung und Geschichte zur Theorie von Mißbrauch und Abhängigkeit. In: Feuerlein W, editor. *Theorie der Sucht*. Berlin; Heidelberg: Springer (1986). pp. 151–64.
5. Leshner AI. Addiction is a brain disease, and it matters. *Science* (1997) 278:45–7.
6. Heyman GM. *Addiction: A Disorder of Choice*. Cambridge, MA: Harvard University Press (2009).
7. Pickard H, Ahmed SH, Foddy B. Alternative models of addiction. *Front Psychiatry* (2015) 4:20. doi: 10.3389/fpsyt.2015.00020
8. Chambers RA. The addiction psychiatrist as dual diagnosis physician: a profession in great need and greatly needed. *J Dual Diagn.* (2013) 9: 260–6. doi: 10.1080/15504263.2013.807072
9. Varela FJ, Thompson E, Rosch E. *The Embodied Mind: Cognitive Science and Human Experience*. Cambridge, MA: MIT Press (1991).
10. Stewart J, Gapenne O, Paolo EAD, editors. *Enaction: Toward a New Paradigm for Cognitive Science*. Cambridge, MA: The MIT Press (2010).
11. Jaspers K. *Allgemeine Psychopathologie*. Berlin; Heidelberg: Springer (1973).
12. Berrios GE. *The History of Mental Symptoms: Descriptive Psychopathology Since the Nineteenth Century*. New York, NY: Cambridge University Press (1996).
13. Häfner H. Descriptive psychopathology, phenomenology, and the legacy of Karl Jaspers. *Dialog Clin Neurosci.* (2015) 17:19–29.
14. Andreasen NC. DSM and the death of phenomenology in America: an example of unintended consequences. *Schizophrenia Bull.* (2007) 33:108–12. doi: 10.1093/schbul/sbl054
15. Ratcliffe M. *Rethinking Commonsense Psychology: A Critique of Folk Psychology, Theory of Mind and Simulation*. Hampshire: Palgrave Macmillan (2007).
16. Flanagan O. The shame of addiction. *Front Psychiatry* (2013) 4:120. doi: 10.3389/fpsyt.2013.00120
17. Schlimme JE. Willensschwäche und Getriebensein - Zugänge zu Jaspers' Verständnis der Sucht. In: Rinofer-Kreidl S, Wiltsche HA, editors. *Karl Jaspers' Allgemeine Psychopathologie zwischen Wissenschaft, Philosophie und Praxis*. Würzburg: Königshausen & Neumann (2008). pp. 147–70.
18. Schott H. Serie-Alkoholismus: Das Alkoholproblem in der Medizingeschichte. *Dtsch Arztebl* (2001) 98:1958–62.
19. Nahmias E, Morris S, Nadelhoffer T, Turner J. Surveying freedom: folk intuitions about free will and moral responsibility. *Philos Psychol.* (2005) 18:561–84. doi: 10.1080/09515080500264180
20. Haggard P. Human volition: towards a neuroscience of will. *Nat Rev Neurosci.* (2008) 9:934–46. doi: 10.1038/nrn2497
21. Clark CJ, Luguri JB, Ditto PH, Knobe J, Shariff AF, Baumeister RF. Free to punish: a motivated account of free will belief. *J Person Soc Psychol.* (2014) 106:501–13. doi: 10.1037/a0035880
22. Lewis M. *The Biology of Desire: Why Addiction is Not a Disease*. New York, NY: Public Affairs (2015).
23. Fuchs T. *Ecology of the Brain: The Phenomenology and Biology of the Embodied Mind*. New York, NY: Oxford University Press (2018).
24. Bandura A. Self-efficacy: toward a unifying theory of behavioral change. *Psychol Rev.* (1977) 84:191–215.
25. Baldwin AS, Rothman AJ, Hertel AW, Linde JA, Jeffery RW, Finch EA, et al. Specifying the determinants of the initiation and maintenance of behavior change: an examination of self-efficacy, satisfaction, and smoking cessation. *Health Psychol.* (2006) 25:626–34. doi: 10.1037/0278-6133.25.5.626
26. Greenfield SF, Hufford MR, Vagge LM, Muenz LR, Costello ME, Weiss RD. The relationship of self-efficacy expectancies to relapse among alcohol dependent men and women: a prospective study. *J Stud Alcoh.* (2000) 61:345–51. doi: 10.15288/jsa.2000.61.345
27. Shiffman S, Balabanis MH, Paty JA, Engberg J, Gwaltney CJ, Liu KS, et al. Dynamic effects of self-efficacy on smoking lapse and relapse. *Health Psychol.* (2000) 19:315–23. doi: 10.1037/0278-6133.19.4.315
28. Pickard H. Responsibility without blame for addiction. *Neuroethics* (2017) 10:169–80. doi: 10.1007/s12152-016-9295-2
29. Pickard H. Denial in addiction. *Mind Lang.* (2016) 31:277–99. doi: 10.1111/mila.12106
30. Shaffer HJ. Denial, ambivalence, and countertransferenctial hate. In: Levin JD, Weiss RH, editors. *The Dynamics and Treatment of Alcoholism: Essential Papers*. Northvale, NJ: Aronson (1994). pp. 421–37.
31. Goldstein RZ, Craig ADB, Bechara A, Garavan H, Childress AR, Paulus MP, et al. The neurocircuitry of impaired insight in drug addiction. *Trends Cogn Sci.* (2009) 13:372–80. doi: 10.1016/j.tics.2009.06.004
32. Le Berre A-P, Sullivan EV. Anosognosia for memory impairment in addiction: insights from neuroimaging and neuropsychological assessment of metamemory. *Neuropsychol Rev.* (2016) 26:420–31. doi: 10.1007/s11065-016-9323-3
33. Moeller SJ, Maloney T, Parvaz MA, Alia-Klein N, Woicik PA, Telang F, et al. Impaired insight in cocaine addiction: laboratory evidence and effects on cocaine-seeking behaviour. *Brain* (2010) 133:1484–93. doi: 10.1093/brain/awq066
34. Sterling P, Eyer J. Allostasis: a new paradigm to explain arousal pathology. In: Fisher S, Reason J, editors. *Handbook of Life Stress, Cognition and Health*. Chichester: John Wiley & Sons (1988). pp. 629–49.
35. Koob GF, Le Moal M. Drug abuse: hedonic homeostatic dysregulation. *Science* (1997) 278:52–8. doi: 10.1126/science.278.5335.52
36. Koob GF, Le Moal M. Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* (2001) 24:97–129. doi: 10.1016/S0893-133X(00)00195-0
37. Ahmed SH, Kenny PJ, Koob GF, Athina M. Neurobiological evidence for hedonic allostasis associated with escalating cocaine use. *Nat Neuroscience* (2002) 5:625–6. doi: 10.1038/nn872
38. Koob GF, Le Moal M. Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. *Nat Neurosci.* (2003) 8:1442–4. doi: 10.1038/nn1105-1442
39. McEwen BS. Protective and damaging effects of stress mediators. *N Engl J Med.* (1998) 338:171–9.

40. Paris J. Is psychoanalysis still relevant to psychiatry? *Can J Psychiatry* (2017) 62:308–12. doi: 10.1177/0706743717692306
41. Insel T, Cuthbert B, Garvey M, Heinssen R, Pine DS, Quinn K, et al. Research Domain Criteria (RDoC): Toward a new classification framework for research on mental disorders. *Am J Psychiatry* (2010) 167:748–51. doi: 10.1176/appi.ajp.2010.09091379
42. Gottesman II, Gould TD. The endophenotype concept in psychiatry: etymology and strategic intentions. *Am J Psychiatry* (2003) 160:636–45. doi: 10.1176/appi.ajp.160.4.636
43. Walter H. The third wave of biological psychiatry. *Front Psychol.* (2013) 4:582. doi: 10.3389/fpsyg.2013.00582
44. Di Paolo EA. Autopoiesis, adaptivity, teleology, agency. *Phenomenology and the Cognitive Sciences* (2005) 4:429–52. doi: 10.1007/s11097-005-9002-y
45. Di Paolo EA, Buhrmann T, Barandiaran XE. *Sensorimotor Life: An Enactive Proposal*. Oxford: Oxford University Press (2017).
46. Di Paolo EA. Extended life. *Topoi* (2009) 28:9–21. doi: 10.1007/s11245-008-9042-3
47. Di Paolo EA. Robotics inspired in the organism. *Intellectica* (2010) 1–2:129–62.
48. James W. *Habit*. New York, NY: Henry Holt and Company (1914).
49. Egbert MD, Barandiaran XE. Modeling habits as self-sustaining patterns of sensorimotor behavior. *Front Hum Neurosci.* (2014) 8:590. doi: 10.3389/fnhum.2014.00590
50. Vernon D, Lowe R, Thill S, Ziemke T. Embodied cognition and circular causality: on the role of constitutive autonomy in the reciprocal coupling of perception and action. *Front Psychol.* (2015) 6:1660. doi: 10.3389/fpsyg.2015.01660

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Larger Numbers of Glial and Neuronal Cells in the Periaqueductal Gray Matter of μ-Opioid Receptor Knockout Mice

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Background: μ-opioid receptor knockout (MOP-KO) mice display baseline hyperalgesia. We have recently identified changes in tissue volume in the periaqueductal gray matter (PAG) using magnetic resonance imaging voxel-based morphometry. Changes in the structure and connectivity of this region might account for some behavior phenotypes in MOP-KO mice, including hyperalgesia.

Methods: Adult male MOP-KO and wild-type (WT) mice were studied. Immunohistochemistry was performed to detect microglia, astrocytes, and neurons in the PAG using specific markers: ionized calcium-binding adaptor molecule 1 (Iba-1) for microglia, glial fibrillary acidic protein (GFAP) for astrocytes, and the neuronal nuclei antigen (NeuN; product of the Rbfox3 gene) for neurons, respectively. Cell counting was performed in the four parallel longitudinal columns of the PAG (dorsomedial, dorsolateral, lateral, and ventrolateral) at three different locations from bregma (−3.5, −4.0, and −4.5 mm).

Results: The quantitative analysis showed larger numbers of well-distributed Iba1-IR cells (microglia), NeuN-IR cells (neurons), and GFAP-IR areas (astrocytes) at all the anatomically distinct regions examined, namely, the dorsomedial (DM) PAG, dorsolateral (DL) PAG, lateral (L) PAG, and ventrolateral (VL) PAG, in MOP-KO mice than in control mice.

Conclusions: The cellular changes in the PAG identified in this paper may underlie aspects of the behavioral alterations produced by MOP receptor deletion, and suggest that alterations in the cellular structure of the PAG may contribute to hyperalgesic states.

Keywords: μ opioid, μ opioid receptor knockout (MOP-KO), periaqueductal gray matter (PAG), microglia, astrocytes, neuron, immunohistochemistry

INTRODUCTION

Studies in knockout (KO) mice have demonstrated that μ-opioid (MOP) receptors play crucial roles in several physiological functions, including nociception, stress responses, tolerance, reward learning, and immune function (1–5). Although these effects of MOP deletion have been generally thought to result simply from elimination of MOP signaling (6), as would be expected from elimination of opiate reinforcement in MOP-KO mice (7, 8), effects on drug reinforcement extend to abused drugs acting through diverse mechanisms [(9–11), see summary in Hall et al. (12)]. This may still just indicate a role for MOP in drug reinforcement generally, just as baseline hyperalgesia may indicate a role of MOP in basal nociception (1, 8). However, there certainly is evidence for neuroadaptations to the elimination of MOP. Most recently, we have found brain volume differences in the periaqueductal gray matter (PAG), olfactory bulb, arcuate nucleus, and several cerebellar regions using magnetic resonance imaging voxel-based morphometry (13).

Several factors are likely to be involved in the brain volume abnormalities in the PAG caused by the deletion of MOP receptors. The brains of several strains of mutant mice exhibit structural changes, that correlate with behavioral consequences of the genetic modifications (14). Since glial cells take up a large portion of neural tissue, it is likely that glial changes may account for some of these differences in tissue volume (although that does not exclude changes in the volume of the neuropil as well, which would be likely to drive changes in glial numbers or volume). Indeed, glial changes are found in altered pain states in the spinal cord, as well as marked changes in specific brain regions (15–17). Changes in some regions may involve a role of opioids in brain development, but others may involve adult plasticity. Hippocampal neurogenesis is affected by MOP deletion (18). Although opioid systems modulate neural stem cell progenitor differentiation and influence aspects of neural development, it is important to note that opioid agonists also affect neurogenesis in adult animals (19, 20), indicating that these effects are not necessarily developmental in nature. Current evidence support genetic factors affect brain functional connectivity and organization (21). MOP receptor gene alters the widespread brain functional connectome and remodels the reward/aversion circuit (22). Such connectivity remodeling may account for brain morphology alterations.

Thus, it is likely that alterations in MOP signaling may have broader effects on developmental and adult neuroplasticity, in addition to simply altering MOP activity. This might be evidenced as altered brain morphology and connectivity. In our previous study, finding increased size of the PAG in MOP-KO mice, histological analysis did not reveal apparent cellular pathological changes, based on conventional hematoxylin and eosin/Klüver-Barrera staining, although there were increased neural cell numbers (13). Consequently, the aim of the present study was to investigate the contribution of different neural cell types to the volume and cell number differences in the PAG resulting from genetic elimination of MOP receptors.

MATERIALS AND METHODS

Animals

All animals were treated in compliance with the "Principles of Laboratory Animal Care" (National Society for Medical Research) and the "Guide for the Care and Use of Laboratory Animals" (National Academies of Sciences). The Animal Care and Use Committee of the Tohoku University Graduate School of Medicine approved this study.

Congenic homozygous male MOP-KO (N = 7) and wild-type (WT, N = 7) mice that had been backcrossed for at least 20 generations to C57BL/6J mice were used (1). All mice were housed at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine, in a colony maintained at an ambient temperature of 22 ± 2°C, on a 12 h light:12 h dark cycle (lights on: 08:00–20:00) with food and water available *ad libitum*. Four to six mice were housed per cage. All mice were 12 weeks old at the time of sacrifice for immunohistochemical analysis.

Immunohistochemistry

Each mouse was anesthetized by intraperitoneal administration of a combination of medetomidine (0.3 mg/kg, Medetomin; Meiji Seika Pharma, Co., Ltd., Tokyo, Japan) and butorphanol (2 mg/kg, Betrorphal; Meiji Seika Pharma, Co., Ltd., Tokyo, Japan). Local anesthesia, with 2% lidocaine (diluted to 0.5%, 3 mg/kg), was performed at the incision site. Animals were perfused transcardially with cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS for 30 min, at rate of 7 mL/min. After perfusion, the brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PBS. After post-fixation, tissues were embedded in paraffin using a specialized automated tissue processing system (Tissue-Tek, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) at 58°C; 5-μm coronal sections were cut from the three anatomically distinct regions of the PAG (bregma: −3.5, −4.0, and −4.5 mm) for each of the brains from MOP-KO and WT mice (23). For each of the three regions, 5 serial sections (total number of sections: 15 per mouse) were collected.

Each formalin-fixed and paraffin wax-embedded tissue section was cleaned in xylene and rehydrated with decreasing concentrations of ethanol. For studying microglia, each section was subjected to a standard antigen retrieval procedure consisting of 5 min autoclaving at 120°C in antigen retrieval buffer, using pretreatment regent (Deparaffinization/Antigen Retrieval Solution, pH 9; Nichirei Bioscience, Tokyo, Japan), for ionized calcium-binding adapter molecule 1 (Iba1). The sections were cooled at 4°C for 30–45 min and incubated with the primary antibody (anti-Iba1 antibody, goat polyclonal, 1:2,000; Abcam, Tokyo, Japan) overnight at 4°C. The next day, the sections were washed three times with 0.01 M PBS (10 min per wash), and endogenous enzyme activity was blocked using 1% H_2O_2 for 20 min. Each section was stained using the indirect immunoperoxidase method (Histofine Simple Stain Max PO (G); Nichirei Bioscience), and a chromogen complex, 3,3′-diaminobenzidine tetrachloride (Simple Stain DAB Solution; Nichirei Bioscience) was used to visualize the targeted

antigens; the sections were then counterstained with hematoxylin (Chroma, Köngen, Germany).

To label astrocytes, a similar protocol was used for polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:2,000; Dako, Tokyo, Japan). Immunoreactivity was examined using the indirect immunoperoxidase method (Histofine Simple Stain Max PO (R); Nichirei Bioscience).

In order to detect neuronal nuclei (NeuN) expression in neurons, the antigen was retrieved by heating the samples in a microwave for 15 min at 100°C in 0.01 mol/L citrate buffer (pH 6.0). After the slides were washed, they were incubated with Blocking Reagent A (Nichirei Bioscience) for 1 h at room temperature. The sections were incubated with primary antibody (Anti-NeuN antibody, mouse monoclonal, 1:1,000; Millipore, CA, USA) overnight at 4°C. The next day, the sections were washed three times with 0.01 M PBS (10 min per wash) and endogenous enzyme activity was blocked using 1% H_2O_2 for 20 min. Each section was stained using the indirect immunoperoxidase method (Histofine Simple Stain Max PO (M); Nichirei Bioscience), and a chromogen complex, 3,3′-diaminobenzidine tetrachloride (Simple Stain DAB Solution, Nichirei Bioscience) was used to visualize the targeted antigens; the sections were then counterstained with hematoxylin, followed by incubation with Blocking Reagent B (Nichirei Bioscience).

The immunoreactivity of each antibody (GFAP, Iba1, and NeuN) for paraffin-embedded sections was confirmed using the procedures recommended in each product's data sheet.

Image Analysis

The number of immunoreactive (IR) nuclei (for Iba1-IR and NeuN-IR, that show distinct cells) and the immunoreactive area (for GFAP-IR, that shows a more diffuse staining) were quantified using a light microscope equipped with a computer-based automated cell counting system (BZ-9000, KEYENCE, Tokyo, Japan) at the four columns in the PAG (dorsomedial: DM, dorsolateral: DL, lateral: L, and ventrolateral: VL) and three different locations from the bregma (−3.5, −4.0, and −4.5 mm) in 300 × 300 μm^2 fields, following standard mouse brain coordinates (23). While viewing the automated cell counting system monitor, upper and lower thresholds of immunostaining gray level were set such that only Iba1-IR, GFAP-IR and NeuN-IR was accurately discriminated from the background in outlined PAG area. The boundaries of each PAG column were defined based on previously published anatomical criteria (24, 25).

Statistical Analysis

A P-value of 0.05 was considered statistically significant. The Mann-Whitney U-test with *post hoc* Bonferroni-Dunn corrected means comparisons were used to evaluate individual group differences. Statistical analyses were performed using IBM SPSS Statistics 2.4 (IBM, Chicago, IL, USA), GraphPad Prism Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA), and SigmaPlot Version 13.0 (Systat Software, Inc., CA, USA). Data is presented as median (interquartile range), unless mentioned otherwise.

RESULTS

The quantitative analysis showed larger numbers of well-distributed microglia (Iba1-IR), neurons (NeuN-IR) and astrocytes (GFAP-IR) at all the anatomically distinct regions examined, namely, the DMPAG, DLPAG, LPAG, and VLPAG, in MOP-KO mice than in control mice. The analysis showed that MOP-KO mice displayed greater numbers of Iba1-IR cells at −3.5 mm from bregma (WT: 3 [2–4] vs. MOP-KO: 8 [7–9], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 3 [2–4] vs. MOP-KO: 7 [6–8], $P < 0.001$, $n = 105$), and −4.5 mm from bregma (WT: 3 [2–4 vs. MOP-KO: 8 [7–9], $P < 0.001$, $n = 105$) in the DMPAG; −3.5 mm from bregma (WT: 3 [2–4] vs. MOP-KO: 7 [6–8], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 3 [2–4] vs. MOP-KO: 8 [7–9], $P < 0.001$, $n = 105$), and −4.5 mm from bregma (WT: 4 [3–5] vs. MOP-KO: 8 [7–9], $P < 0.001$, $n = 105$) in the DLPAG; −3.5 mm from bregma (WT: 2 [1–3] vs. MOP-KO: 6 [5–7], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 2 [1–3] vs. MOP-KO: 6 [5–7], $P < 0.001$, $n = 105$), −4.5 mm from bregma (WT: 3 [2–4] vs. MOP-KO: 7 [6–8], $P < 0.001$, $n = 105$) in the LPAG; and −4.5 mm from bregma (WT: 3 [2–4] vs. MOP-KO: 8 [7–9], $P < 0.001$, $n = 105$) in the VLPAG. These data are illustrated in **Figure 1** (also see the representative photomicrographs in **Figure 4**).

MOP-KO mice displayed larger GFAP-IR area (μm^2) at −3.5 mm from bregma (WT: 471.0 [455.8–486.0] vs. MOP-KO: 699.0 [685.0–711.3], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 548.0 [538.8–554.3] vs. MOP-KO: 854.5 [843.8–866.3], $P < 0.001$, $n = 105$), and −4.5 mm from bregma (WT: 351.0 [344.0–357.3] vs. MOP-KO: 923.0 [899.0–934.0], $P < 0.001$, $n = 105$) in the DMPAG; −3.5 mm from bregma (WT: 60.0 [58–62.0] vs. MOP-KO: 158.0 [153.0–164.3], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 63.0 [59.8–66.0] vs. MOP-KO: 248.5 [243.0–251.0], $P < 0.001$, $n = 105$), and −4.5 mm from bregma (WT: 51.0 [49.0–53.0] vs. MOP-KO: 350.5 [349.0–354.3], $P < 0.001$, $n = 105$) in the DLPAG; −3.5 mm from bregma (WT: 102.0 [99.0–108.3] vs. MOP-KO: 451.0 [450.0–455.0], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 99.0 [94.0–102.0] vs. MOP-KO: 437.0 [429.8–450.0], $P < 0.001$, $n = 105$), and −4.5 mm from bregma (WT: 131.0 [128.0–137.0] vs. MOP-KO: 609.5 [600.8–617.0], $P < 0.001$, $n = 105$) in the LPAG; and −4.5 mm from bregma (WT: 62.5 [59.8–68.0] vs. MOP-KO: 395.0 [388.8–403.0], $P < 0.001$, $n = 105$) in the VLPAG. These data are illustrated in **Figure 2** (also see the representative photomicrographs in **Figure 4**).

The analysis showed that MOP-KO mice had larger numbers of NeuN-IR cells at −3.5 mm from bregma (WT: 65.0 [60.5–69.0] vs. MOP-KO: 121.0 [116.5–127.0], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 98.0 [91.5–102.5] vs. MOP-KO: 142.0 [130.5–147.0], $P < 0.001$, $n = 105$), and −4.5 mm from bregma (WT: 105.0 [102.0–109.0] vs. MOP-KO: 150.0 [144.0–154.5], $P < 0.001$, $n = 105$) in the DMPAG; −3.5 mm from bregma (WT: 80.0 [75.0–82.0] vs. MOP-KO: 122.0 [117.0–126.0], $P < 0.001$, $n = 105$), −4.0 mm from bregma (WT: 90.0 [86.0–97.0] vs. MOP-KO: 143.0 [138.5–149.0], $P < 0.001$, $n = 105$), and −4.5 mm from

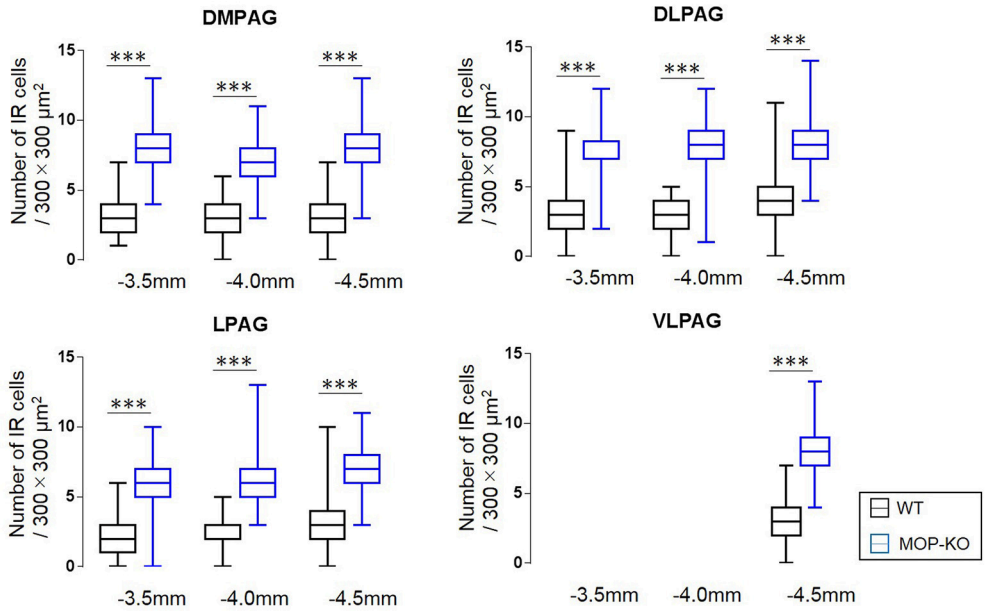

FIGURE 1 | Median values and interquartile ranges for the number of Iba1-IR cells in the examined fields (300 × 300 μm^2) in the DMPAG (−3.5, −4.0, and −4.5 mm from bregma), DLPAG (−3.5, −4.0, and −4.5 mm from bregma), LPAG (−3.5, −4.0, and −4.5 mm from bregma), and VLPAG (−4.5 mm from bregma). Fifteen slides per brain tissue block (N = 7) were designated for image analysis. The asterisk indicates significant differences (MOP-KO vs. WT mice). Iba1, ionized calcium-binding adapter molecule 1; IR, immunoreactive; DMPAG, dorsomedial periaqueductal gray matter (PAG); DLPAG, dorsolateral PAG; LPAG, lateral PAG; VLPAG, ventrolateral PAG. ***P < 0.001.

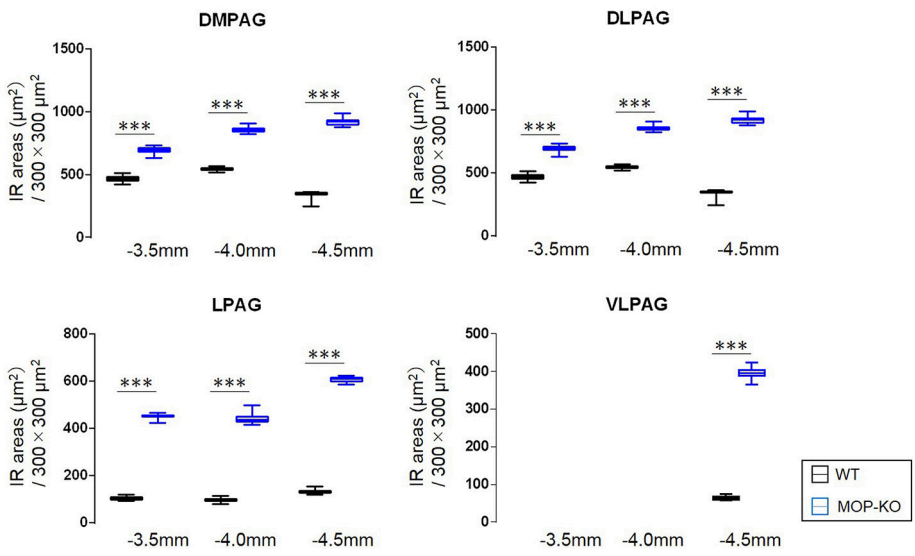

FIGURE 2 | Median values and interquartile ranges of the GFAP-IR areas (μm^2) in the examined fields (300 × 300 μm^2) in the DMPAG (−3.5, −4.0, and −4.5 mm from bregma), DLPAG (−3.5, −4.0, and −4.5 mm from bregma), LPAG (−3.5, −4.0, and −4.5 from bregma), and VLPAG (−4.5 mm from bregma). Fifteen slides per brain tissue block (N = 7) were designated for image analysis. The asterisk indicates significant differences (MOP-KO vs. WT). GFAP, glial fibrillary acidic protein; DMPAG, dorsomedial periaqueductal gray matter (PAG); DLPAG, dorsolateral PAG; LPAG, lateral PAG; VLPAG, ventrolateral PAG. ***P < 0.001.

bregma (WT: 84.0 [80.0–86.0] vs. MOP-KO: 147.0 [141.5–151.0], P < 0.001, n = 105) in the DLPAG; −3.5 mm from bregma (WT: 85.0 [81.0–90.0] vs. MOP-KO: 160.0 [154.0–165.0], P < 0.001, n = 105), −4.0 mm from bregma (WT: 75.0 [72.0–79.0] vs. MOP-KO: 130.0 [128.0–136.0], P < 0.001, n = 105), and −4.5 mm from bregma (WT: 100.0 [98.0– 105.0] vs. MOP-KO: 140 [133.0–145.5], P < 0.001, n = 105) in the LPAG; and −4.5 mm (WT: 90.0 [88.0–93.0] vs. MOP-KO: 151.0 [146.0–155.0], P < 0.001, n = 105) in the VLPAG. These data are illustrated in **Figure 3** (also see the representative photomicrographs in **Figure 4**).

DISCUSSION

The aim of this study was to determine the contribution of changes in the numbers of different neural cell types (or area for astrocytes) to the volume changes in the PAG of MOP-KO mice. Immunohistochemical analysis revealed that enlarged brain size was accompanied by an increase in the number of microglia and neurons, and area of astrocyte immunoreactivity, in all of the anatomically distinct regions of the PAG that were examined (these included three different locations from the bregma [−3.5, −4.0, and −4.5 mm] and four different columns in the PAG [dorsomedial, dorsolateral, lateral, and ventrolateral].

Regulation of neurodevelopment by the endogenous opioid system is an important concept for the interpretation of our

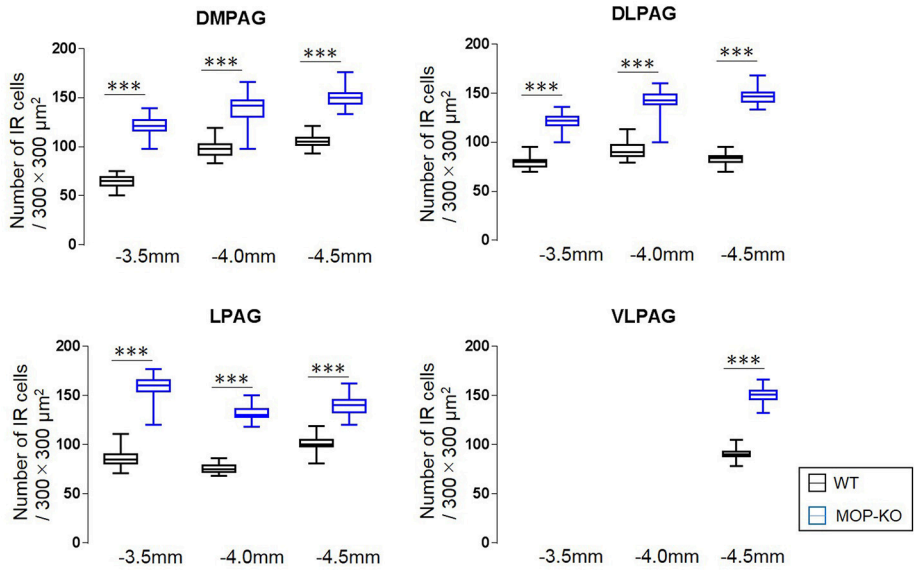

FIGURE 3 | Median values and interquartile ranges for the number of NeuN-IR cells in the examined fields (300 × 300 μm^2) in the DMPAG (−3.5, −4.0, and −4.5 mm from bregma), DLPAG (−3.5 mm, −4.0 mm, and −4.5 mm from bregma), LPAG (−3.5, −4.0, and −4.5 mm from bregma), and VLPAG (−4.5 mm from bregma). Fifteen slides per brain tissue block (N = 7) were designated for image analysis. The asterisk indicates significant differences (MOP-KO vs. WT). NeuN, neuronal nuclei; DMPAG, dorsomedial periaqueductal gray matter (PAG); DLPAG, dorsolateral PAG; LPAG, lateral PAG; VLPAG, ventrolateral PAG. ***P < 0.001.

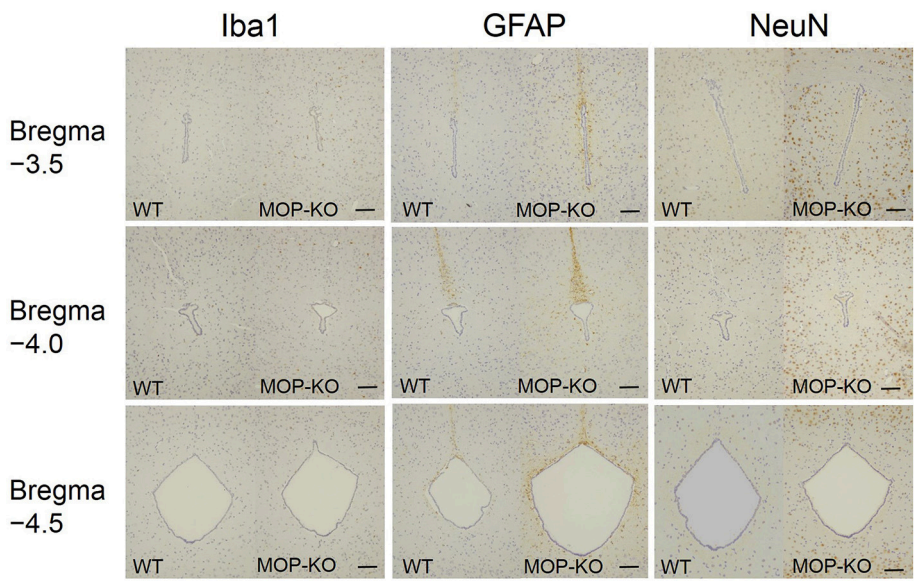

FIGURE 4 | Representative photomicrographs of Iba1-IR cells, GFAP-IR areas, and NeuN-IR cells in the PAG. Iba1, ionized calcium-binding adapter molecule 1; IR, immunoreactive; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; PAG, periaqueductal gray matter. Scale bars indicate 100 μm.

findings. Firstly, it is of interest to note that opioid receptor blockade increases DNA synthesis in germinal neural cells (26). Opioid antagonists exert a marked, stereospecific influence on the growth of neural tissues, depending on the duration of opioid receptor blockade (27). Continuous daily blockade of opioid receptors increases the number of cells in the cerebellum, whereas intermittent opioid receptor blockade decreases the number of these cells (26). Quantitative analysis has demonstrated that all cerebellar cell types, including granule cells, Purkinje cells, and glial cells, contribute to this cerebellar plasticity (28). Given these facts, our findings are in line with the cerebellar structural changes induced by continuous daily blockade of opioid receptor using naltrexone in postnatal rats (27).

However, the specific mechanisms by which MOP receptors regulate neural development in specific brain areas is yet to be determined, and likely to involve more than just MOP receptor density. Distribution and levels of MOP receptors in the brain do not predict those areas in which increased cell numbers are observed, such as the cerebellum and PAG, since neither of those areas contain high levels of MOP receptors (29), and many regions with high levels of MOP expression do not seem to be affected.

Our results indicate that structural abnormalities in the PAG may not relate to the anatomical locations and cellular organization of PAG circuits because increased cell numbers were observed in all cell types, in all PAG columns—there is just more of everything. Each anatomical subdivision of the PAG does have a role in distinct physiological functions that include the control and expression of pain, analgesia, fear, and anxiety (30, 31). Briefly, dorsolateral PAG stimulation evokes active coping strategies, such as fight/flight behaviors, non-opioid-mediated analgesia, hypertension, and tachycardia. The lateral PAG appears to coordinate non-opioid analgesia, active defensive behaviors, and exerts a hypertensive effect. Ventrolateral stimulation, on the other hand, evokes passive defensive behaviors, such as quiescence, opioid-mediated analgesia, hypotension, and bradycardia (25, 31, 32). Our findings do not necessarily indicate a large shift in these behavioral circuits mediated by PAG since all areas were affected, although these areas were not entirely equally affected. It will require more work to determine if these small differences affect the activity of individual PAG subregions and the behavior circuits that they influence.

A number of studies have demonstrated the importance of several brain sites in modulating nociception and/or mediating analgesic effects, including the PAG (33, 34), thalamus (35), hypothalamus, and amygdala (34). It is well-known that the endogenous descending pain modulatory circuit originates in the PAG and includes neurons in the rostral ventromedial medulla and spinal cord dorsal horn (36). Evidence indicates that glial plasticity in specific brain areas affect pain states, beyond the changes in numbers of glia occurring in the spinal cord and the peripheral nerves (15–17). Marked structural glial modifications may also occur in the PAG of MOP-KO mice that have baseline hyperalgesia, but the present study demonstrates that increased numbers of neuronal cells also contribute to volume abnormalities in that brain region. Neuronal changes in specific brain areas associated with chronic pain states, including hyperalgesia and allodynia, remain to be fully elucidated, so the extent to which these changes are specifically involved in hyperalgesia remains to be seen.

Our study has some limitations. Firstly, the examination was performed at 12 weeks of age, and our findings require corroboration from investigations at earlier stages of life. Secondly, increased regional gray matter volume in MOP-KO mice was observed in other brain regions, such as the hypothalamus and olfactory bulb (13). Further investigation of these brain regions is warranted to determine whether changes in all neural cell types are also detected in these regions as well. Thirdly, we did not examine baseline cytokine levels, or other potential mediators beyond MOP in MOP-KO mice that show baseline hyperalgesia. Specific cytokines may influence the numbers of glial cells observed in the PAG due to the deletion of MOP receptors. Finally, further study is warranted to clarify the sex-bias in each genotype since we did not analyze the data by sex.

In conclusion, the present study shows that increased numbers of microglia, and neurons, and greater astrocytic area, in the PAG in MOP-KO mice might result from developmental roles of by the endogenous opioid system. Further investigations based on the present findings are necessary to elucidate whether structural changes are observed earlier in life, and if so, why these changes are induced in specific brain areas, and what phenotypic outcomes are mediated by these cellular changes.

AUTHOR CONTRIBUTIONS

KS study design, immunohistochemistry, data interpretation, statistical analysis, and preparation of the manuscript. FH, GU, and IS data interpretation and preparation of the manuscript. All authors have read and approved the manuscript.

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REFERENCES

1. Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, et al. Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci USA.* (1997) 94:1544–9.
2. Wang J, Charboneau R, Barke RA, Loh HH, Roy S. μ-opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. *J Immunol.* (2002) 169:3630–6. doi: 10.4049/jimmunol.169.7.3630
3. Ide S, Minami M, Satoh M, Uhl GR, Sora I, Ikeda K. Buprenorphine antinociception is abolished, but naloxone-sensitive reward is retained, in mu-opioid receptor knockout mice. *Neuropsychopharmacology* (2004) 29:1656–63. doi: 10.1038/sj.npp.1300463
4. Ide S, Sora I, Ikeda K, Minami M, Uhl GR, Ishihara K. Reduced emotional and corticosterone responses to stress in mu-opioid receptor knockout mice. *Neuropharmacology* (2010) 58:241–7. doi: 10.1016/j.neuropharm.2009.07.005
5. Komatsu H, Ohara A, Sasaki K, Abe H, Hattori H, Hall FS, et al. Decreased response to social defeat stress in mu-opioid-receptor knockout mice. *Pharmacol Biochem Behav.* (2011) 99:676–82. doi: 10.1016/j.pbb.2011.06.008
6. Mizoguchi H, Wu HE, Narita M, Sora I, Hall SF, Uhl GR, et al. Lack of μ-opioid receptor-mediated G-protein activation in the spinal cord of mice lacking Exon 1 or Exons 2 and 3 of the MOR-1 gene. *J Pharmacol Sci.* (2003) 93:423–9. doi: 10.1254/jphs.93.423
7. Sora I, Elmer G, Funada M, Pieper J, Li XF, Hall FS, et al. Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu receptor reserve. *Neuropsychopharmacology* (2001) 25:41–54. doi: 10.1016/s0893-133x(00)00252-9
8. Hall FS, Li XF, Goeb M, Roff S, Hoggatt H, Sora I, et al. Congenic C57BL/6 μ opiate receptor (MOR) knockout mice: baseline and opiate effects. *Genes Brain Behav.* (2003) 2:114–21. doi: 10.1034/j.1601-183X.2003.00016.x
9. Hall FS, Sora I, Uhl GR. Ethanol consumption and reward are decreased in μ-opiate receptor knockout mice. *Psychopharmacology* (2001) 154:43–9. doi: 10.1007/s002130000622
10. Hall FS, Goeb M, Li XF, Sora I, Uhl GR. mu-Opioid receptor knockout mice display reduced cocaine conditioned place preference but enhanced sensitization of cocaine-induced locomotion. *Brain Res Mol Brain Res.* (2004) 121:123–30. doi: 10.1016/j.molbrainres.2003.10.024
11. Moriya Y, Kasahara Y, Hall FS, Sakakibara Y, Uhl GR, Tomita H, et al. Sex differences in the effects of adolescent social deprivation on alcohol consumption in mu-opioid receptor knockout mice. *Psychopharmacology* (2015) 232:1471–82. doi: 10.1007/s00213-014-3784-y
12. Hall FS, Sora I, Uhl GR. Molecular mechanisms underlying behavioral effects of cocaine: lessons from knockout mice. *Curr Pharmacogenom.* (2004) 2:185–202. doi: 10.2174/1570160043377673
13. Sasaki K, Sumiyoshi A, Nonaka H, Kasahara Y, Ikeda K, Hall FS, et al. Specific regions display altered grey matter volume in mu-opioid receptor knockout mice: MRI voxel-based morphometry. *Br J Pharmacol.* (2015) 172:654–67. doi: 10.1111/bph.12807
14. Nieman BJ, Lerch JP, Bock NA, Chen XJ, Sled JG, Henkelman RM. Mouse behavioral mutants have neuroimaging abnormalities. *Hum Brain Mapp.* (2007) 28:567–75. doi: 10.1002/hbm.20408
15. Kuzumaki N, Narita M, Narita M, Hareyama N, Niikura K, Nagumo Y, et al. Chronic pain-induced astrocyte activation in the cingulate cortex with no change in neural or glial differentiation from neural stem cells in mice. *Neurosci Lett.* (2007) 415:22–7. doi: 10.1016/j.neulet.2006.12.057
16. Zhao P, Waxman SG, HainsBC. Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J Neurosci.* (2007) 27:8893–902. doi: 10.1523/jneurosci.2209-07.2007
17. Miyamoto K, Kume K, Ohsawa M. Role of microglia in mechanical allodynia in the anterior cingulate cortex. *J Pharmacol Sci.* (2017) 134:158–65. doi: 10.1016/j.jphs.2017.05.010
18. Harburg GC, Hall FS, Harrist AV, Sora I, Uhl GR, Eisch AJ. Knockout of the mu opioid receptor enhances the survival of adult-generated hippocampal granule cell neurons. *Neuroscience* (2007) 144:77–87. doi: 10.1016/j.neuroscience.2006.09.018
19. Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ. Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci USA.* (2000) 97:7579–84. doi: 10.1073/pnas.120552597
20. Arguello AA, Harburg GC, Schonborn JR, Mandyam CD, Yamaguchi M, Eisch AJ. Time course of morphine's effects on adult hippocampal subgranular zone reveals preferential inhibition of cells in S phase of the cell cycle and a subpopulation of immature neurons. *Neuroscience* (2008) 157:70–9. doi: 10.1016/j.neuroscience.2008.08.064
21. Thompson PM, Ge T, Glahn DC, Jahanshad N, Nichols TE. Genetics of the connectome. *Neuroimage* (2013) 80:475–88. doi: 10.1016/j.neuroimage.2013.05.013
22. Mechling AE, Arefin T, Lee HL, Bienert T, Reisert M, Ben Hamida S, et al. Deletion of the mu opioid receptor gene in mice reshapes the reward-aversion connectome. *Proc Natl Acad Sci USA.* (2016) 113:11603–8. doi: 10.1073/pnas.1601640113
23. Franklin K, Paxinos, G. The Mouse Brain in Stereotaxic Coordinates 3rd ed. Boston, MA: Elsevier Academic Press (2004).
24. Bandler R, Shipley MT. Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci.* (1994) 17:379–89.
25. Bandler R, Keay KA, Floyd N, Price J. Central circuits mediating patterned autonomic activity during active vs. passive emotional coping. *Brain Res Bull.* (2000) 53:95–104. doi: 10.1016/S0361-9230(00)00313-0
26. Zagon IS, McLaughlin PJ. Endogenous opioid systems regulate cell proliferation in the developing rat brain. *Brain Res.* (1987) 412:68–72.
27. Zagon IS, McLaughlin PJ. Naltrexone modulates body and brain development in rats: a role for endogenous opioid systems in growth. *Life Sci.* (1984) 35:2057–64.
28. Zagon IS, McLaughlin PJ. Opioid antagonist (naltrexone) modulation of cerebellar development: histological and morphometric studies. *J Neurosci.* (1986) 6:1424–32.
29. Gackenheimer SL, Suter TM, Pintar JE, Quimby SJ, Wheeler WJ, Mitch CH, et al. Localization of opioid receptor antagonist [3H]-LY255582 binding sites in mouse brain: comparison with the distribution of mu, delta and kappa binding sites. *Neuropeptides* (2005) 39:559–67. doi: 10.1016/j.npep.2005.09.006
30. Behbehani MM. Functional characteristics of the midbrain periaqueductal gray. *Prog Neurobiol.* (1995) 46:575–605.
31. Linnman C, Moulton EA, Barmettler G, Becerra L, Borsook D. Neuroimaging of the periaqueductal gray: state of the field. *Neuroimage* (2012) 60:505–22. doi: 10.1016/j.neuroimage.2011.11.095
32. Dampney R. Emotion and the cardiovascular system: postulated role of inputs from the medial prefrontal cortex to the dorsolateral periaqueductal gray. *Front Neurosci.* (2018) 12:343. doi: 10.3389/fnins.2018.00343
33. Lewis VA, Gebhart GF. Evaluation of the periaqueductal central gray (PAG) as a morphine-specific locus of action and examination of morphine-induced and stimulation-produced analgesia at coincident PAG loci. *Brain Res.* (1977) 124:283–303.
34. Manning BH, Franklin KB. Morphine analgesia in the formalin test: reversal by microinjection of quaternary naloxone into the posterior hypothalamic area or periaqueductal gray. *Behav. Brain Res.* (1998) 92:97–102.
35. Cohen SR, Melzack, R. Morphine injected into the habenula and dorsal posteromedial thalamus produces analgesia in the formalin test. *Brain Res.* (1985) 359:131–9.
36. Osborne PB, Vaughan CW, Wilson H I, Christie MJ. Opioid inhibition of rat periaqueductal grey neurones with identified projections to rostral ventromedial medulla *in vitro*. *J Physiol.* (1996) 490 (Pt 2):383–9.

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Synthesis, Biological Evaluation, and SAR Studies of 14β-phenylacetyl Substituted 17-cyclopropylmethyl-7, 8-dihydronoroxymorphinones Derivatives: Ligands With Mixed NOP and Opioid Receptor Profile

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A series of 14β-acyl substituted 17-cyclopropylmethyl-7,8-dihydronoroxymorphinone compounds has been synthesized and evaluated for affinity and efficacy for mu (MOP), kappa (KOP), and delta (DOP) opioid receptors and nociceptin/orphanin FQ peptide (NOP) receptors. The majority of the new ligands displayed high binding affinities for the three opioid receptors, and moderate affinity for NOP receptors. The affinities for NOP receptors are of particular interest as most classical opioid ligands do not bind to NOP receptors. The predominant activity in the [^{35}S]GTPγS assay was partial agonism at each receptor. The results are consistent with our prediction that an appropriate 14β side chain would access a binding site within the NOP receptor and result in substantially higher affinity than displayed by the parent compound naltrexone. Molecular modeling studies, utilizing the recently reported structure of the NOP receptor, are also consistent with this interpretation.

Keywords: opioid, nociceptin, ORL-1, analgesics, kappa opioid receptor, mu opioid receptors

INTRODUCTION

There are three classical opioid receptors mu (MOP), delta (DOP), and kappa (KOP), which play important physiological and pharmacological roles especially in pain regulation. In addition to these, the NOP receptor (earlier ORL1) was identified as a fourth member of the opioid receptor family. This G-protein coupled receptor (1) has significant homology with classical opioid receptors; however none of the endogenous opioid ligands show high affinity to NOP. The endogenous ligand for this receptor, nociceptin/orphanin FQ (N/OFQ) (2, 3) is a 17 amino acid peptide having sequence similarity to the opioid peptides, particularly dynorphin, but it itself does not have high affinity for other opioid receptors. Various early studies indicated that the NOP receptor may play an important role in pain regulation (4), the cardiovascular system (5, 6), opioid tolerance (7), learning and memory (8–10), anorexia (11), anxiety (12), and others (6). However, the development of new therapeutics targeting NOP receptors has not proven easy and it has become clear that the biological actions of NOP receptor ligands vary enormously depending on species, route of administration and dose (13).

For example, the pharmacological action of nociceptin on the perception of pain is not straightforward. Early studies on nociceptin provided mutually contradictory results of either increasing or decreasing perception of pain, depending on dose, site and method of administration (14, 15). Whereas Meunier et al. (3) reported nociceptin induced hyperalgesia in the hot plate test when injected intracerebroventricularly (i.c.v) in mice, Rossi et al. (16) found that i.c.v nociceptin produced a transient hyperalgesia followed by analgesia in the tail flick test in mice. More recent evidence from studies using non-human primates, which may have greater translational validity than studies using rodents, appears to confirm that NOP agonists have analgesic effects comparable to morphine (17–19), though variations in level of response have been reported (20).

Often medicinal chemistry programs aim to develop ligands with ever greater selectivity for a particular target so as to decrease the possibility of side effects. More recently there has been a move to rationally design drugs having a multi receptor affinity profile, recognizing the complexity of many disease states (21, 22). The continued development of Cebranopadol, now in multiple clinical trials is an example of this approach (23, 24). Cebranopadol is a potent, full agonist at both MOP and NOP receptors but is reported to have an improved safety profile over standard MOP receptor agonist analgesics. In a similar vein our groups have been interested in the development of compounds with a mixed affinity profile, including MOP partial agonist/NOP receptor partial agonists and separately MOP partial agonist/KOP partial agonists (25–27). In particular, MOP/NOP partial agonists are expected to be analgesic but with reduced side effect profile, including less respiratory depression, low abuse potential and less tolerance development (27–30).

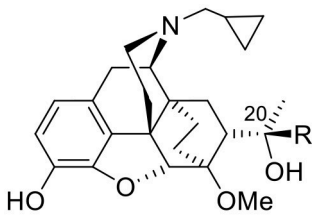
The orvinol, buprenorphine (**1**) is a partial MOP receptor agonist with modest affinity for the NOP receptor (31). Its efficacy in the treatment of pain may involve a NOP receptor component (32). The close homolog of buprenorphine, BU08028 (**2**) (26, 27), displays significant affinity and partial agonist activity for NOP receptors *in vitro* and SAR from this series of orvinols provides evidence that the region of space occupied by the *t*-butyl group in buprenorphine is key to good NOP receptor activity (26). Subsequently, similar NOP activity was found in the related phenethyl orvinols (**3**) (33) further highlighting the importance of the C20 group in the orvinol series.

The 14β-hydroxymorphinan-6-ones naltrexone (**4**) and naloxone are MOP receptor antagonists used in clinical practice. It is known that substituting the C14-oxygen can have a dramatic effect on the opioid receptor profile of these compounds (34, 35). Thus, while 14-O methyl & ethyl derivatives (36, 37) of naltrexone and naloxone are nonselective opioid receptor antagonists, 14-phenylpropyloxymorphinan-6-ones (38) have shown powerful agonist properties. We have previously reported on cinnamoyl esters of naltrexone as MOP receptor antagonists-partial agonists (39). From molecular modeling studies, it is clear that a suitable substituent attached to the C14-oxygen of naltrexone could access the same region of space as the *t*-butyl group of buprenorphine and it therefore seemed possible that such a series of ligands might display the mixed MOP/NOP receptor partial agonist activity desired.

Abbreviations: MOP receptor, mu opioid receptor; NOP receptor, nociceptin/orphanin FQ receptor; DOP receptor, delta opioid receptor; KOP receptor, kappa opioid receptor; MPE, maximum percent effect; ANOVA, analysis of variance; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; DPDPE, [D-Pen2,D-Pen5]enkephalin.

CHEMISTRY

The 3-hydroxy group of **4** was protected with tert-butyldimethylsilyl chloride in order to carry out selective esterification of the 14-hydroxy group. The tendency of the C6-carbonyl to exist in its enol form meant that clean esterification was not possible with acyl chlorides but could be achieved with the appropriate anhydrides which were synthesized from the corresponding phenylacetic acid and triphosgene. Thereafter the 3-hydroxy group was regenerated using a 1:1 mixture of methanol and HCl (6N) to give the target esters (**7**) (**Scheme 1**).

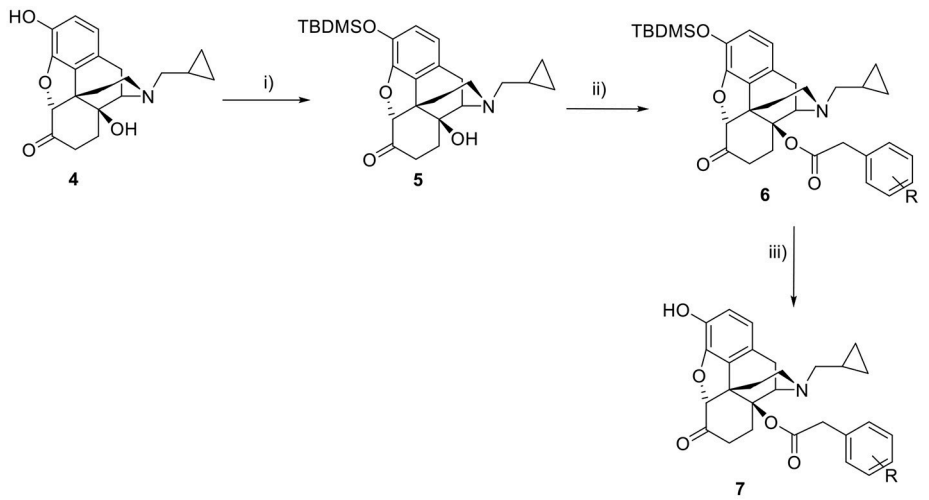


1: R = $C(CH_3)_3$, buprenorphine
2: R = $C(CH_3)_2CH_2CH_3$, BU08028
3: R = $CH_2CH_2C_6H_5$

RESULTS

Affinities for the individual opioid receptors were determined in displacement binding assays in recombinant human opioid receptors transfected into Chinese hamster ovary (CHO) cells as previously described (31). The displaced selective radioligands were [^{3}H]N/OFQ (NOP), [^{3}H]DAMGO (MOP), [^{3}H]Cl-DPDPE (DOP), and [^{3}H]U69593 (KOP). All of the ligands displayed high affinity binding in the subnanomolar to nanomolar range toward MOP, KOP, and DOP receptors, with 1–2 orders of magnitude lower affinity at NOP (**Table 1**). No selectivity in binding between MOP, KOP, and DOP receptors was expected or seen with this series of compounds; similarly there was no substantial effect on the affinities of the ligands at MOP, KOP, and DOP receptors on introduction of a substituent to the aryl ring of the phenylacetyl group. At NOP receptors, it appears that a substituent on the ring may be beneficial to affinity with a two- to four-fold increase in affinity on addition of a single substituent (compare unsubstituted **7a** to substituted analog **7b**-**7k**). When compared with the parent compound **4**, these ligands displayed a substantial increase in binding affinity toward the NOP receptor, a small increase in affinity at DOP (two- to eight-fold) and no change at MOP and KOP. Affinities were almost identical to those of buprenorphine (**1**).

The *in vitro* assay used to determine opioid receptor functional activity was the [^{35}S]GTPγS binding stimulation



SCHEME 1 | Synthesis of 14-*O*-phenylacetylnaltrexone and analogs. Reagents and conditions: (i) TBDMSiCl, imidazole, DCM, rt; (ii) R-phenylacetic anhydride, toluene, 125°C; (iii) MeOH-HCl (6N) 1:1, reflux.

TABLE 1 | Binding affinities of new compounds to human opioid receptor transfected into CHO cells[a].

Cpd	R	Ki/nM			
		NOP	**MOP**	**DOP**	**KOP**
7a	H	127 ± 17	0.86 ± 0.18	3.10 ± 0.60	1.14 ± 0.46
7b	4-CH_3	36.3 ± 4.2	1.87 ± 0.09	1.70 ± 0.20	1.56 ± 0.28
7c	2-CH_3	44.1 ± 3.1	3.59 ± 0.86	1.97 ± 0.19	1.69 ± 1.2
7d	3-CH_3	49.9 ± 3.5	1.91 ± 0.51	5.58 ± 0.22	2.80 ± 0.91
7e	3-OCH_3	50.1 ± 2.7	1.10 ± 0.08	3.61 ± 0.34	1.44 ± 0.33
7f	4-OCH_3	49.8 ± 7.1	1.08 ± 0.33	2.56 ± 0.48	1.90 ± 0.42
7g	3,4-OCH_2O-	94.3 ± 28	0.99 ± 0.35	1.25 ± 0.20	1.20 ± 0.42
7h	2-OCH_3	62.3 ± 4.9	3.77 ± 0.90	2.12 ± 0.59	3.52 ± 0.90
7i	2-F	69.7 ± 2.4	2.59 ± 1.1	4.07 ± 0.91	4.55 ± 0.70
7j	4-Cl	51.3 ± 14	1.78 ± 0.03	4.95 ± 1.1	3.95 ± 0.59
7k	2-Cl	32.6 ± 2.3	4.66 ± 1.8	3.24 ± 0.15	1.34 ± 0.50
3	–	>10K	0.66 ± 0.10	10.7 ± 0.82	1.10 ± 0.22
1	–	77.4 ± 16	1.5 ± 0.8	6.1 ± 0.4	2.5 ± 1.2

[a]*Data are the average ± SD from two experiments, each carried out in triplicate. Tritiated ligands were [^{3}H]DAMGO (MOP), [^{3}H]N/OFQ (NOP), [^{3}H]Cl-DPDPE (DOP), and [^{3}H]U69593 (KOP).*

assay, which, like the binding assays, was performed in human receptor transfected CHO cells as described previously (31). Agonist efficacy at these opioid receptors was determined in comparison to the standard selective agonists N/OFQ (NOP), DAMGO (MOP), DPDPE (DOP), and U69593 (KOP) (**Table 2**). The ligands were predominantly low efficacy agonists at MOP receptors. **7f**, **7g,** and **7j** were also evaluated as MOP receptor antagonists with **7f** and **7g** proving to be very potent competitive antagonists (pA2 values of 10.58 ± 0.13 and 10.29 ± 0.24, respectively, **Table 3**), whereas **7j** was non-competitive, as determined by a Schild analysis with a slope different than −1. Similar results were obtained at the other receptors with partial agonism being the standard activity. **7f** had sufficiently low efficacy at KOP, DOP, and NOP receptors to warrant evaluation as an antagonist at each. Whilst competitive at the MOP receptor, inhibition was non-competitive at the other receptors, with IC_{50} values of 12.4 ± 2.25, 12.2 ± 0.11, 48.1± 14.06, and 5,637 ± 2,242 nM at MOP, KOP, DOP, and NOP, respectively.

7f was evaluated in CD1 mice using the tail flick assay with an analgesia instrument (Stoetling) that uses radiant heat. Methods were as reported previously (27, 31). The overall ANOVA indicated that there was a significant interaction effect [$F_{(6,56)} = 3.96$, $P < 0.05$]. The positive control morphine (3 mg/kg) produced the anticipated increase in %MPE at all time points. At the doses tested (1 and 3 mg/kg) **7f** produced low levels of antinociception, consistent with partial agonist activity demonstrated in the [^{35}S]GTPγS binding assay. The 1 mg/kg dose of **7f** produced a significant increase in tail flick latency compared to vehicle controls at the 60- and 120-min time points, whereas the 3.0 mg/kg dose produced significant antinociception at the 30- and 120-min time points (**Figure 1A**). Given that both doses of **7f** produced similar levels of antinociception, we examined whether the lower dose would alter morphine-induced analgesia. As evident in **Figure 1B**, when **7f** was given as a pretreatment to morphine, morphine-induced antinociception was attenuated at the 30- and 60-min time points ($P < 0.05$).

DISCUSSION

Substitution at 14-*O* position of naltrexone (**4**) has a significant impact on the pharmacological profile. Lia et al. (40) reported on a series of 14-*O* heterocyclic esters of **4** as selective MOP receptor antagonists with subnanomolar to nanomolar binding affinities. Similarly we have reported (39) that the predominant activity of 14-*O* cinnamoyl esters of **4** was MOP partial agonism/antagonism both *in vitro* and *in vivo*. In contrast, the

TABLE 2 | Opioid agonist stimulation of [^{35}S]GTPγS binding in recombinant human opioid receptor[a].

	NOP		MOP		DOP		KOP	
Cpd	**EC_{50}/nM**	**% stim**	**EC_{50}/nM**	**% stim**	**EC_{50}/nM**	**% stim**	**EC_{50}/nM**	**% stim**
7a	401 ± 161	28.1 ± 4.8	3.8 ± 1.8	31.6 ± 4.2	5.6 ± 0.4	30.5 ± 5.5	1.2 ± 0.6	44.5 ± 13
7b	169 ± 3.4	22.3 ± 1.4	1.6 ± 0.9	34.9 ± 2.0	3.8 ± 1.1	27.0 ± 4.8	6.3 ± 2.2	11.5 ± 2.7
7c	106 ± 32.2	21.1 ± 1.4	2.2 ± 0.2	41.9 ± 1.4	201 ± 59	12.6 ± 3.1	2.9 ± 1.8	86.3 ± 6.5
7d	855 ± 185	59.3 ± 2.4	5.2 ± 2.0	40.1 ± 4.4	21.3 ± 2.2	15.8 ± 0.1	1.0 ± 0.3	46.4 ± 3.6
7e	374 ± 81.6	36.1 ± 0.3	13.3 ± 2.2	11.3 ± 1.3	9.9 ± 0.2	35.7 ± 0.7	1.7 ± 0.4	46.3 ± 8.1
7f	61.8 ± 20.1	8.9 ± 1.0	*	7.8 ± 3.5	59.4 ± 19.6	18.4 ± 1.3	*	6.5 ± 3.3
7g	562 ± 67.5	43.5 ± 9.4	*	12.1 ± 3.7	*	----	5.4 ± 0.1	41.1 ± 0.9
7h	479 ± 33.3	40.8 ± 5.4	0.5 ± 0.1	37.3 ± 0.7	4.15 ± 1.85	19.2 ± 4.9	1.6 ± 0.3	50.4 ± 6.0
7i	94.1 ± 24.7	14.5 ± 2.9	2.0 ± 0.7	27.9 ± 2.8	*	----	4.0 ± 2.0	43.8 ± 4.7
7j	298 ± 18.6	18.9 ± 2.7	*	8.2 ± 7.2	2.91 ± 10.6	37.2 ± 0.2	5.4 ± 1.3	37.1 ± 0.2
7k	808 ± 45.8	49.9 ± 0.7	2.4 ± 0.6	39.3 ± 4.7	*	----	13.9 ± 6.4	31.3 ± 5.6
1	116 ± 88.0	21.0 ± 8.4	10.2 ± 2.2	28.7 ± 1.1	>10,000	----	>10,000	----
DAMGO	----	----	35.3 ± 0.5	100	----	----	----	----
Nociceptin	8.1 ± 1.4	100	----	----		----	----	----
DPDPE	----	----	----	----	6.9 ± 0.4	100	----	----
U69,593	----	----	----	----	----	----	78.5 ± 8.8	100

[a]*Data are the average ± SD from at least two experiments, each carried out in triplicate.*
**Too little stimulation (if <15% an EC_{50} was not always determined).*
N/OFQ, Nociceptin/orphinan FQ.

equivalent phenylpropyl ether was a potent agonist in a battery of thermal nociceptive assays (38); thus substitution at the 14-*O* position of **4** plays a critical role in modulating activity, and predominantly efficacy, of the ligands at the traditional opioid receptors MOP, DOP, and KOP. In the current study, this SAR is further explored and extended to include activity at the NOP receptor. The new ligands, substituted 14-*O*-phenylacetyl esters of **4**, were evaluated for binding affinities and efficacies at MOP, DOP, and KOP, and NOP receptors. Phenylacetyl substitution at the 14-oxygen had little effect on affinity at MOP, DOP, and KOP receptors, but did substantially increase the binding affinity at NOP receptors. Addition of a substituent to the aryl ring of the phenylacetyl group further increased affinity for NOP receptors leading to a series of compounds with binding profiles directly comparable to buprenorphine (**1**). This provides support for our hypothesis that the group, in this case phenylacetyl, attached to the 14-*O* of **4** can access the same space as the *t*-butyl group in **1**, leading to moderate affinity at NOP receptors. The non-competitive binding seen with **7f** and **7j** may relate to the increased lipophilicity of these esters relative to **4**. The calculated logPs of **7j** (logP 4.41 ± 0.57) and **7f** (3.73 ± 0.57) (calculated using ACD/I-lab 2.0) are similar to those found with the orvinols—a series for which there is evidence for pseudo-irreversible binding in *in vitro* bioassays (25, 41).

Recently the structure of the NOP receptor in complex with the peptide mimetic C-24 has been determined (42). As part of the current study, **7c** was docked to the binding site of the crystal structure using GOLD. The docked pose of **7c** that best fit with the known interactions of C-24 with the protein is illustrated in **Figure 2**. Key interactions are between the basic nitrogen and Asp130, while the cyclopropylmethyl group occupies, but not fully, a lipophilic site accessed by the dihydroisobenzofuran head group of C-24. Most interestingly, the phenylacetyl type side chain of **7c** extends into the same region occupied by the pyrolidine ring of C-24 (including the amino acid residues Gln107, Asp110, Trp116, and Val126) and perhaps explains the substantial increase in affinity for these new ligands relative to the parent compound **3**, which cannot access this region. The Schrödinger software was then used to superimpose buprenorphine on the minimized structure of **7c** in the protein-ligand complex resulting in the same interactions between the basic nitrogen and the cyclopropylmethyl group with the protein and now with the bulky *t*-butyl group accessing the same region as the phenylacetyl group of **7c** (**Figure 3**). We have shown previously that minor changes to the *t*-butyl group of **1** can have a significant impact on binding affinity and efficacy at the NOP receptor (26, 27) and again, the interaction of this group with the site defined by, amongst other residues, Gln107, Asp110, Trp116, and Val126 could explain this finding. This docking pose would also help explain the lack of effect on NOP affinity on substituting the aromatic A-ring of **1** with halogens (26) as the A-ring extends into a very large, open region of the binding pocket, making no close interactions with receptor residues.

The predominant activity in the [^{35}S]GTPγS assay was of partial agonism at each of the receptors under study. Thus, when compared to the parent compound **4**, an antagonist, introduction of the phenylacetyl side chain has increased efficacy at each receptor. The effect was most pronounced at the KOP where one compound, **7c**, had high efficacy (86% of the standard) and a number of others fell in the 40–50%

TABLE 3 | Antagonist activity of selected compounds at the MOP receptor in the [^{35}S]GTPγS binding assay.

	MOP	
Compound	**Ke**	**pA2**
7f	0.026 ± 0.008	10.58 ± 0.14
7g	0.051 ± 0.018	10.29 ± 0.24
7j		Non-competitive

*Schild analysis indicated that compound **7j** had a slope significantly different than −1.0. This indicated non-competitive antagonism and a pA2 could not be determined. In an inhibition assay it had an IC_{50} value of 5.9 ± 1.84 nM when inhibiting DAMGO stimulation of [^{35}S]GTPγS binding. Results represent cumulative data from at least three separate experiments.*

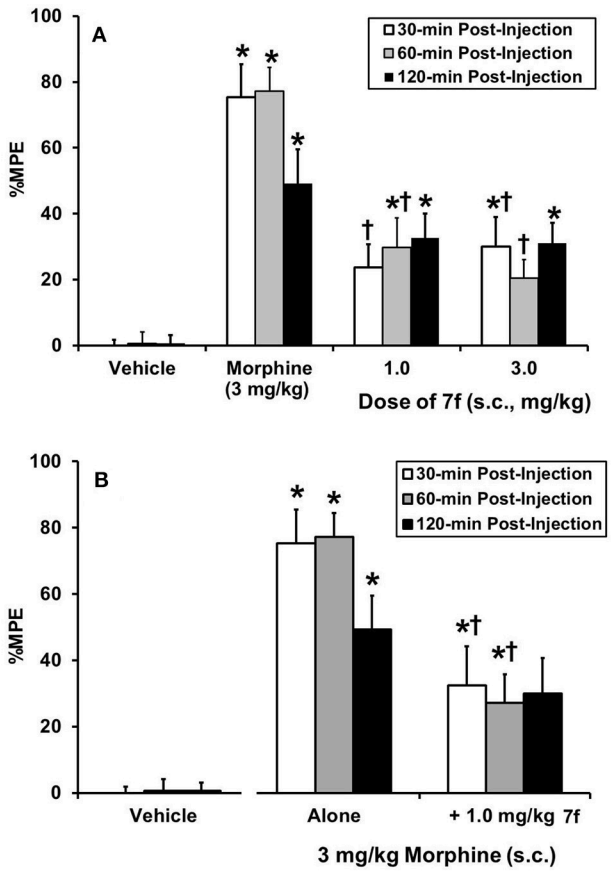


FIGURE 1 | Acute thermal antinociceptive effect of **7f** alone **(A)** or as a pretreatment to morphine **(B)**, using the mouse tail flick assay ($N = 8$/group). Data are mean %MPE (± SEM). *Significant difference from vehicle control; †Significant difference from morphine alone ($P < 0.05$). Behavioral results were analyzed by use of repeated measures ANOVAs with drug treatment (**7f**, morphine) as between group variables and post-injection time (30, 60, and 120 min) as the repeated measure followed by Tukey/Kramer *post-hoc* tests where appropriate.

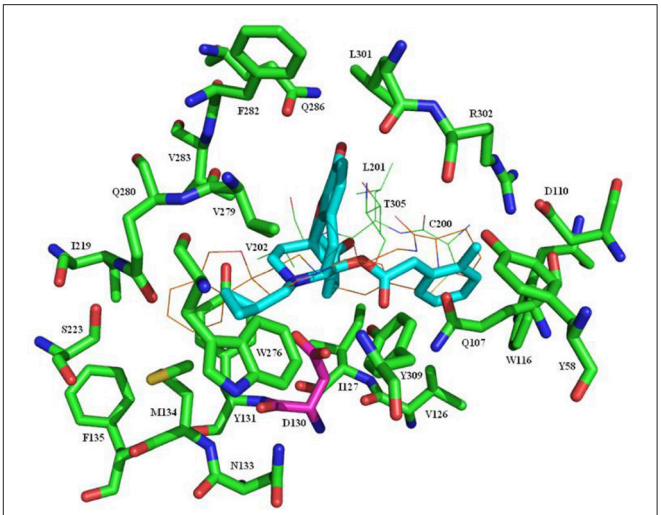


FIGURE 2 | Docking of **7c** in the binding site of the NOP receptor. **7c** is shown with cyan carbons. The protein is shown with green carbons apart from D130 which has purple carbons. For clarity, residues C200, L201, V202, and T305 are shown as lines rather than sticks. The crystal structure ligand, C-24, is shown as lines with brown carbons.

range. In this assay, efficacies were somewhat lower at MOP and lower still at DOP receptors. Some consistent SAR does emerge, with *ortho* substitution tending to give the highest efficacy, followed by *meta* and then *para* at both MOP and KOP receptors. A similar trend is observed at NOP receptors, where the *ortho* and *meta*-substituted ligands were typically higher efficacy than their *para* substituted equivalents. At MOP and NOP receptors a number of the new ligands had profiles somewhat similar to **1**, though typically with more selectivity for MOP. The most substantial difference to **1** was at the KOP receptor where the potent partial agonism of many of the current series contrasts with the potent antagonism characteristic of **1**. Compared to the closely related cinnamoyl esters reported previously (39), these phenylacetyl esters have similar affinities, but higher efficacies at MOP, DOP, and KOP receptors (NOP receptor activity was not measured for the cinnamoyl esters).

The 4-methoxy substituted analog **7f** was of interest due to its good affinity and very low level stimulation of all the receptors in the [^{35}S]GTPγS assay. Agonists for KOP and DOP receptors have been, and continue to be, evaluated as potential analgesics either as selective ligands (43) or dual-acting (44). In an extension of the argument made earlier for the development of mixed MOP/NOP agonists, it could be envisaged that ligands displaying low efficacy at each of the receptors might provide analgesia with very little in the way of side-effect profile. The low level of antinociception observed in the mouse tail flick assay and the ability to act as a morphine antagonist is consistent with this hypothesis and **7f** may provide a useful lead in the development of new, safer analgesics.

CONCLUSION

The hypothesis that introduction of a lipophilic group to the 14-oxygen of **4** would introduce NOP receptor affinity has been validated by the present study. Moderate affinity,

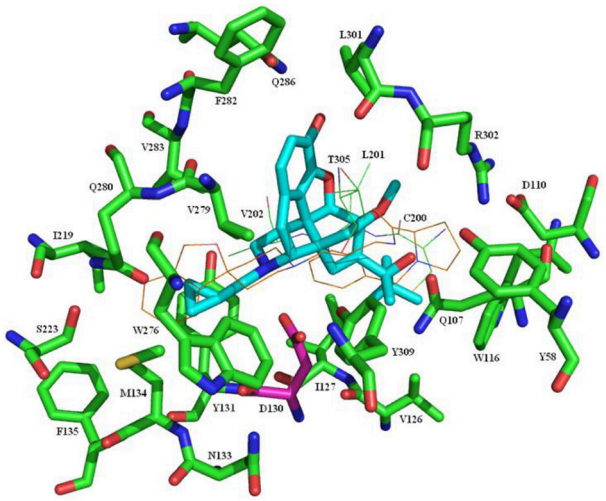


FIGURE 3 | Docking of Buprenorphine (**1**) in the binding site of the NOP receptor. **1** is shown with cyan carbons. The protein is shown with green carbons apart from D130 which has purple carbons. For clarity, residues C200, L201, V202, and T305 are shown as lines rather than sticks. The crystal structure ligand, C-24, is shown as lines with brown carbons.

equivalent to that of the orvinol buprenorphine (**1**), was seen alongside low efficacy partial agonism, supporting our belief that the *t*-butyl group of **1** and the phenylacetyl group of the current series might access the same region of the NOP receptor. This is reinforced by docking studies, to the recently solved crystal structure of the NOP receptor that provide a rationale for the moderate affinity shown by the ligands reported here and also, help explain the SAR of close analogs of **1** (26). As expected, introduction of the 14-*O* side chain also raised efficacy relative to **4** at the standard opioid receptors.

METHODS

Reagents and solvents were purchased from Sigma-Aldrich or Alfa Aesar and used as received. ^{1}H and ^{13}C NMR spectra were obtained with a Brucker-400-MHz instrument (^{1}H at 400 MHz, ^{13}C at 100 MHz); δ in ppm, J in Hz with TMS as an internal standard. ESIMS: microTOF (BRUKER). Microanalysis: Perkin-Elmer 240C analyzer. Column Chromatography was performed using pre-packed column in combi flash instrument. Ligands were tested as their hydrochloride salts, prepared by adding 5 equivalent of HCl (1 N solution in diethyl ether) in a solution of compound in anhydrous methanol. All reactions were carried out under an inert atmosphere of nitrogen unless otherwise indicated. All compounds were >95% pure.

General Procedure. 14-*O*-Esterification

To a solution of TBDMS-protected naltrexone (**5**) (0.4 mmol) in anhydrous toluene, optionally substituted phenylacetic anhydride (0.8 mmol), and DMAP (0.04 mmol) were added and the reaction mixture refluxed for 16 h. After completion saturated sodium bicarbonate (15 mL) was added and the aqueous layer extracted with EtOAc (3 × 10 mL). Organic layer was washed with water (2 × 20 mL), brine (10 mL) and dried over magnesium sulfate and evaporated *in vacuo* to obtain crude product which was purified by flash chromatography using methanol:dichloromethane (0.5:99.5).

General Procedure. TBDMS Deprotection

The substrate (0.3 g) was dissolved in 6 mL (1:1) solution of methanol:hydrochloric acid (6N) and refluxed for 5 h. The reaction mixture was cooled to 0°C and neutralized with saturated aq. sodium bicarbonate. The organic layer was extracted with ethyl acetate (3 × 20 ml), washed with water (2 × 25 mL), brine (25 mL), dried over magnesium sulfate and evaporated to obtained crude product which was purified by flash chromatography using methanol:dichloromethane:ammonium hydroxide (2:97.5:0.5).

14β-phenylacetyl-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7a)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.09–0.12 (2H, m), 0.51–0.54 (2H, m), 0.78–0.83 (1H, m), 1.41–1.45 (1H, m), 1.57 (1H, dt, J = *3.72* and *14.44Hz*), 2.09–2.21 (2H, m), 2.23–2.52 (4H, m), 2.58–2.67 (2H, m), 2.79–2.82 (1H, m), 3.07 (1H, d, J = *18.2 Hz*), 3.77 (2H, m), 4.44 (1H, d, J = *5.52 Hz*), 4.51 (1H, s), 6.59 (1H, d, J = *8.0 Hz*), 6.72 (1H, d, J = *8.0 Hz*), 7.24–7.31 (2H, m), 7.36–7.41 (3H, m); HRMS, m/z for ($C_{28}H_{30}NO_5$) $[MH]^+$, calcd- 460.2124, found- 460.2103.

14β-(4′-methylphenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7b)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.09–0.12 (2H, m), 0.50–0.54 (2H, m), 0.74–0.80 (1H, m), 1.44–1.48 (1H, m), 1.61 (1H, dt, J = *3.72* and *14.44Hz*), 2.14–2.22 (2H, m), 2.27–2.51 (8H, m), 2.57–2.61 (1H, m), 2.78–2.82 (1H, m), 3.07 (1H, d, J = *18.2 Hz*), 3.74–3.76 (2H, m), 4.48 (1H, d, J = *5.52 Hz*), 4.53 (1H, s), 6.60 (1H, d, J = *8.0 Hz*), 6.72 (1H, d, J = *8.0 Hz*), 7.16 (2H, d, J = *8.0 Hz*), 7.27 (2H, d, J = *8.0 Hz*); HRMS, m/z for ($C_{29}H_{32}NO_5$) $[MH]^+$, calcd-474.2280, found-474.2329.

14β-(2′-methylphenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7c)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.09–0.14 (2H, m), 0.51–0.55 (2H, m), 0.78–0.84 (1H, m), 1.32–1.35 (1H, m), 1.51 (1H, dt, J = *3.72* and *14.44Hz*), 2.10–2.50 (10H, m), 2.62–2.68 (1H, m), 2.74–2.81 (1H, m), 3.03 (1H, d, J = *18.2 Hz*), 3.76–3.88 (2H, m), 4.42 (1H, s), 4.51 (1H, d, J = *5.52 Hz*), 5.90 (1H, bd), 6.59 (1H, d, J = *8.0 Hz*), 6.72 (1H, d, J = *8.0 Hz*), 7.20–7.23 (3H, m), 7.29–7.33 (1H, m); HRMS, m/z for ($C_{29}H_{32}NO_5$) $[MH]^+$, calcd- 474.2280, found- 474.2258.

14β-(3′-methylphenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7d)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.09–0.12 (2H, m), 0.50–0.0.54 (2H, m), 0.70–0.75 (1H, m), 1.45–1.49 (1H, dd, *J* = *4.00* and *12.1 Hz*), 1.59–1.66 (1H, dt, *J* = *3.72* and *14.44Hz*), 2.14–2.31 (2H, m), 2.32–2.40 (8H, m), 2.67 (1H, dd, *J* = *4.0* and *12.1 Hz*), 2.76–2.81 (1H, m), 3.07 (1H, d, *J* = *18.4 Hz*), 3.69 (2H, dd, *J* = *8.0* and *18.4 Hz*), 4.49 (1H, d, *J* = *4.0 Hz*), 4.53 (1H, s), 5.75 (1H, bd), 6.60 (1H, d, *J* = *8.0 Hz*), 6.73 (1H, d, *J* = *8.0 Hz*), 7.11 (1H, d, *J* = *6.1 Hz*), 7.17 (3H, d, *J* = *8.1 Hz*); HRMS, m/z for ($C_{29}H_{32}NO_5$) $[MH]^+$, calcd- 474.2280, found- 474.2288.

14β-(3′-methoxyphenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7e)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.09–0.14 (2H, m), 0.50–0.0.54 (2H, m), 0.69–0.74 (1H, m), 1.45–1.49 (1H, dd, *J* = *4.00* and *12.1 Hz*), 1.59–1.66 (1H, dt, *J* = *3.72* and *14.44Hz*), 2.14–2.31 (2H, m), 2.31–2.41 (5H, m), 2.67 (1H, dd, *J* = *4.0* and 12.1 *Hz*), 2.76–2.81 (1H, m), 3.07 (1H, d, *J* = *18.4 Hz*), 3.71 (2H, dd, *J* = *8.0* and *18.4 Hz*), 3.83 (3H, s), 4.49 (1H, d, *J* = *4.0 Hz*), 4.54 (1H, s), 5.79 (1H, bd), 6.60 (1H, d, *J* = *8.0 Hz*), 6.73 (1H, d, *J* = *8.0 Hz*), 6.83 (1H, dd, *J* = *4.0* and 8.1 *Hz*), 6.95–6.99 (2H, m), 7.28–7.30 (1H, m); HRMS, m/z for ($C_{29}H_{32}NO_6$) $[MH]^+$, calcd- 490.2230, found- 490.2278.

14β-(4′-methoxyphenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7f)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.05–0.08 (2H, m), 0.46–0.50 (2H, m), 0.70–0.73 (1H, m), 1.42–1.45 (1H, m), 1.56 (1H, dt, *J* = *3.76* and *14.44Hz*), 2.11–2.16 (2H, m), 2.18–2.47 (5H, m), 2.61–2.67 (1H, m), 2.74–2.79 (1H, m), 3.03 (1H, d, *J* = *18.2 Hz*), 3.64–3.73 (2H, m), 3.79 (3H, s), 4.44 (1H, d, *J* = *5.52 Hz*), 4.52 (1H, s), 6.56 (1H, d, *J* = *8.0 Hz*), 6.69 (1H, d, *J* = *8.0 Hz*), 6.85 (2H, d, *J* = *8.0 Hz*), 7.25 (2H, d, *J* = *8.0 Hz*); HRMS, m/z for ($C_{29}H_{32}NO_6$) $[MH]^+$, calcd- 490.2230, found- 490.2200.

14β-(3′,4′-dioxymethylenephenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7g)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.09–0.14 (2H, m), 0.51–0.54 (2H, m), 0.89–0.95 (1H, m), 1.46–1.51 (1H, m), 1.57 (1H, dt, *J* = *3.76* and *14.44Hz*), 2.11–2.31 (3H, m), 2.36–2.46 (4H, m), 2.65–2.72 (1H, m), 2.76–2.82 (1H, m), 3.08 (1H, d, *J* = *18.2 Hz*), 3.68–3.72 (2H, m), 4.49 (1H, d, *J* = *5.52 Hz*), 4.58 (1H, s), 5.50 (1H, bd), 5.97 (2H, s), 6.61 (1H, d, *J* = *8.0 Hz*), 6.73 (1H, d, *J* = *8.0 Hz*), 6.79–6.82 (2H, m), 6.92 (1H, s); HRMS, m/z for ($C_{29}H_{30}NO_7$) $[MH]^+$, calcd- 504.2022, found- 504.2069.

14β-(2′-methoxyphenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7h)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.08–0.11 (2H, m), 0.49–0.52 (2H, m), 0.78–0.84 (1H, m), 1.32–1.35 (1H, m), 1.51 (1H, dt, *J* = *3.72* and *14.44Hz*), 2.10–2.45 (6H, m), 2.55–2.65 (2H, m), 2.74–2.81 (1H, m), 3.03 (1H, d, *J* = *18.2 Hz*), 3.76 (2H, m), 3.81 (3H, s), 4.34 (1H, s), 4.43 (1H, d, *J* = *5.52 Hz*), 5.61 (1H, bd), 6.55 (1H, d, *J* = *8.0 Hz*), 6.68 (1H, d, *J* = *8.0 Hz*), 6.89 (1H, d, *J* = *8.0 Hz*), 6.93 (1H, m), 7.24–7.28 (2H, m); HRMS, m/z for ($C_{29}H_{32}NO_6$) $[MH]^+$, calcd- 490.2230, found- 490.2228.

14β-(2′-fluorophenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7i)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.06–0.09 (2H, m), 0.48–0.0.51 (2H, m), 0.71–0.75 (1H, m), 1.33–1.36 (1H, m), 1.56–1.64 (1H, dt, *J* = *3.72* and *14.44Hz*), 2.04–2.42 (6H, m), 2.46–2.62 (2H, m), 2.76–2.80 (1H, m), 3.07 (1H, d, *J* = *18.4 Hz*), 3.72 (2H, m), 4.42 (1H, d, *J* = *4.0 Hz*), 4.44 (1H, s), 5.65 (1H, bd), 6.56 (1H, d, *J* = *8.0 Hz*), 6.68 (1H, d, *J* = *8.0 Hz*), 7.08–7.14 (2H, m), 7.25–7.29 (1H, m) 7.35–7.37 (1H, m); HRMS, m/z for ($C_{28}H_{29}FNO_5$) $[MH]^+$, calcd- 478.2030, found- 478.2073.

14β-(4′-chlorophenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7j)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.04–0.10 (2H, m), 0.47–0.50 (2H, m), 0.65–0.69 (1H, m), 1.43–1.46 (1H, m), 1.59 (1H, dt, *J* = *3.72* and *14.44Hz*), 2.08–2.16 (1H, m), 2.21–2.34 (4H, m), 2.39–2.48 (2H, m), 2.62–2.65 (1H, m), 2.74–2.81 (1H, m), 3.04 (1H, d, *J* = *18.2 Hz*), 3.72–3.74 (2H, m), 4.43 (1H, d, *J* = *5.52 Hz*), 4.53 (1H, s), 6.56 (1H, d, *J* = *8.0 Hz*), 6.70 (1H, d, *J* = *8.0 Hz*), 7.29–7.31 (4H, m); HRMS, m/z for ($C_{28}H_{29}ClNO_5$) $[MH]^+$, calcd- 494.1734, found- 494.1734.

14β-(2′-chlorophenylacetyl)-17-cyclopropylmethyl-7,8-dihydronoroxymorphinone (7k)

White Solid; ^{1}H NMR ($CDCl_3$) δ 0.08–0.10 (2H, m), 0.49–0.53 (2H, m), 0.76–0.81 (1H, m), 1.30–1.33 (1H, m), 1.56 (1H, dt, *J* = *3.72* and *14.44Hz*), 2.04–2.09 (2H, m), 2.25–2.32 (3H, m), 2.41–2.46 (1H, m), 2.53–2.59 (2H, m), 2.76–2.81 (1H, m), 3.03 (1H, d, *J* = *18.2 Hz*), 3.90 (2H, m), 4.41 (1H, d, *J* = *4.0 Hz*), 4.43 (1H, s), 5.48 (1H, bd), 6.55 (1H, d, *J* = *8.0 Hz*), 6.68 (1H, d, *J* = *8.0 Hz*), 7.24–7.26 (2H, m), 7.40–7.43 (2H, m); HRMS, m/z for ($C_{28}H_{29}ClNO_5$) $[MH]^+$, calcd- 494.1734, found- 494.1729.

Molecular Modeling Methods

The 4EA3 crystal structure (42) of NOP was the starting point. The structure was run through the Protein Preparation Wizard in the Schrödinger software suite running under Maestro version 9.3.023. Buprenorphine (**1**) and **7c** were built and minimized using the same software. Both ligands were docked into the binding site using GOLD. The docked pose of **7c** that seemed to best fit with the known interactions of the ligand with the protein was subjected to 1,000 rounds of minimization using the Schrödinger MacroModel software with the constraint that the ligand nitrogen be 2.8 Å from the nearest acidic oxygen of D130. GOLD failed to bind **1** with a sensible pose so the Schrödinger software was used to

superimpose **1** on the minimized structure of **7c** in the protein-ligand complex. The protein-**1** complex was then subjected to 1,000 rounds of minimisation. Figures were prepared using PyMOL.

In vitro Characterization

Cell culture

All receptors were individually expressed in CHO cells stably transfected with human receptor cDNA, The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin, in 100-mm polystyrene culture dishes. For binding assays, the cells were scraped off the plate at confluence. Receptor expression levels were 1.2, 1.6, 1.8, and 3.7 pmol per mg protein for the NOP, MOP, KOP, and DOP receptors, respectively.

Receptor binding

Binding to cell membranes was conducted in a 96-well format, as described previously (45, 46). Briefly, cells were removed from the plates, homogenized in 50 mM Tris pH 7.5, using a Polytron homogenizer, then centrifuged once and washed by an additional centrifugation at 27,000 × g for 15 min. The final pellet was re suspended in Tris, and the suspension incubated with [^{3}H]DAMGO (51 Ci/mmol, 1.6 nM), [^{3}H]Cl-DPDPE (42 Ci/mmol, 1.4 nM), [^{3}H]U69593 (41.7 Ci/mmol, 1.9 nM), or [^{3}H]N/OFQ (120 Ci/mmol, 0.2 nM) for binding to, MOP, DOP, KOP, and NOP receptors, respectively. Non-specific binding was determined with 1 μM of unlabeled DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin), DPDPE ([D-Pen2,D-Pen5]Enkephalin), ethylketocyclazocine, and N/OFQ, respectively. Samples were incubated for 60 min at 25°C in a total volume of 1.0 ml, with 15 μg protein per well. The reaction was terminated by filtration using a Tomtec 96 harvester (Orange, CT) through glass fiber filters and radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ). IC_{50} values were calculated using Graphpad/Prism (ISI, San Diego, CA) and Ki values were determined by the method of Cheng and Prusoff (47).

[^{35}S]GTPγS binding

[^{35}S]GTPγS binding was conducted basically as described by Traynor and Nahorski (48). Cells were scraped from tissue culture dishes into 20 mM Hepes, 1 mM EDTA, then centrifuged at 500x *g* for 10 min. Cells were resuspended in this buffer and homogenized using a Polytron Homogenizer. The homogenate was centrifuged at 27,000 × g for 15 min, and the pellet re suspended in Buffer A, containing: 20 mM Hepes, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4. The suspension was re centrifuged at 27,000 × g and suspended once more in Buffer A. For the binding assay, membranes (8–15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μM), and the appropriate compound, in a total volume of 1.0 ml, for 60 min at 25°C. Samples were filtered over glass fiber filters and counted as described for the binding assays. Statistical analysis was conducted using the program Prism. For the antagonist assay, various concentrations of 7f, 7g, and 7j were incubated in the presence of 100 nM N/OFQ, or 1 μM DAMGO, DPDPE or U69593 to determine antagonist potency at NOP, MOP, DOP, and KOP receptors, respectively. Schild analysis was also conducted at MOP receptors using various concentrations of the inhibitor in the present of a full DAMGO dose response curve.

In vivo Testing

Animals

Male ICR mice weighing 25–30 g at the start of the experiment were used. Animals were group-housed (N = 10/cage) under standard laboratory conditions using nestlets as environmental enrichment in their cages and were kept on a 12:12-hr day/night cycle (lights on at 7:00 a.m.). Testing was conducted during the animals' light cycle between 9 a.m. and 2 p.m. Animals were handled for 3–4 days before the experiments were conducted. On behavioral test days, animals were transported to the testing room and acclimated to the environment for 1 h. Mice were maintained in accordance with the guidelines of SRI International and of the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (49). Prior to any *in vivo* testing, approval for the behavioral protocols was obtained from the institutional ACUC of SRI International.

Drugs

7f was dissolved in 1–2% Dulbecco's modified Eagle's medium and 0.5% aqueous hydroxypropyl cellulose. Morphine hydrochloride (Eli Lilly & Co., Indianapolis, IN) was dissolved in water. Drugs were injected subcutaneously (s.c.) in a volume of 0.1 ml/30 g. Controls received 0.1 ml/30 g of the appropriate vehicle.

Assessment of acute thermal nociception

Tail-flick assay. Acute nociception was assessed using the tail flick assay with an analgesia instrument (Stoelting) that uses radiant heat. This instrument is equipped with an automatic quantification of tail flick latency, and a 15 s cutoff to prevent damage to the animal's tail. During testing, the focused beam of light was applied to the lower half of the animal's tail, and tail flick latency was recorded.

Baseline values for tail flick latency were determined before drug administration in each animal. The mean basal tail flick latency was 5.39 ± 0.09 SEM. After baseline measures, animals received a subcutaneous injection of their assigned dose of drug(s) and were tested for tail-flick latencies at 30, 60, and 120 min following the last drug injection. Controls received vehicle prior to testing.

Drug regimen. In the first series of experiments, animals (N = 8/group) received injections of **7f** (1 and 3 mg/kg s.c.) or morphine (3 mg/kg). Given that both 1 and 3 mg/kg **7f** produced similar levels of antinociception, we chose to examine the effects of 1 mg/kg **7f** given as a pretreatment to morphine. In these experiments, animals received 1 mg/kg **7f** or vehicle and 10 min later received an injection of morphine. A group of animals

served as vehicle controls. Testing was conducted as described above.

Statistical analyses. Antinociception (% maximum potential effect; % MPE) was quantified by the following formula: % MPE = 100[(test latency–baseline latency)/(15–baseline latency)]. If the animal did not respond before the 15-s cutoff, the animal was assigned a score of 100%. Behavioral results were analyzed by use of repeated measures ANOVAs with drug treatment **(7f,** morphine) as between group variables and post-injection time (30, 60, and 120 min) as the repeated measure followed by Tukey/Kramer *post-hoc* tests where appropriate. The level of significance was set at $P = 0.05$.

AUTHOR CONTRIBUTIONS

SH, LT, and TK participated in the research design. TK, VK, GC-K, MT, and WP conducted the experimental work. SH wrote the manuscript with input from all co-authors.

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REFERENCES

1. Mollereau C, Parmentier M, Mailleux P, Butour J, Moisand C, Chalon P, et al. ORL-1, a novel member of the opioid receptor family. cloning, functional expression and localization. *FEBS Lett*. (1994) 341:33–8. doi: 10.1016/0014-5793(94)80235-1
2. Reinsceid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, et al. (1995). Orphanin FQ: a neuropeptide that activates an opioid like G protein-coupled receptor. *Science* 270:792–4. doi: 10.1126/science.270.5237.792
3. Meunier JC, Mollereau C, Toll L, Suaudeu C, Moisand C, Alvinerie P, et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL-1 receptor. *Nature* 377:532–5. doi: 10.1038/377532a0
4. Mogil JS, Grisel JE, Reinscheid RK, Civelli O, Belknap JK, Grandy DK. (1996). Orphanin FQ is a functional anti-opioid peptide. *Neuroscience* 75:333–7. doi: 10.1016/0306-4522(96)00338-7
5. Gumusel B, Hao Q, Hyman A, Chang JK, Kapusta DR, Lippton H. (1997). Nociceptin: an endogenous agonist for central opioid like1 (ORL1) receptors possesses systemic vasorelaxant properties. *Life Sci.* 60:141–5. doi: 10.1016/S0024-3205(96)00696-0
6. Champion HC, Wang R, Hellstrom WJG, Kadowitz PJ. (1997). Nociceptin, a novel endogenous ligand for ORL1 receptor, has potent erectile activity in the cat. *Am J Physiol.* 273:E214–9. doi: 10.1152/ajpendo.1997.273.1.E214
7. Ueda H, Yamaguchi T, Tokuyama S, Inoue M, Nishi M, Takeshima H. (1997). Partial loss of tolerance liability to morphine analgesia in mice lacking the nociceptin receptor gene. *Neuroscience Lett*. 237:136–8. doi: 10.1016/S0304-3940(97)00832-X
8. Yu TP, Fein J, Phan T, Evans CJ, Xie CW. (1997). Orphanin FQ inhibits synaptic transmission and long-term potentiation in rat hippocampus. *Hippocampus* 7:88–94. doi: 10.1002/(SICI)1098-1063(1997)7:1<88::AID-HIPO9>3.0.CO;2-3
9. Sandin J, Georgieva J, Schött PA, Ogren SO, Terenius L. (1997). Nociceptin/Orphanin FQ microinjected into hippocampus impairs spinal learning in rats. *Eur J Neurosci.* 9:194–7. doi: 10.1111/j.1460-9568.1997.tb01367.x
10. Manabe T, Noda Y, Mamiya T, Katagiri H, Houtani T, Nishi M, et al. (1998). Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. *Nature* 394:577–81. doi: 10.1038/29073
11. Pomonis JD, Billington CJ, Levine AS. Orphanin FQ agonist of orphan opioid receptor ORL1, stimulates feeding in rats. *Neuro Rep.* (1996) 8:369–71. doi: 10.1097/00001756-199612200-00072
12. Jenck F, Moreau JL, Martin JR, Kilpatrick GJ, Reinscheid RK, Monsma FJJr, et al. (1997). OrphaninFQ acts as an anxiolytic to attenuate behavioral response to stress. *Proc Natl Acad Sci USA*. 94:14854–8. doi: 10.1073/pnas.94.26.14854
13. Toll L, Bruchas MR, Calo G, Cox BM, Zaveri NT. (2016). Nociceptin/Orphanin FQ receptor structure, signalling, ligands, functions and interactions with opioid systems. *Pharmacol Rev*. 68:419–57. doi: 10.1124/pr.114.009209
14. Zeilhofer HU, Calo G. (2003). Nociceptin/orphinan FQ and its receptor-potential targets for pain therapy. *J Pharmacol Exp Ther.* 306:423–9. doi: 10.1124/jpet.102.046979
15. Meunier JC. Utilising functional genomics to identify new pain treatments: the example of nociceptin. *Am J Pharmacogenom.* (2003) 3:117–30. doi: 10.2165/00129785-200303020-00005
16. Rossi GC, Leventhal L, Pasternak GW. (1996). Naloxone sensitive orphanin FQ-indiced analgesia in mice. *Eur J Pharmacol.* 311:R7–8. doi: 10.1016/0014-2999(96)00578-X
17. Hu E, Calò G, Guerrini R, Ko MC. (2010). Long-lasting antinociceptive spinal effects in primates of the novel nociceptin/orphanin FQ receptor agonist UFP-112, *Pain* 148:107–13. doi: 10.1016/j.pain.2009.10.026
18. Ko MC, Naughton NN. (2009). Antinociceptive effects of nociceptin/orphanin FQ administered intrathecally in monkeys, *J Pain* 10:509–16. doi: 10.1016/j.jpain.2008.11.006
19. Rizzi A, Sukhtankar DD, Ding H, Hayashida K, Ruzza C, Guerrini R, et al. (2015). Spinal antinociceptive effects of the novel NOP receptor agonist PWT2- nociceptin/orphanin FQ in mice and monkeys. *Br J Pharmacol*. 172:3661–70. doi: 10.1111/bph.13150
20. Saccone PA, Zelenock KA, Lindsey AM, Sulima A, Rice KC, Prinssen EP, et al. Characterization of the discriminative stimulus effects of a NOP receptor agonist Ro 64-6198 in rhesus monkeys. *J Pharmacol Exp Ther*. (2016) 357:17–23. doi: 10.1124/jpet.115.231134
21. Woodcock J, Witter J, Dionne RA. (2007). Stimulating the development of mechanism-based individualized pain therapies. *Nat Rev Drug Discov*. 6:703–10. doi: 10.1038/nrd2335
22. Günther T, Dasgupta P, Mann A, Miess E, Kliewer A, Fritzwanker S, et al. (2017). Targeting multiple opioid receptors – improved analgesics with reduced side effects? *Brit J Pharmacol.* 175:2857–68. doi: 10.1111/bph.13809
23. Raffa RB, Burdge G, Gambrah J, Kinecki HE, Lin F, Lu B, et al. Cebranopadol: novel dual opioid/NOP receptor agonist analgesic. *J Clin Pharm Ther*. (2017) 42:8–17. doi: 10.1111/jcpt.12461
24. Christoph A, Eerdekens MH, Kok M, Volkers G, Freynhagen R. Cebranopadol, a novel first-in-class analgesic drug candidate: first experience in patients with chronic low back pain in a randomized clinical trial. *Pain* (2017) 158:1813–24. doi: 10.1097/j.pain.0000000000000986
25. Greedy B, Bradbury F, Thomas MP, Grivas K, Cami-Kobeci G, Aarchambeau A, et al. (2013). Orvinols with mixed kappa/mu opioid receptor agonist activity. *J Med Chem.* 56:3207–16. doi: 10.1021/jm301543e
26. Cami-Kobeci G, Polgar WE, Khroyan TV, Toll L, Husbands SM. (2011). Structural determinants of opioid and NOP receptor activity in derivatives of buprenorphine. *J Med Chem.* 54:6531–7. doi: 10.1021/jm2003238
27. Khroyan TV, Polgar WE, Cami-Kobeci G, Husbands SM, Zaveri NT, Toll L. (2011). The first universal opioid ligand, (2S)-2-(5R, 6R,

7R, 14S)-N-cyclopropylmethyl-4, 5-epoxy-6, 14-ethano-3-hydroxy-6-methoxymorphinan-7-yl−3,3-dimethylpentan-2-ol (BU08028): characterization of the *in vitro* profile and *in vivo* behavioral effects in mouse models of acute pain and cocaine-induced reward. *J Pharmacol Exp Ther*. 336:952–61. doi: 10.1124/jpet.110.175620

28. Sukhtankar DD, Zaveri NT, Husbands SM, Ko MC. Effects of spinally administered bifunctional nociceptin/orphanin FQ peptide receptor/μ-opioid receptor ligands in mouse models of neuropathic and inflammatory pain. *J Pharmacol Exp Ther*. (2013) 346:11–22. doi: 10.1124/jpet.113.203984
29. Ding H, Czoty PW, Kiguchi N, Cami-Kobeci G, Sukhtankar DD, Nader MA, et al. (2016). A novel orvinol analog, BU08028, as a safe opioid analgesic without abuse liability in primates *Proc Natl Acad Sci USA*. 113:E5511–8. doi: 10.1073/pnas.1605295113
30. Kiguchi N, Ding H, Ko MC. Central N/OFQ-NOP receptor system in pain modulation. *Adv Pharmacol.* (2016) 75:217–43. doi: 10.1016/bs.apha.2015.10.001
31. Spagnolo B, Calo G, Polgar WE, Jiang F, Olsen CM, Berzetei-Gurske I, et al. (2008). Activities of mixed NOP and μ-opioid receptor ligands. *Br J Pharmacol.* 153:609–19. doi: 10.1038/sj.bjp.0707598
32. Huang P, Kehner GB, Cowan A. (2001). Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *J Pharmacol Exp Ther*. 297:688–95.
33. Khroyan T, Wu J, Polgar W, Cami-Kobeci G, Fotaki N, Husbands S, et al. (2015). BU08073 a buprenorphine analogue with partial agonist activity at μ-receptors *in vitro* but long-lasting opioid antagonist activity *in vivo* in mice. *Br J Pharmacol.* 172:668–80. doi: 10.1111/bph.12796
34. Schmidhammer H, Spetea M. Synthesis of 14-alkoxymorphinan derivatives and their pharmacological actions. *Top Curr Chem.* (2011) 299:63–91. doi: 10.1007/128_2010_77
35. Lewis JW, Husbands SM. (2011). 14-Amino-4,5-epoxymorphinan derivatives and their pharmacological actions. *Top Curr Chem.* 299:93–119. doi: 10.1007/128_2010_89
36. Schmidhammer H, Aeppli L, Atwell L, Fritsch F, Jacobson AE, Nebuchla M, et al. (1984). Synthesis and biological evaluation of 14-alkoxymorphinans. 1. Highly potent opioid agonists in the series of (-)-14-methoxy-*N*-methylmorphinan-6-ones. *J Med Chem.* 27:1575–9. doi: 10.1021/jm00378a009
37. Schmidhammer H, Burkard WP, Eggstein-Aeppli L. (1989). Synthesis and biological evaluation of 14-alkoxymorphinans. 4. Opioid agonists and partial opioid agonists in a series of *N*-(cyclobutylmethyl)-14-methoxymorphinan-6-ones. *Helv Chim Acta* 72:1233–40. doi: 10.1002/hlca.19890720607
38. Greiner E, Spetea M, Krassnig R, Schüllner F, Aceto M, Harris LS, et al. (2003). Synthesis and biological evaluation of 14-alkoxymorphinans. 18. *N*-substituted 14-phenylpropyloxymorphinan-6-ones with unanticipated agonist properties: extending the scope of common structure-activity relationships. *J Med Chem*. 46:1758–63. doi: 10.1021/jm021118o
39. Moynihan H, Jales AR, Greedy BM, Rennison D, Broadbear JH, Purington L, et al. (2009). 14-*O*-Cinnamoylnaltrexone and related dihydrocodeinones are mu opioid receptor partial agonists with predominant antagonist activity. *J Med Chem.* 52:1553–7. doi: 10.1021/jm8012272
40. Lia G, Lindsey A, Schenbach CK, He H, Selley DE, Zhang Y. (2009). 14-*O*-Heterocyclic-substituted naltrexone derivatives as non-peptide mu opioid receptor selective antagonists: design, synthesis, and biological studies. *Bioorg Med Chem Lett.* 19:1825–9. doi: 10.1016/j.bmcl.2008.12.093
41. Lewis JW, Husbands SM. (2004). The orvinols and related opioids – high affinity ligands with diverse efficacy profiles. *Curr Pharm Des.* 10:717–32. doi: 10.2174/1381612043453027
42. Thompson AA, Liu W, Chun E, Katritch V, Wu H, Vardy E, et al. (2012). Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* 485:395–9. doi: 10.1038/nature11085
43. Vanderah TW. Delta and kappa opioid receptors as suitable drug targets for pain. *Clin J Pain*. (2010) 26:S10–5. doi: 10.1097/AJP.0b013e3181c49e3a
44. Váradi A, Marrone GF, Eans SO, Ganno ML, Subrath JJ, Rouzic VL, et al. Synthesis and characterization of a dual kappa-delta opioid receptor agonist analgesic blocking cocaine reward behavior. *ACS Chem Neurosci.* (2015) 6:1813–24. doi: 10.1021/acschemneuro.5b00153
45. Dooley CT, Spaeth CG, Berzetei-Gurske IP, Craymer K, Adapa ID, Brandt SR, et al. (1997). Binding and *in vitro* activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL1. *J Pharmacol Exp Ther.* 283:735–41.
46. Toll L, Berzetei-Gurske IP, Polgar WE, Brandt SR, Adapa ID, Rodriguez L, et al. (1998). Standard binding and functional assays related to medications development division testing for potential cocaine and opiate narcotic treatment medications. *NIDA Res Monogr.* 178:440–66.
47. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol.* (1973) 22:3099–108. doi: 10.1016/0006-2952(73)90196-2
48. Traynor JR, Nahorski SR. (1995). Modulation by mu-opioid agonists of guanosine-5′-O-(3-[S-35]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol.* 47:848–54.
49. National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research. *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research.* Washington, DC: National Academies Press (2003).

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Episodic Ethanol Exposure in Adolescent Rats Causes Residual Alterations in Endogenous Opioid Peptides

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Adolescent binge drinking is associated with an increased risk of substance use disorder, but how ethanol affects the central levels of endogenous opioid peptides is still not thoroughly investigated. The aim of this study was to examine the effect of repeated episodic ethanol exposure during adolescence on the tissue levels of three different endogenous opioid peptides in rats. Outbred Wistar rats received orogastric (i.e., gavage) ethanol for three consecutive days per week between 4 and 9 weeks of age. At 2 h and 3 weeks, respectively, after the last exposure, beta-endorphin, dynorphin B and Met-enkephalin-Arg^{6}Phe^7 (MEAP) were analyzed with radioimmunoassay. Beta-endorphin levels were low in the nucleus accumbens during ethanol intoxication. Remaining effects of adolescent ethanol exposure were found especially for MEAP, with low levels in the amygdala, and high in the substantia nigra and ventral tegmental area three weeks after the last exposure. In the hypothalamus and pituitary, the effects of ethanol on beta-endorphin were dependent on time from the last exposure. An interaction effect was also found in the accumbal levels of MEAP and nigral dynorphin B. These results demonstrate that repeated episodic exposure to ethanol during adolescence affected opioid peptide levels in regions involved in reward and reinforcement as well as stress response. These alterations in opioid networks after adolescent ethanol exposure could explain, in part, the increased risk for high ethanol consumption later in life.

Keywords: beta-endorphin, dynorphin B, enkephalin, rat model, developing brain, alcohol

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INTRODUCTION

During adolescence, social interactions with peers become highly important and increased frequencies in behaviors like risk-taking, impulsivity and novelty-seeking can be observed in experimental models (1). In the western world, many adolescents begin experimentation with ethanol during this period of life (2, 3). Exposure to ethanol may pose risks as indicated by findings showing that early onset of drug consumption can increase later susceptibility for drug abuse and addiction (4–7). This vulnerability could be a result of three factors (8). Firstly, adolescents frequent environments in which drugs are used. Secondly, early use could be a consequence of an inherited vulnerability for drugs of abuse. Thirdly, as the adolescent brain continually matures, early use might shape the brain toward a vulnerability state, which consequently leads to later susceptibility for drug use. To investigate the third factor, an adolescent rat model was used to study the endogenous opioid system after episodic binges of ethanol.

Ethanol has an unspecific mechanism of action as it does not have a specific target protein. Instead, ethanol acts on several receptors and ion channels in a number of transmitter networks, including the endogenous opioid system (9, 10). The endogenous opioids regulate other neurotransmitters of importance for reward and reinforcement (e.g., dopamine and γ-aminobutyric acid). Most drugs of abuse affect the endogenous opioid system and their effects on different brain target areas differ depending on the drug and also on the phase in the addiction cycle, i.e., binge/intoxication, withdrawal/negative affect, preoccupation/anticipation (9, 11, 12). The classical endogenous opioid system consists of three G-protein coupled receptors (μ-, δ-, and κ-receptors) and their corresponding ligands (endorphins, enkephalins and dynorphins). The endogenous opioid peptides are derived from precursors i.e., prohormones (13, 14). Beta-endorphin that binds to μ-receptors is generated from proopiomelanocortin (15). Dynorphin B is cleaved from prodynorphin (16) and binds to κ-receptors. Met-enkephalin-Arg^6-Phe^7 (MEAP) is derived from proenkephalin (17) and binds predominantly to δ-receptors but also to μ-receptors (18).

This study used adolescent male Wistar rats to evaluate the effects of episodic binge-like exposure of ethanol on brain levels of the following three opioid peptides; beta-endorphin, dynorphin B, and MEAP. Levels were measured at 2 h, to investigate the effects of intoxication, and at 3 weeks, to study long-term, residual changes (**Figure 1**).

METHODS

Animals and Experimental Design

All animal experiments were performed with the approval of the Uppsala Animal Ethical Committee and according to the principles of the Guide for the Care and Use of Laboratory Animals, the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56), and the EU Parliament and the Council Directive of 22 September 2010 (2010/63/EU).

Two sets of time-mated Wistar rats (Harlan Laboratories B.V., Horst, the Netherlands) arrived at the animal facility in Uppsala, Sweden, at gestation day 15. The dams were housed individually in a standard cage (59 × 38 × 20 cm) with wood chip bedding and nesting material under standard conditions (22°C, 50 ± 10% humidity, 12 h light-dark cycle commencing at 07:00, *ad libitum* access to pellet food and tap water, and background noise masking). The pregnant females were transported during the least sensitive phase of the gestation. No signs of negative impact of the travel were noticed and the delivery was normal in all females. To avoid biological littermates, the litters were cross-fostered and mixed on the day of birth (postnatal day, PND, 0) so each litter contained four females and six males. Previous studies have shown that single housing affects brain levels of endogenous opioid peptides in adolescent rats (19, 20), so on PND 21 the pups were weaned and group housed (2–3 rats per cage) to avoid confounding factors.

Adolescent Ethanol Exposure

Between 4 and 9 weeks of age, the male rats received orogastric administration of water ($n = 20$) or ethanol, 2 g/kg, 20% v/v ethanol diluted with tap water, ($n = 20$).

Administrations were given at 09:00 on three consecutive days, followed by 4 days without treatment. Orogastric administration was used since it does not require single housing and this route of administration resemble the oral ingestion of ethanol by humans. Unpublished data from our pilot study and published data from others (21) have shown that 2 g/kg produces blood alcohol concentration reaching the National Institute on Alcohol Abuse and Alcoholism criterion for binge drinking (i.e., >0.08 g/dl in 2 h). The rats were housed under standard conditions as described above except that the light and dark cycles were reversed at weaning. The rats were euthanized by decapitation either 2 h or 3 weeks after the last ethanol exposure.

Tissue Stabilization and Sampling

The pituitary glands was snap frozen on dry ice whereas the whole brains were immediately frozen in an isopentane bath (−20°C for 2 min). The tissues were stored at −80°C. One day prior to stabilization, the whole brains and the pituitaries were moved to a −20°C freezer to reduce the temperature gradient before stabilization. The tissue samples were stabilized by heat denaturation (95°C) with a bench-top Stabilizor T1 (Denator AB, Uppsala, Sweden) according to the manufacturer's manual. The stabilization process involves a combination of conductive heat transfer and pressure under vacuum to prevent enzymatic degradation (e.g., of peptides) during freeze-thawing (22). Whole brains were placed in a Maintainor Tissue card (Denator AB, Uppsala, Sweden) and stabilized in the "frozen structure preserve mode" and thereafter in the "fresh structure preserve mode" to ensure an adequate treatment. After stabilization, the brains were dissected according to Paxinos and Watson (23) to separate the hypothalamus, medial prefrontal cortex, cingulate cortex, dorsal striatum, nucleus accumbens, amygdala, hippocampus, ventral tegmental area (VTA) and substantia nigra. The pituitaries were individually placed in a pre-chilled Maintainor Tissue card and stabilized in the "frozen quick compress mode". Stabilized tissues were thereafter stored at −80°C.

Peptide Extraction

The tissues were moved from −80°C and heated in 95°C acetic acid (1M) for 5 min, then placed on ice and homogenized by sonication using a Branson Sonifier (Danbury, CT, USA). The homogenates were centrifuged for 15 min at 4°C, 12,000 × *g* in a Beckman GS-15R centrifuge (Fullerton, CA, USA) and supernatants were purified by cation exchange chromatography procedure (24). The purified samples were dried in a vacuum centrifuge and stored at −20°C.

Abbreviations: ANOVA, Analysis of variance; MEAP, Met-Enkephalin-Arg^6-Phe^7; VTA, Ventral tegmental area.

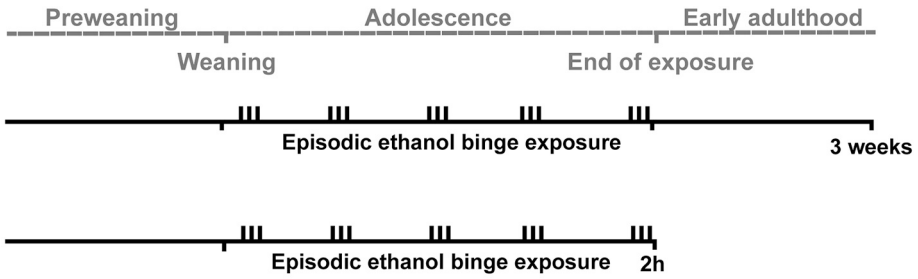

FIGURE 1 | A schematic overview of the experiment. Adolescent male Wistar rats were exposed to episodic binges of ethanol three times per week (indicated by vertical bars) during adolescence. Two hours (in the intoxicated state) or three weeks (to measure residual effects) after the last exposure of ethanol, three endogenous opioids (beta-endorphin, dynorphin B and Met-Enkephalin-Arg^6-Phe^7) were measured in several brain areas. The gray dotted line indicates the timeline of the experiment.

TABLE 1 | Immunoreactive levels (fmol/mg) of beta-endorphin.

	Water intoxication	Ethanol intoxication	Water residual effects	Ethanol residual effects	Two-factor ANOVA			η_p^2
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Treatment	Time	Treatment × Time	
Pit	27.982 ± 1.653	33.940 ± 2.890	31.685 ± 2.783	25.800 ± 2.054	$F_{(1,35)} < 0.01$; $p = 0.99$	$F_{(1,35)} = 0.84$; $p = 0.37$	$\mathbf{F_{(1,35)} = 5.99; p = 0.020}$	0.17
Ht	41.0 ± 1.3	44.4 ± 1.6	45.0 ± 3.7	37.5 ± 2.2	$F_{(1,35)} = 0.69$; $p = 0.41$	$F_{(1,35)} = 0.35$; $p = 0.56$	$\mathbf{F_{(1,35)} = 5.16; p = 0.029}$	0.15
AMY	3.5 ± 0.3	2.9 ± 0.2	3.1 ± 0.4	2.8 ± 0.2	$F_{(1,35)} = 2.84$; $p = 0.10$	$F_{(1,35)} = 0.79$; $p = 0.38$	$F_{(1,35)} = 0.36$; $p = 0.55$	0.10
NAc	2.2* ± 0.5	1.0# ± 0.1	0.9 ± 0.1	1.1 ± 0.2	$F_{(1,35)} = 3.23$; $p = 0.081$	$\mathbf{F_{(1,35)} = 4.89; p = 0.034}$	$\mathbf{F_{(1,35)} = 5.32; p = 0.027}$	0.28
VTA	2.9 ± 0.3	2.6 ± 0.2	2.7 ± 0.3	2.8 ± 0.3	$F_{(1,34)} = 0.14$; $p = 0.71$	$F_{(1,34)} < 0.01$; $p = 0.99$	$F_{(1,34)} = 0.37$; $p = 0.55$	0.016
dStr	0.6 ± 0.1	0.4 ± 0.04	0.4 ± 0.04	0.4 ± 0.02	$F_{(1,34)} = 3.87$; $p = 0.057$	$\mathbf{F_{(1,34)} = 5.84; p = 0.021}$	$F_{(1,34)} = 1.55$; $p = 0.22$	0.25

*Rats were repeatedly exposed to ethanol or water during adolescence. Two hours (during ethanol intoxication) or three weeks (residual effects) after the last exposure, the immunoreactive levels of beta-endorphin were measured in different brain regions. Amy, amygdala; ANOVA, analysis of variance; Ht, hypothalamus; NAc, nucleus accumbens; η_p^2, partial eta-squared; Pit, pituitary; Str, striatum; VTA, ventral tegmental area. Tukey's post hoc test; *$p < 0.05$ intoxication effects (2 h) compared to the residual effects (3 weeks) of the same treatment; #$p < 0.05$ ethanol compared to water at the same time-point. Bold letters highlights statistically significant results.*

Radioimmunoassay

Measurement of the immunoreactive levels of dynorphin B and MEAP was performed according to Nylander et al. (25, 26) with antisera generated in rabbits. The dynorphin B antiserum (113+) was used at a final dilution of 1:500,000. The cross-reactivity with DYNB 29 is 1% and with big dynorphin (DYN 32) 100%, whereas no other opioid peptide cross-reacts in the assay. The detection range in the dynorphin B assay is 1–70 fmol in 25 µl of the sample. The MEAP antiserum (90:3D II) was used at a final dilution of 1:140,000. The cross-reactivity with Met-enkephalin, Met-enkephalin-Arg^6, Met-enkephalin-Arg^{6}Gly^{7}Leu^8, Leu- enkephalin and dynorphin A (1–6) is less than 0.1% and no other opioid peptide cross-reacts in the assay. The detection range in the MEAP assay is 2-100 fmol in 25 µl of the sample. Antibody-bound peptides in the dynorphin B assay were separated from free peptides by adding goat-anti-rabbit-IgG and normal rabbit serum. For the MEAP assay, separation was performed by adding charcoal suspension (Sigma-Aldrich, MO, USA).

For the beta-endorphin, a commercial kit was used according to the manufacturer's instructions (Phoenix Pharmaceutical, Inc., Burlingame, CA, USA). Cross-reactivity was reported to be 100% with alpha-endorphin, 40% with human beta-endorphin but none with alpha-MSH, ACTH, PACAP 38, Met- or Leu-enkephalin and the detection range was 1–128 pg in 100 µl of the sample.

Statistics

One-way analysis of variance (ANOVA) was used to investigate overall differences between the groups and effect size was estimated with the partial eta-squared test. Factorial ANOVAs were used to test the effects of treatment (adolescent exposure to ethanol or water), time (2 h or 3 weeks after the last exposure) or interaction (time × treatment). The factor time also represents a factor of age since the rats were 9 or 12 weeks of age at the time-point for decapitation, i.e., 2 h or 3 weeks after the last exposure. Significant levels were set to $p < 0.05$; Tukey's *post hoc* test was used to analyze between-group differences. Extreme values (1.5 standard deviation) within each treatment group were excluded from the analyses.

RESULTS

The statistical results for beta-endorphin, dynorphin B and MEAP in all brain tissues and the pituitary are given in **Tables 1–3** respectively.

TABLE 2 | Immunoreactive levels (fmol/mg) of dynorphin B.

	Water intoxication	Ethanol intoxication	Water residual effects	Ethanol residual effects	Two-factor ANOVA			η_p^2
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Treatment	Time	Treatment × Time	
Pit	570.1° ± 23.1	703.4 ± 32.6	677.8 ± 30.3	748.4 ± 48.7	**$F_{(1,34)} = 8.09$; $p = 0.01$**	**$F_{(1,34)} = 4.54$; $p = 0.04$**	$F_{(1,34)} = 0.76$; $p = 0.39$	0.28
Ht	22.6 ± 2.3	21.8 ± 1.6	28.3 ± 1.4	29.2 ± 1.9	$F_{(1,36)} = 0.02$; $p = 0.88$	**$F_{(1,36)} = 4.76$; $p = 0.04$**	$F_{(1,36)} = 0.25$; $p = 0.62$	0.20
AMY	9.8 ± 1.0	9.2 ± 0.8	8.9 ± 1.1	10.4 ± 0.8	$F_{(1,35)} = 0.22$; $p = 0.65$	$F_{(1,35)} = 0.03$; $p = 0.87$	$F_{(1,35)} = 1.23$; $p = 0.27$	0.040
NAc	36.7* ± 2.4	30.0 ± 1.6	28.7 ± 1.1	29.0 ± 2.2	$F_{(1,35)} = 2.73$; $p = 0.11$	**$F_{(1,35)} = 5.49$; $p = 0.02$**	$F_{(1,35)} = 3.24$; $p = 0.08$	0.25
VTA	5.3 ± 0.6	5.7 ± 0.8	6.2 ± 1.2	8.3 ± 1.5	$F_{(1,34)} = 1.24$; $p = 0.27$	$F_{(1,34)} = 2.62$; $p = 0.11$	$F_{(1,34)} = 0.54$; $p = 0.47$	0.12
SN	83.2 ± 7.9	85.4 ± 8.9	62.5 ± 9.9	101.4# ± 9.3	**$F_{(1,35)} = 5.21$; $p = 0.03$**	$F_{(1,35)} = 0.07$; $p = 0.79$	**$F_{(1,35)} = 4.13$; $p = 0.05$**	0.22
dStr	16.1 ± 0.8	14.7 ± 1.5	15.5 ± 0.9	13.4 ± 0.6	$F_{(1,35)} = 2.91$; $p = 0.10$	$F_{(1,35)} = 0.86$; $p = 0.36$	$F_{(1,35)} = 0.13$; $p = 0.72$	0.097
Hc	22.6 ± 2.3	21.8 ± 1.6	26.6 ± 2.7	28.1 ± 2.7	$F_{(1,36)} = 0.02$; $p = 0.88$	**$F_{(1,36)} = 4.76$; $p = 0.04$**	$F_{(1,36)} = 0.25$; $p = 0.62$	0.12
CCx	1.4 ± 0.3	1.0 ± 0.2	1.2 ± 0.2	1.5 ± 0.3	$F_{(1,35)} = 0.02$; $p = 0.90$	$F_{(1,35)} = 0.23$; $p = 0.64$	$F_{(1,35)} = 1.83$; $p = 0.18$	0.055
MPFCx	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	$F_{(1,36)} = 0.14$; $p = 0.71$	$F_{(1,36)} = 0.06$; $p = 0.82$	$F_{(1,36)} = 0.42$; $p = 0.52$	0.017

*Rats were repeatedly exposed to ethanol or water during adolescence. Two hours (during ethanol intoxication) or three weeks (residual effects) after the last exposure, the immunoreactive levels of dynorphin B were measured in different brain regions. Amy, amygdala; CCx, cingulate cortex; Hc, hippocampus; Ht, hypothalamus; DynB; mPFCx, medial prefrontal cortex; NAc, nucleus accumbens; η_p^2, partial eta squared; Pit, pituitary; SN, substantia nigra; Str, striatum; VTA, ventral tegmental area. Tukeys post hoc test; *$p < 0.05$ intoxication effects (2 h) compared to the residual effects (3 weeks) of the same treatment; #$p < 0.05$ ethanol compared to water at the same time-point; °$p = 0.06$ ethanol compared to water at the same time-point. Bold letters highlights statistically significant results.*

TABLE 3 | Immunoreactive levels (fmol/mg) of Met-Enkephalin-Arg^6-Phe^7 (MEAP).

	Water intoxication	Ethanol intoxication	Water residual effects	Ethanol residual effects	Two-factor ANOVA			η_p^2
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Treatment	Time	Treatment x Time	
Pit	12.6 ± 2.1	33.9° ± 9.1	17.1 ± 3.1	17.6 ± 3.1	$F_{(1,33)} = 3.88$; $p = 0.06$	$F_{(1,33)} = 1.15$; $p = 0.3$	$F_{(1,33)} = 3.58$; $p = 0.07$	0.12
Ht	129.2* ± 4.4	125.3* ± 6.5	107.1 ± 6.9	99.3 ± 5.2	$F_{(1,35)} = 0.98$; $p = 0.3$	**$F_{(1,35)} = 17.07$; $p < 0.001$**	$F_{(1,35)} = 0.11$; $p = 0.8$	0.34
AMY	88.0 ± 7.2	65.9 ± 11.3	92.2 ± 10.8	68.9 ± 5.3	**$F_{(1,36)} = 6.33$; $p = 0.02$**	$F_{(1,36)} = 0.16$; $p = 0.7$	$F_{(1,36)} = 0.0041$; $p = 0.9$	0.15
NAc	93.6 ± 7.6	121.6 ± 6.8	106.4 ± 10.5	100.4 ± 7.7	$F_{(1,34)} = 1.79$; $p = 0.2$	$F_{(1,34)} = 0.26$; $p = 0.6$	**$F_{(1,34)} = 4.31$; $p = 0.05$**	0.16
VTA	20.7 ± 1.3	23.9 ± 3.2	16.9 ± 1.9	25.6 ± 2.6	**$F_{(1,34)} = 6.20$; $p = 0.02$**	$F_{(1,34)} = 0.18$; $p = 0.7$	$F_{(1,34)} = 1.27$; $p = 0.3$	0.18
SN	11.6 ± 0.9	15.8 ± 1.3	11.7 ± 1.6	15.6 ± 1.9	**$F_{(1,35)} = 7.15$; $p = 0.01$**	$F_{(1,35)} = 0.0016$; $p = 0.9$	$F_{(1,35)} = 0.0089$; $p = 0.9$	0.16
dStr	85.1* ± 5.8	81.5 ± 6.1	60.9 ± 4.9	71.5 ± 6.1	$F_{(1,34)} = 0.36$; $p = 0.6$	**$F_{(1,34)} = 8.60$; $p = 0.006$**	$F_{(1,34)} = 1.50$; $p = 0.2$	0.23
Hc	9.6 ± 0.9	10.5 ± 0.7	9.4 ± 1.3	9.8 ± 1.2	$F_{(1,36)} = 0.35$; $p = 0.6$	$F_{(1,36)} = 0.18$; $p = 0.7$	$F_{(1,36)} = 0.069$; $p = 0.8$	0.016
CCx	2.0 ± 0.4	1.8 ± 0.3	1.6 ± 0.3	2.3 ± 0.5	$F_{(1,35)} = 0.37$; $p = 0.5$	$F_{(1,35)} = 0.014$; $p = 0.9$	$F_{(1,35)} = 1.33$; $p = 0.3$	0.048
MPFCx	5.8 ± 0.5	7.1 ± 0.6	7.0 ± 1.7	5.9 ± 0.4	$F_{(1,36)} = 0.023$; $p = 0.9$	$F_{(1,36)} = 0.0014$; $p = 0.9$	$F_{(1,36)} = 1.91$; $p = 0.2$	0.043

*Rats were exposed to ethanol or water during adolescence. Two hours (during ethanol intoxication) or three weeks (residual effects) after the last exposure, the immunoreactive levels of MEAP were measured in different brain regions. Amy, amygdala; CCx, cingulate cortex; Hc, hippocampus; Ht, hypothalamus; mPFCx, medial prefrontal cortex; NAc, nucleus accumbens; Pit, pituitary; SN, substantia nigra; Str, striatum; VTA, ventral tegmental area; w, weeks. Tukeys post hoc test; *$p < 0.05$ intoxication effects (2 h) compared to the residual effects (3 weeks) of the same treatment; °$p = 0.052$ compared to water at the same time-point. Bold letters highlights statistically significant results.*

Beta-Endorphin

In the nucleus accumbens, differences in beta-endorphin levels between the ethanol-treated rats and water controls were indicated by an interaction between time and treatment [$F_{(1,35)} = 5.32$; $p = 0.03$]. Beta-endorphin levels were lower in the intoxicated state (i.e., after 2 h) than for their time-matched water controls; this effect was not present 3 weeks after the last exposure of ethanol (**Figure 2**).

An effect of time [$F_{(1,35)} = 4.89$; $p = 0.03$] was also found in the nucleus accumbens and was driven by the higher beta-endorphin in the water group at 2 h. Interactions between treatment and time were also found in the hypothalamus [$F_{(1,35)} = 5.16$; $p = 0.03$] and in pituitary [$F_{(1,35)} = 5.99$; $p = 0.02$] but the Tukey's *post-hoc* test did not reveal any between-group differences, see **Figure 3**. An overall effect between the treatment groups was found in the dorsal striatum [$F_{(3,34)} = 3.85$; $p = 0.03$], but *post hoc* analyses failed to identify any statistical between-group differences in beta-endorphin. Furthermore, the two-way ANOVA analysis showed an effect of time [$F_{(1,34)} = 5.84$; $p = 0.02$] in the dorsal striatum (**Table 1**).

Dynorphin B

In the pituitary, there was an overall effect of treatment [$F_{(1,34)} = 8.09$; $p = 0.01$] and time [$F_{(1,34)} = 4.54$; $p = 0.04$], and a trend ($p = 0.06$) of increased dynorphin B was seen in ethanol-intoxicated rats (**Table 2**). In the substantia nigra,

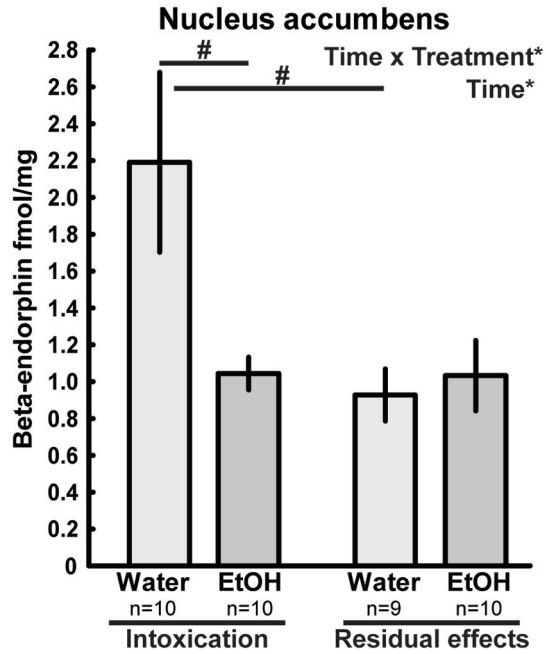

FIGURE 2 | Beta-endorphin (fmol/mg tissue) in the nucleus accumbens after repeated adolescent ethanol (EtOH) exposure. Levels were measured in an ethanol-intoxicated state (2 h after last exposure) and 3 weeks after the exposure (residual effects). Data expressed as mean ± SEM. "Treatment × time" and "Treatment" indicates a significant interaction effect and an effect of treatment, respectively, $^{*}p < 0.05$ two-factor ANOVA. $^{\#}p < 0.05$ Tukey's HSD *post hoc* test.

there was an effect of treatment [$F_{(1,35)} = 5.21$; $p = 0.03$] as well as an interaction effect [$F_{(1,35)} = 4.13$; $p = 0.05$]. In the intoxicated state (2 h), there was no difference between the ethanol-treated group and water controls, but higher dynorphin B were found in the substantia nigra of the ethanol treated group at 3 weeks (**Figure 4**). For dynorphin B, an effect of time was present in the hypothalamus [$F_{(1,36)} = 4.76$; $p = 0.04$], the nucleus accumbens [$F_{(1,35)} = 5.49$; $p = 0.02$] and the hippocampus [$F_{(1,36)} = 4.76$; $p = 0.04$] (**Table 2**).

Met-Enkephalin-Arg^6-Phe^7

In several brain areas, the effects of ethanol exposure on MEAP levels persisted 3 weeks after the last exposure to ethanol. In the amygdala, an effect of treatment [$F_{(1,36)} = 6.33$; $p = 0.02$] was found with lower MEAP after ethanol exposure (**Figure 5A**). In the VTA [$F_{(1,34)} = 6.20$; $p = 0.02$] and substantia nigra [$F_{(1,35)} = 7.15$; $p = 0.01$], the levels were higher in ethanol-exposed rats (**Figures 5B,C**). In the above-mentioned structures, Tukey's *post hoc* test did not reveal any between-group differences. There was a significant overall effect [$F_{(3,33)} = 2.97$; $p = 0.05$] in the pituitary, but *post hoc* analysis showed only a strong trend ($p = 0.053$) of higher MEAP in the ethanol-intoxicated rats (**Table 3**). In the nucleus accumbens, an interaction effect [$F_{(1,34)} = 4.31$; $p = 0.05$] showed that the levels of MEAP varied, depending on both treatment and time but there

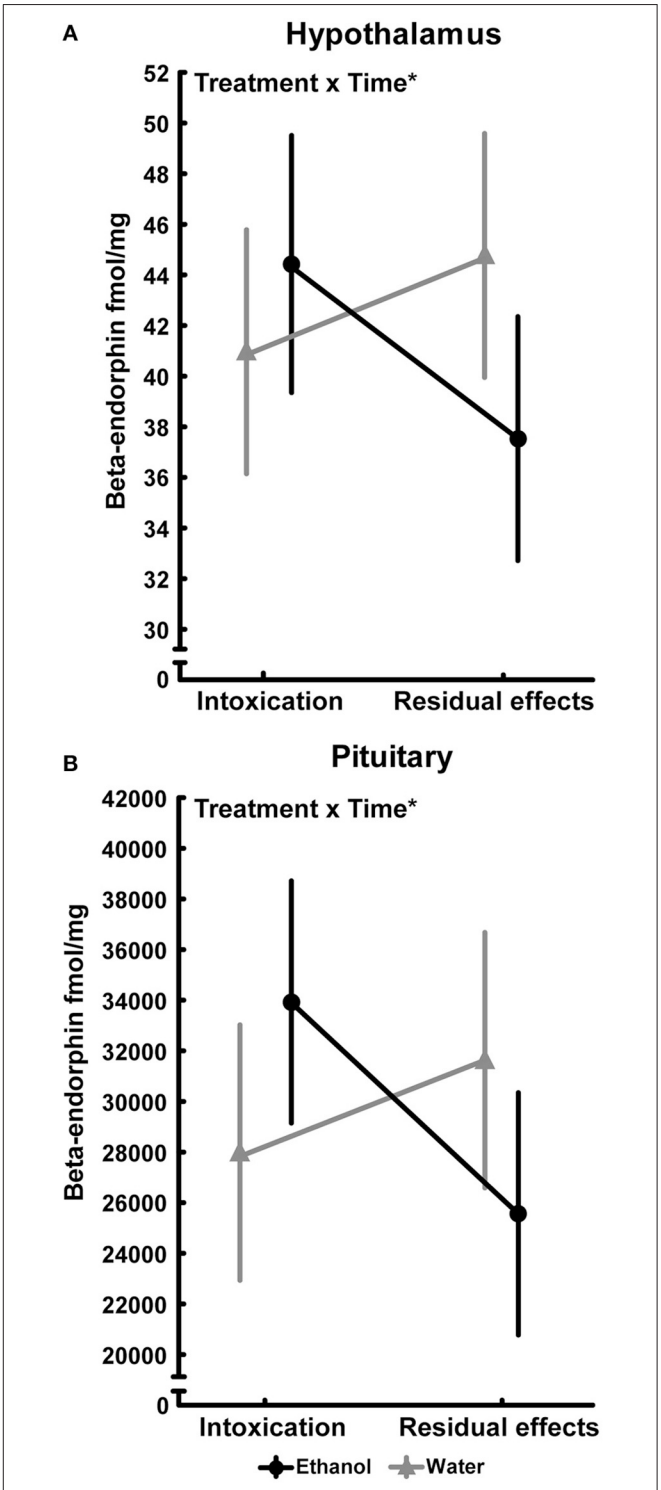

FIGURE 3 | Beta-endorphin (fmol/mg tissue) in **(A)** hypothalamus (Intoxication; water, $n = 10$; ethanol, $n = 9$ and residual effects; water, $n = 10$; ethanol, $n = 10$) and **(B)** pituitary (Intoxication; water, $n = 9$; ethanol $n = 10$ and Residual effects; water, $n = 9$; ethanol, $n = 10$) after repeated adolescent ethanol exposure. Levels were measured in an ethanol-intoxicated state (2 h after last exposure) and 3 weeks after the last exposure (residual effects). Data expressed as mean ± SEM. "Treatment × time" indicates a significant inter-action effect ($^{*}p < 0.05$ two-factor ANOVA).

was no significant differences between the groups (**Figure 6**). An effect of time, i.e., 2 h or 3 weeks after the last ethanol exposure, was found in the hypothalamus [$F_{(1,35)} = 17.07$; $p < 0.001$]. MEAP levels were found to be lower at 3 weeks than at 2 h for both the ethanol and control groups (**Table 3**). The same effect of time was also present in the dorsal striatum [$F_{(1,34)} = 8.60$; $p = 0.006$] (**Table 3**).

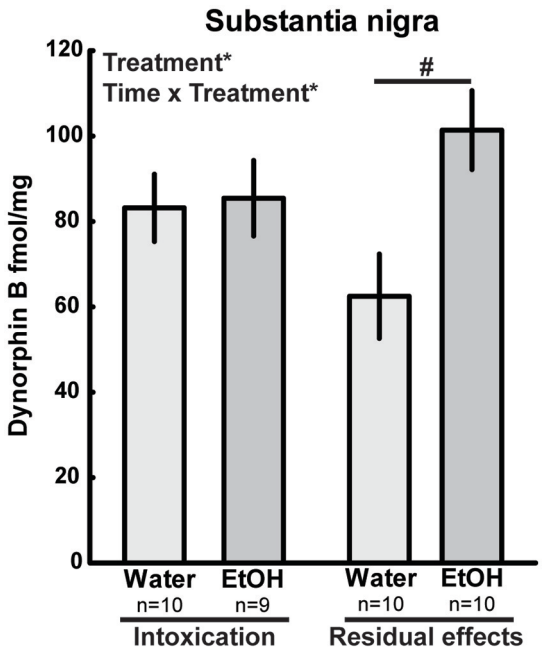

FIGURE 4 | Dynorphin B (fmol/mg tissue) in substantia nigra after repeated adolescent ethanol (EtOH) exposure. Levels were measured in an ethanol-intoxicated state (2 h after last exposure) and 3 weeks after the last exposure (residual effects). Data expressed as mean ± SEM. "Treatment × time" and "Treatment" indicates a significant interaction effect and an effect of treatment, respectively, $^{*}p < 0.05$ two-factor ANOVA. $^{\#}p < 0.05$ Tukey's HSD *post hoc* test.

DISCUSSION

The neurobiological consequences of ethanol exposure during adolescence have been a neglected field in preclinical research until only recently [for recent reviews see (27, 28)]. There is, for example, still a knowledge gap in how ethanol affects the endogenous opioids in the adolescent brain, and the literature regarding the effect of repeated ethanol exposure during adolescence is almost nonexistent. To our knowledge, this is the first study to investigate the pharmacological effects of repeated adolescent ethanol exposure on the endogenous opioids, including both intoxication effects and residual effects 3 weeks after the exposure.

Previous studies from our laboratory have reported the effects of ethanol on endogenous opioids in adult rats as a function of strain, housing condition and ethanol administration paradigm (29–31). The differences for adult vs. adolescent rats must be compared carefully as they could be due to age, ethanol administration model, or both. In both adult and adolescent rats, the central levels of endogenous opioids interact with housing conditions (i.e., single or group housed) and ethanol intake (19, 20). These aforementioned studies show the profound importance of the experimental settings when working with ethanol models in rats. Therefore, to evaluate the pharmacological effects of ethanol exposure during adolescence, our rats were housed in groups and the ethanol was administered orogastrically by gavage to control the doses received. The present study focused on the effects in male rats and how ethanol affects the endogenous opioid peptides in females remains to be examined.

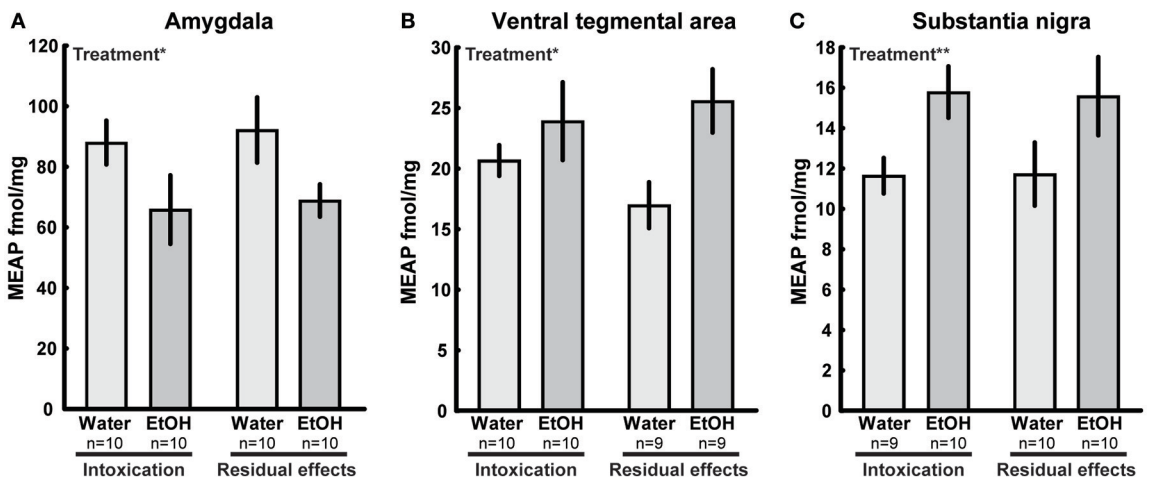

FIGURE 5 | Met-Enkephalin-Arg^{6}-Phe^{7} (MEAP) (fmol/mg tissue) in **(A)** amygdala, **(B)** ventral tegmental area, and **(C)** substantia nigra after repeated adolescent ethanol (EtOH) exposure. Levels were measured in an ethanol-intoxicated state (2 h after last exposure) and 3 weeks after the last exposure (residual effects). Data expressed as mean ± SEM. "Treatment" indicates a significant interaction effect of treatment, $^{*}p < 0.05$, $^{**}p < 0.01$ two-factor ANOVA.

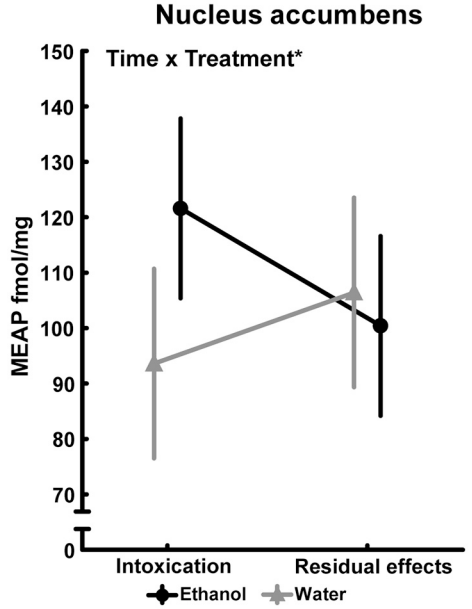

FIGURE 6 | Met-Enkephalin-Arg^6-Phe^7 (MEAP) (fmol/mg tissue) in the nucleus accumbens after repeated adolescent ethanol exposure. Levels were measured in an ethanol-intoxicated state (2 h after last exposure) and 3 weeks after the last exposure (residual effects). Data expressed as mean ± SEM. "Treatment × time" indicates a significant interaction effect, $^*p < 0.05$ two-factor ANOVA. Intoxication; water, $n = 10$; ethanol, $n = 10$ and residual effects; water, $n = 9$; ethanol, $n = 10$.

Repeated Ethanol Exposure During Adolescence and Intoxication Effects

In the present study, MEAP levels were increased in the pituitary 2 h after the last exposure. Palm and Nylander (20) presented similar results with increased MEAP 2 h after last drinking session in both single and group housed rats. This indicates a pharmacological effect of increased MEAP in the pituitary during intoxication. A trend ($p = 0.06$) toward increased dynorphin B was also found in the pituitary of the ethanol intoxicated rats. These changes may reflect ethanol involvement in stress axis activation as previously been reported [for review see Zhou and Kreek (32)].

Effects of intoxication were also found in nucleus accumbens where beta-endorphin was lower in the ethanol-exposed rats. This finding is in contrast to studies on adult rats, that report increased beta-endorphin in the nucleus accumbens after acute ethanol exposure (33, 34). This difference could be due to the choice of methodology, i.e., measuring peptide content in dialysate vs. tissue content, or it could be due to the effect of intoxication after just a single exposure vs. repeated exposure as in our study. The low beta-endorphin could be an indirect effect caused by ethanol-induced alterations in social behavior. Social play behavior activates the endogenous opioid system (35), specifically, the μ-receptors in the nucleus accumbens (36, 37). Ethanol has been shown to interfere with social play behavior; at low doses (0.25–0.75 g/kg) this behavior increase whereas at higher doses (1–4 g/kg) it decrease (38). In the present study, 2 g/kg ethanol was administered during the age interval (4–9 weeks of age) in which play behavior is reported to peak (39). Hence, the ethanol exposure could have affected the normal play behavior and thus social development, which could explain the differences in beta-endorphin levels in the nucleus accumbens. Another plausible explanation is that the high levels in water controls at the 2-h time-point is a consequence of stress-induced activation of beta-endorphin networks by the orogastric administration, considered a mild stressor (40), and that this effect is blunted by ethanol in the intoxicated animals. Differences between the ethanol-treated rats and water controls were not seen at the other time-point when 3 weeks had passed from the handling procedure. Changes in beta-endorphin were also seen as an interaction effect between time and treatment in the hypothalamus and pituitary, indicating possible effects of the handling procedure. Intermittent exposure of ethanol in adolescence have been reported to increase the expression of *pomc* in hypothalamus along with an increase of histone acetylation of the gene promotor (41).

An interaction between treatment (ethanol or water) and time (2 h or 3 weeks after the last exposure) was seen in the accumbal levels of MEAP, with the highest levels occurring in the intoxicated state. Previous studies have shown that ethanol intoxication increase enkephalins in the nucleus accumbens of adult rats. An increase of *Penk* expression and δ-receptor binding in shell and core of accumbens can be seen 2 h after ethanol administration (42, 43). Awake rats have increased accumbal levels of Met-enkephalin when injected with 1.6 g/kg ethanol, whereas higher (2.4 or 3.2 g/kg) or lower (0.8 g/kg) doses have no effect on Met-enkephalin (44). In anesthetized rats, the highest dose of ethanol (2.5 g/kg) leads to a peak of Met-enkephalin at 30 min, but lower doses (0.5 or 1.0 g/kg) delay the peak 90 and 60 min respectively (45). Furthermore, adolescent ethanol exposure alters the expression of *Penk* in the nucleus accumbens after an acute ethanol challenge in adult rats (46).

Residual Effects After Repeated Ethanol Exposure During Adolescence

An interesting finding was the residual effects of adolescent ethanol exposure on MEAP, such as the lower MEAP in the amygdala observed 3 weeks after the last exposure. The enkephalin system in amygdala is involved in emotional processing of states such as anxiety and stress (47) and *Oprd1* and *Penk* knock-out mice show increased anxiety and depressive like behaviors in a variety of tests (48–50). Pharmacological studies with systemic administrations and local injections of δ-receptor agonists into the amygdala decrease anxious behavior (51–53). Likewise, the administration of antagonists (51, 52, 54, 55) increases anxiety-like behaviors.

The scope herein was not to study behavioral manifestations *per se*, but the finding of residual low levels of enkephalin in the amygdala after adolescent exposure to ethanol indeed indicates long-lasting consequences that could relate to the increase in anxiety-like behaviors reported by others (41, 56, 57). δ-receptor knockout mice have an increased consumption of ethanol (58) and their elevated intake may be a way to reduce

their elevated anxiety level (47). The low enkephalin tone after adolescent ethanol exposure may therefore constitute a risk factor for elevated intake of ethanol later in life. As noted above, an interaction between time and treatment was found in beta-endorphin in the hypothalamus and in the pituitary—these brain areas, along with the amygdala, are important in the regulation of the stress response.

Repeated exposure of ethanol during adolescence increased MEAP and dynorphin B in substantia nigra and MEAP in the VTA. The substantia nigra and VTA contain dopaminergic neurons that extend into the striatal, limbic and cortical areas (59). Importantly, endogenous opioids are highly involved in regulating dopamine output (60–62) and residual effects after adolescent ethanol exposure may have consequences for opioid regulation of dopamine pathways. Adolescent ethanol exposure has been shown to alter the dopamine dynamics in the dorsal striatum (63, 64), nucleus accumbens (65–67) and medial prefrontal cortex (68). An interesting aspect for future research would be to investigate the relationships between dopamine and opioid changes after adolescent ethanol exposure.

CONCLUSION

Intoxication after repeated ethanol exposure during adolescence altered the levels of MEAP and beta-endorphin in the accumbens and dynorphin B and MEAP in the pituitary. Especially noteworthy is the observation of long-term consequences of the adolescent ethanol exposure, particularly MEAP in the amygdala and beta-endorphin in the hypothalamus and pituitary as these regions are involved in the response to anxiety and stress. Furthermore, residual effects were noted in the substantia nigra and VTA, areas important for opioid regulation of dopaminergic projections in the reward circuitry. It has been postulated that changes in stress circuits and in dopaminergic activity increase the susceptibility for alcohol use disorders. Hopefully, the data presented herein on the alterations in endogenous opioids after adolescent ethanol exposure can contribute in the understanding of how adolescent ethanol exposure increases the risk of elevated alcohol consumption later in life.

AUTHOR CONTRIBUTIONS

IN, LG, and LS, experimental design. LG and LS, experimental work. LG, statistical analyses. LG, writing of the first draft. LS and IN, critical revision of the manuscript. LG, LS, and IN, finalization and approval of manuscript content.

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REFERENCES

1. Spear LP. Adolescents and alcohol: acute sensitivities, enhanced intake, and later consequences. *Neurotoxicol Teratol*. (2014) 41:51–9. doi: 10.1016/j.ntt.2013.11.006
2. HBSC. Growing up unequal: gender and socioeconomic differences in young people's health and well-being [Online]. *World Health Organization*. (2013/2014) (Accessed 23 Nov, 2017).
3. Johnston L, O'malleyP, Miech R, Bachman J, Schulenberg J. *Monitoring the Future National Survey Results on Drug Use, 1975-2016: Overview, Key Findings on Adolescent Drug Use*. [Online]. Ann Arbor, MI: Institute for Social Research, University of Michigan (2017).
4. Grant BF, Dawson DA. Age of onset of drug use and its association with DSM-IV drug abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. *J Subst Abuse* (1998) 10:163–73. doi: 10.1016/S0899-3289(99)80131-X
5. Dewit DJ, Adlaf EM, Offord DR, Ogborne AC. Age at first alcohol use: a risk factor for the development of alcohol disorders. *Am J Psychiatry* (2000) 157:745–50. doi: 10.1176/appi.ajp.157.5.745
6. York JL, Welte J, Hirsch J, Hoffman JH, Barnes G. Association of age at first drink with current alcohol drinking variables in a national general population sample. *Alcohol Clin Exp Res*. (2004) 28:1379–87. doi: 10.1097/01.ALC.0000139812.98173.A4
7. Merline A, Jager J, Schulenberg JE. Adolescent risk factors for adult alcohol use and abuse: stability and change of predictive value across early and middle adulthood. *Addiction* (2008) 103(Suppl. 1):84–99. doi: 10.1111/j.1360-0443.2008.02178.x
8. Spear LP. Adolescent alcohol exposure: are there separable vulnerable periods within adolescence? *Physiol Behav*. (2015) 148:122–30. doi: 10.1016/j.physbeh.2015.01.027
9. Trigo JM, Martin-Garcia E, Berrendero F, Robledo P, Maldonado R. The endogenous opioid system: a common substrate in drug addiction. *Drug Alcohol Depend*. (2010) 108:183–94. doi: 10.1016/j.drugalcdep.2009.10.011
10. Söderpalm B, Ericson M. Neurocircuitry involved in the development of alcohol addiction: the dopamine system and its access points. *Curr Top Behav Neurosci*. (2013) 13:127–61. doi: 10.1007/978-3-642-28720-6_170
11. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev*. (2009) 89:1379–412. doi: 10.1152/physrev.00005.2009
12. Koob GF, Volkow ND. Neurocircuitry of addiction. *Neuropsychopharmacology* (2010) 35:217–38. doi: 10.1038/npp.2009.110
13. Akil H, Owens C, Gutstein H, Taylor L, Curran E, Watson S. Endogenous opioids: overview and current issues. *Drug Alcohol Depend*. (1998) 51:127–40. doi: 10.1016/S0376-8716(98)00071-4
14. Hallberg M. Neuropeptides: metabolism to bioactive fragments and the pharmacology of their receptors. *Med Res Rev*. (2015) 35:464–519. doi: 10.1002/med.21323
15. Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, et al. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* (1979) 278:423–7. doi: 10.1038/278423a0
16. Kakidani H, Furutani Y, Takahashi H, Noda M, Morimoto Y, Hirose T, et al. Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor. *Nature* (1982) 298:245–9. doi: 10.1038/298245a0
17. Comb M, Seeburg PH, Adelman J, Eiden L, Herbert E. Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. *Nature* (1982) 295:663–6. doi: 10.1038/295663a0
18. Yaksh T, Wallace M. Opioids, Analgesia, and Pain Management. In: Brunton Ll, Knollman B, editors. *Goodman and Gillman's The Pharmacological Basis of Therapeutics*. New York, NY: McGraw-Hill (2011). 481–526.

19. Granholm L, Roman E, Nylander I. Single housing during early adolescence causes time-, area- and peptide-specific alterations in endogenous opioids of rat brain *Br J Pharmacol*. (2014) 172:606–14. doi: 10.1111/bph.12753
20. Palm S, Nylander I. Alcohol-induced changes in opioid Peptide levels in adolescent rats are dependent on housing conditions. *Alcohol Clin Exp Res*. (2014a) 38:2978–87. doi: 10.1111/acer.12586
21. Walker BM, Ehlers CL. Age-related differences in the blood alcohol levels of Wistar rats. *Pharmacol Biochem Behav*. (2009) 91:560–5. doi: 10.1016/j.pbb.2008.09.017
22. Segerström L, Gustavsson J, Nylander I. Minimizing postsampling degradation of peptides by a thermal benchtop tissue stabilization method. *Biopreserv Biobank*. (2016) 14:172–9. doi: 10.1089/bio.2015.0088
23. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. San Diego, CA: Academic Press (1997).
24. Christensson-Nylander I, Nyberg F, Ragnarsson U, Terenius L. A general procedure for analysis of proenkephalin B derived opioid peptides. *Regul Pept*. (1985) 11:65–76. doi: 10.1016/0167-0115(85)90032-1
25. Nylander I, Vlaskovska M, Terenius L. Brain dynorphin and enkephalin systems in Fischer and Lewis rats: effects of morphine tolerance and withdrawal. *Brain Res*. (1995) 683:25–35. doi: 10.1016/0006-8993(95)00279-Y
26. Nylander I, Stenfors C, Tan-No K, Mathe AA, Terenius L. A comparison between microwave irradiation and decapitation: basal levels of dynorphin and enkephalin and the effect of chronic morphine treatment on dynorphin peptides. *Neuropeptides* (1997) 31:357–65. doi: 10.1016/S0143-4179(97)90072-X
27. Crews FT, Vetreno RP, Broadwater MA, Robinson DL. Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. *Pharmacol Rev*. (2016) 68:1074–109. doi: 10.1124/pr.115.012138
28. Spear LP. Effects of adolescent alcohol consumption on the brain and behavior. *Nat Rev Neurosci*. (2018) 19:197–214. doi: 10.1038/nrn.2018.10
29. Palm S, Roman E, Nylander I. Differences in basal and ethanol-induced levels of opioid peptides in Wistar rats from five different suppliers. *Peptides* (2012) 36:1–8. doi: 10.1016/j.peptides.2012.04.016
30. Palm S, Daoura L, Roman E, Nylander I. Effects of rearing conditions on behavior and endogenous opioids in rats with alcohol access during adolescence. *PLoS ONE* (2013) 8:e76591. doi: 10.1371/journal.pone.0076591
31. Momeni S, Segerstrom L, Roman E. Supplier-dependent differences in intermittent voluntary alcohol intake and response to naltrexone in Wistar rats. *Front Neurosci*. (2015) 9:424. doi: 10.3389/fnins.2015.00424
32. Zhou Y, Kreek MJ. Alcohol: a stimulant activating brain stress responsive systems with persistent neuroadaptation. *Neuropharmacology* (2014) 87:51–8. doi: 10.1016/j.neuropharm.2014.05.044
33. Marinelli PW, Quirion R, Gianoulakis C. An *in vivo* profile of beta-endorphin release in the arcuate nucleus and nucleus accumbens following exposure to stress or alcohol. *Neuroscience* (2004) 127:777–84. doi: 10.1016/j.neuroscience.2004.05.047
34. Lam MP, Nurmi H, Rouvinen N, Kiianmaa K, Gianoulakis C. Effects of acute ethanol on beta-endorphin release in the nucleus accumbens of selectively bred lines of alcohol-preferring AA and alcohol-avoiding ANA rats. *Psychopharmacology (Berl)* (2010) 208:121–30. doi: 10.1007/s00213-009-1733-y
35. Vanderschuren LJ, Achterberg EJ, Trezza V. The neurobiology of social play and its rewarding value in rats. *Neurosci Biobehav Rev*. (2016) 70:86–105. doi: 10.1016/j.neubiorev.2016.07.025
36. Trezza V, Damsteegt R, Achterberg EJ, Vanderschuren LJ. Nucleus accumbens mu-opioid receptors mediate social reward. *J Neurosci*. (2011) 31:6362–70. doi: 10.1523/JNEUROSCI.5492-10.2011
37. Manduca A, Lassalle O, Sepers M, Campolongo P, Cuomo V, Marsicano G, et al. Interacting cannabinoid and opioid receptors in the nucleus accumbens core control adolescent social play. *Front Behav Neurosci*. (2016) 10:211. doi: 10.3389/fnbeh.2016.00211
38. Trezza V, Baarendse PJ, Vanderschuren LJ. On the interaction between drugs of abuse and adolescent social behavior. *Psychopharmacology (Berl)* (2014) 231:1715–29. doi: 10.1007/s00213-014-3471-z
39. Burke AR, Miczek KA. Stress in adolescence and drugs of abuse in rodent models: role of dopamine, CRF, and HPA axis. *Psychopharmacology (Berl)* (2014) 231:1557–80. doi: 10.1007/s00213-013-3369-1
40. Rivier C. Role of hypothalamic corticotropin-releasing factor in mediating alcohol-induced activation of the rat hypothalamic-pituitary-adrenal axis. *Front Neuroendocrinol*. (2014) 35:221–33. doi: 10.1016/j.yfrne.2013.10.005
41. Kokare DM, Kyzar EJ, Zhang H, Sakharkar AJ, Pandey SC. Adolescent alcohol exposure-induced changes in alpha-melanocyte stimulating hormone and neuropeptide Y pathways via histone acetylation in the brain during adulthood. *Int J Neuropsychopharmacol*. (2017) 20:758–68. doi: 10.1093/ijnp/pyx041
42. Mendez M, Morales-Mulia M. Ethanol exposure differentially alters pro-enkephalin mRNA expression in regions of the mesocorticolimbic system. *Psychopharmacology (Berl)* (2006) 189:117–24. doi: 10.1007/s00213-006-0503-3
43. Oliva JM, Ortiz S, Perez-Rial S, Manzanares J. Time dependent alterations on tyrosine hydroxylase, opioid and cannabinoid CB1 receptor gene expressions after acute ethanol administration in the rat brain. *Eur Neuropsychopharmacol*. (2008) 18:373–82. doi: 10.1016/j.euroneuro.2007.09.001
44. Marinelli PW, Bai L, Quirion R, Gianoulakis C. A microdialysis profile of Met-enkephalin release in the rat nucleus accumbens following alcohol administration. *Alcohol Clin Exp Res*. (2005) 29:1821–8. doi: 10.1097/01.alc.0000183008.62955.2e
45. Mendez M, Barbosa-Luna IG, Perez-Luna JM, Cupo A, Oikawa J. Effects of acute ethanol administration on methionine-enkephalin expression and release in regions of the rat brain. *Neuropeptides* (2010) 44:413–20. doi: 10.1016/j.npep.2010.05.001
46. Alaux-Cantin S, Warnault V, Legastelois R, Botia B, Pierrefiche O, Vilpoux C, et al. Alcohol intoxications during adolescence increase motivation for alcohol in adult rats and induce neuroadaptations in the nucleus accumbens. *Neuropharmacology* (2013) 67:521–31. doi: 10.1016/j.neuropharm.2012.12.007
47. Chu Sin Chung P, Kieffer BL. Delta opioid receptors in brain function and diseases. *Pharmacol Ther*. (2013) 140:112–20. doi: 10.1016/j.pharmthera.2013.06.003
48. Konig M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ, et al. Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* (1996) 383:535–8. doi: 10.1038/383535a0
49. Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F, et al. Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet*. (2000) 25:195–200. doi: 10.1038/76061
50. Ragnauth A, Schuller A, Morgan M, Chan J, Ogawa S, Pintar J, et al. Female preproenkephalin-knockout mice display altered emotional responses. *Proc Natl Acad Sci USA*. (2001) 98:1958–63. doi: 10.1073/pnas.98.4.1958
51. Saitoh A, Kimura Y, Suzuki T, Kawai K, Nagase H, Kamei J. Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents. *J Pharmacol Sci*. (2004) 95:374–80. doi: 10.1254/jphs.FPJ04014X
52. Perrine SA, Hoshaw BA, Unterwald EM. Delta opioid receptor ligands modulate anxiety-like behaviors in the rat. *Br J Pharmacol*. (2006) 147:864–72. doi: 10.1038/sj.bjp.0706686
53. Randall-Thompson JF, Pescatore KA, Unterwald EM. A role for delta opioid receptors in the central nucleus of the amygdala in anxiety-like behaviors. *Psychopharmacology (Berl)* (2010) 212:585–95. doi: 10.1007/s00213-010-1980-y
54. Saitoh A, Yoshikawa Y, Onodera K, Kamei J. Role of delta-opioid receptor subtypes in anxiety-related behaviors in the elevated plus-maze in rats. *Psychopharmacology (Berl)* (2005) 182:327–34. doi: 10.1007/s00213-005-0112-6
55. Narita M, Kuzumaki N, Narita M, Kaneko C, Hareyama N, Miyatake M, et al. Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical delta-opioid receptor dysfunction. *J Neurochem*. (2006) 97:1369–78. doi: 10.1111/j.1471-4159.2006.03824.x
56. Van Skike CE, Diaz-Granados JL, Matthews DB. Chronic intermittent ethanol exposure produces persistent anxiety in adolescent and adult rats. *Alcohol Clin. Exp Res*. (2015) 39:262–71. doi: 10.1111/acer.12617
57. Varlinskaya EI, Kim EU, Spear LP. Chronic intermittent ethanol exposure during adolescence: effects on stress-induced social alterations and social drinking in adulthood. *Brain Res*. (2016) 1654(Pt B):145–56. doi: 10.1016/j.brainres.2016.03.050

58. Roberts AJ, Gold LH, Polis I, Mcdonald JS, Filliol D, Kieffer BL, et al. Increased ethanol self-administration in delta-opioid receptor knockout mice. *Alcohol Clin Exp Res*. (2001) 25:1249–56. doi: 10.1111/j.1530-0277.2001.tb02344.x
59. Björklund A, Dunnett SB. Dopamine neuron systems in the brain: an update. *Trends Neurosci*. (2007) 30:194–202. doi: 10.1016/j.tins.2007.03.006
60. Christensson-Nylander I, Herrera-Marschitz M, Staines W, Hokfelt T, Terenius L, Ungerstedt U, et al. Striato-nigral dynorphin and substance P pathways in the rat. I Biochemical and immunohistochemical studies. *Exp Brain Res*. (1986) 64:169–92. doi: 10.1007/BF00238213
61. Herrera-Marschitz M, Christensson-Nylander I, Sharp T, Staines W, Reid M, Hokfelt T, et al. Striato-nigral dynorphin and substance P pathways in the rat. II Functional analysis. *Exp Brain Res*. (1986) 64:193–207. doi: 10.1007/BF00238214
62. Fields HL, Margolis EB. Understanding opioid reward. *Trends Neurosci*. (2015) 38:217–25. doi: 10.1016/j.tins.2015.01.002
63. Palm S, Nylander I. Dopamine release dynamics change during adolescence and after voluntary alcohol intake. *PLoS ONE* (2014b) 9:e96337. doi: 10.1371/journal.pone.0096337
64. Granholm L, Rowley S, Ellgren M, Segerstrom L, Nylander I. Impact of adolescent ethanol exposure and adult amphetamine self-administration on evoked striatal dopamine release in male rats. *Psychopharmacology (Berl)* (2015) 232:4421–31. doi: 10.1007/s00213-015-4070-3
65. Philpot RM, Wecker L, Kirstein CL. Repeated ethanol exposure during adolescence alters the developmental trajectory of dopaminergic output from the nucleus accumbens septi. *Int J Dev Neurosci*. (2009) 27:805–15. doi: 10.1016/j.ijdevneu.2009.08.009
66. Zandy SL, Matthews DB, TokunagaS, Miller AD, Blaha CD, Mittleman G. Reduced dopamine release in the nucleus accumbens core of adult rats following adolescent binge alcohol exposure: age and dose-dependent analysis. *Psychopharmacology (Berl)* (2014) 232:777–84. doi: 10.1007/s00213-014-3712-1
67. Shnitko TA, Spear LP, Robinson DL. Adolescent binge-like alcohol alters sensitivity to acute alcohol effects on dopamine release in the nucleus accumbens of adult rats. *Psychopharmacology (Berl)* (2016) 233:361–71. doi: 10.1007/s00213-015-4106-8
68. Trantham-Davidson H, Centanni SW, Garr SC, New NN, Mulholland PJ, Gass JT, et al. Binge-like alcohol exposure during adolescence disrupts dopaminergic neurotransmission in the adult prelimbic cortex. *Neuropsychopharmacology* (2017) 42:1024–36. doi: 10.1038/npp.2016.190
69. Granholm L. *Stress, Drugs and Neuroscience: Neurobiological Effects of Social Stressors and Drug Exposure in Young and Adolescent Rats.* Dissertation, Uppsala University (2018).

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In vitro and *in vivo* Pharmacological Activities of 14-*O*-Phenylpropyloxymorphone, a Potent Mixed Mu/Delta/Kappa-Opioid Receptor Agonist With Reduced Constipation in Mice

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Pain, particularly chronic pain, is still an unsolved medical condition. Central goals in pain control are to provide analgesia of adequate efficacy and to reduce complications associated with the currently available drugs. Opioids are the mainstay for the treatment of moderate to severe pain. However, opioid pain medications also cause detrimental side effects, thus highlighting the need of innovative and safer analgesics. Opioids mediate their actions via the activation of opioid receptors, with the mu-opioid receptor as the primary target for analgesia, but also for side effects. One long-standing focus of drug discovery is the pursuit for new opioids exhibiting a favorable dissociation between analgesia and adverse effects. In this study, we describe the *in vitro* and *in vivo* pharmacological profiles of the 14-*O*-phenylpropyl substituted analog of the mu-opioid agonist 14-*O*-methyloxymorphone (14-OMO). The consequence of the substitution of the 14-*O*-methyl in 14-OMO with a 14-*O*-phenylpropyl group on *in vitro* binding and functional activity, and *in vivo* behavioral properties (nociception and gastrointestinal motility) was investigated. In binding studies, 14-*O*-phenylpropyloxymorphone (POMO) displayed very high affinity at mu-, delta-, and kappa-opioid receptors (K_i values in nM, mu:delta:kappa = 0.073:0.13:0.30) in rodent brain membranes, with complete loss of mu-receptor selectivity compared to 14-OMO. In guinea-pig ileum and mouse vas deferens bioassays, POMO was a highly efficacious and full agonist, being more potent than 14-OMO. In the [^{35}S]GTPγS binding assays with membranes from CHO cells expressing human opioid receptors, POMO was a potent mu/delta-receptor full agonist and a kappa-receptor partial agonist. *In vivo*, POMO was highly effective in acute thermal nociception (hot-plate test, AD_{50} = 0.7 nmol/kg) in mice after

subcutaneous administration, with over 70- and 9000-fold increased potency than 14-OMO and morphine, respectively. POMO-induced antinociception is mediated through the activation of the mu-opioid receptor, and it does not involve delta- and kappa-opioid receptors. In the charcoal test, POMO produced fourfold less inhibition of the gastrointestinal transit than 14-OMO and morphine. In summary, POMO emerges as a new potent mixed mu/delta/kappa-opioid receptor agonist with reduced liability to cause constipation at antinociceptive doses.

Keywords: pain, analgesia, constipation, opioid agonist, opioid receptor, morphinans, binding affinity

INTRODUCTION

Pain, particularly chronic pain, remains an ongoing global health and socioeconomical problem (Severino et al., 2018), affecting more people than cancer, heart disease, and diabetes combined (Skolnick and Volkow, 2016). Furthermore, comorbidity of chronic pain with mood disorders (e.g., depression, anxiety) in pain patients is well-recognized (Nicholson and Verma, 2004; Tsang et al., 2008; Miller and Cano, 2009). Opioids are the most effective drugs for the treatment of moderate to severe pain (Pasternak, 2014; Stein, 2016). However, their wide use is hampered by unwanted side effects, including constipation, apnea, sedation, nausea, tolerance, and dependence (Benyamin et al., 2008; Imam et al., 2018). A huge increase in medical use and abuse of prescription opioids with raised opioid-related morbidity and mortality has been reported in the past years (Skolnick and Volkow, 2016; Severino et al., 2018). Ongoing monitoring of pain patients receiving opioids to ensure appropriate use and effectiveness is of major importance. The central goal is to balance the patient's pain relief, potential harmful consequences of opioids, and quality of life. Opioids induce their actions via the activation of opioid receptors, that is, mu (MOR), delta (DOR), and kappa (KOR), as members of the large family of G protein-coupled receptors (GPCRs) with seven transmembrane domains (Kieffer and Evans, 2009; Shang and Filizola, 2015). Opioid receptors modulate neurotransmission in neuronal circuits that subserve pain both at central and peripheral sites (Stein and Machelska, 2011). One long-standing focus of opioid drug discovery is the pursuit for safe and effective analgesics with more favorable pharmacological features. Different approaches are therefore being evaluated to mitigate the deleterious effects of opioid analgesics, with extended reports into the field over the past years (Stein and Machelska, 2011; Aldrich and McLaughlin, 2012; Albert-Vartanian et al., 2016; Del Vecchio et al., 2017; Günther et al., 2017; Madariaga-Mazón et al., 2017; Schmid et al., 2017; Yekkirala et al., 2017; Livingston and Traynor, 2018; Pergolizzi et al., 2018).

The MOR is the primary target for analgesia, but also for side effects of opioid analgesics (Pasternak and Pan, 2013). The present understanding of the MOR function is persistently increasing with the crystal (active and inactive) structures of the MOR available (Filizola, 2018). Among clinically used opioids, morphinans including morphine, oxycodone, and oxymorphone, are of key importance as potent MOR agonists (Fürst and Hosztafi, 2008; Spetea et al., 2013). Modifications at position 14 of the morphinan skeleton were targeted by us and others with the prospect of designing novel MOR analgesics, which retain their opioid analgesic properties, but with fewer or no adverse effects (Fürst and Hosztafi, 2008; Lewis and Husbands, 2011; Spetea and Schmidhammer, 2012; Spetea et al., 2013). We have reported that the introduction of a 14-methoxy group in oxymorphone leading to 14-*O*-methyloxymorphone (14-OMO, **Figure 1**) (Schmidhammer et al., 1984) not only increased binding affinity and agonist potency at the MOR, but also resulted in a significant increase in antinociceptive potency in various pain models in rodents (Schmidhammer et al., 1984; Lattanzi et al., 2005; Spetea et al., 2010; Dumitrascuta et al., 2017). However, 14-OMO induces the typical opioid-like side effects (Schmidhammer et al., 1984; Lattanzi et al., 2005). In this study, we describe the *in vitro* and *in vivo* pharmacological profiles of the 14-*O*-phenylpropyl substituted analog of 14-OMO, namely 14-*O*-phenylpropyloxymorphone (POMO, **Figure 1**), which emerges as a new potent mixed mu/delta/kappa-opioid receptor agonist with reduced propensity to cause constipation at antinociceptive doses.

MATERIALS AND METHODS

Drugs and Chemicals

Cell culture media and supplements were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, United States) or Life Technologies (Carlsbad, CA, United States). Radioligands, [^{3}H][D-Ala^2,N-Me-Phe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO), [^{3}H]5α,7α,8β-(-)N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5) dec-8-yl]benzeneacetamide ([^{3}H]U69,593), and guanosine 5′-O-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS), were purchased from PerkinElmer (Boston, MA, United States). [^{3}H][Ile^{5,6}]deltorphin II was obtained from the Institute of Isotopes Co. Ltd. (Budapest, Hungary). Guanosine diphosphate (GPD), GTPγS and opioid ligands, naloxone, DAMGO, [D-Pen^2,D-Pen^5]enkephalin (DPDPE), U69,593 and naltrindole, were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, United States). Nor-binaltorphimine (nor-BNI) was purchased from Tocris (Abingdon, United Kingdom). Morphine hydrocloride was obtained from S.A.L.A.R.S. (Como, Italy). Dermorphin and deltorphin I were synthesized as previously described (Erspamer et al., 1989; Negri et al., 1992). 14-OMO and POMO were prepared as described earlier (Schmidhammer et al., 1984; Spetea

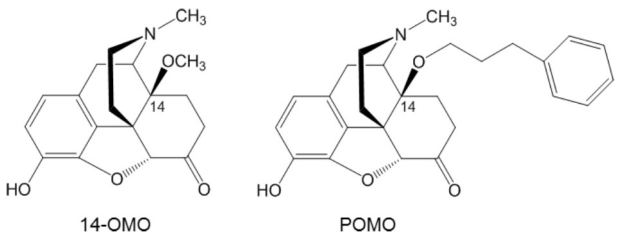

FIGURE 1 | Structures of 14-*O*-methyloxymorphone (14-OMO) and 14-*O*-phenylpropyloxymorphone (POMO).

et al., 2004). All other chemicals were of analytical grade and obtained from standard commercial sources.

Animals

Male CD-1 mice (20–25 g) and guinea-pigs (400–500 g) were obtained from Charles River (Lecco, Italy, or Sulzfeld, Germany). Animals were housed at 22°C with food and water *ad libitum* and a 12-h light/dark cycle. Animals were used after 4–5 days of acclimatization to the housing conditions. All animal studies were conducted in accordance with ethical guidelines and animal welfare standards according to Italian and Austrian regulations for animal research and were approved by the Animal Care and Use Committee of the Italian Ministry of Health and the Austrian Federal Ministry of Science and Research. All efforts were made to minimize animal suffering and to reduce the number of animals used. For behavioral studies, compounds were dissolved in sterile saline solution, and administered subcutaneously (s.c.) to mice. Separate groups of mice received the respective dose of compound, and individual mice were only used once for behavioral testing.

Radioligand Binding Assays

Membranes were prepared from Sprague–Dawley rat brains or guinea-pig brains obtained frozen from Labortierkunde und Laborgenetik, Medizinische Universität Wien, Himberg, Austria according to the described procedure (Lattanzi et al., 2005). Protein content of brain homogenates was determined by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). Binding experiments were performed in 50 mM Tris-HCl buffer (pH 7.4.) in a final volume of 1 ml containing 0.3–0.5 mg protein and various concentrations of test compound as described previously (Lattanzi et al., 2005). Rat brain membranes were incubated either with [^{3}H]DAMGO (1 nM, 45 min, 35°C) or [^{3}H][Ile^{5,6}]deltorphin II (0.5 nM, 45 min, 35°C) for labeling MOR and DOR, respectively. Guinea-pig brain membranes were incubated with [^{3}H]U69,593 (1 nM, 30 min, 30°C) for labeling the KOR. Nonspecific binding was determined using 10 μM naloxone. After incubation, reactions were terminated by rapid filtration through Whatman glass fiber filters. Filters were washed three times with 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Brandel M24R cell harvester (Gaithersburg, MD, United States). Radioactivity retained on the filters was counted by liquid scintillation counting using a Beckman Coulter LS6500 (Beckman Coulter Inc., Fullerton, CA, United States). All experiments were performed in duplicate and repeated at least three times.

[^{35}S]GTPγS Binding Assays

Chinese hamster ovary (CHO) cells stably expressing the human opioid receptors, MOR, DOR, or KOR (CHO-hMOR, CHO-hDOR, and CHO-hKOR cell lines) were kindly provided by Dr. Lawrence Toll (SRI International, Menlo Park, CA, United States). The CHO-hMOR and CHO-hDOR cell lines were maintained in Dulbecco's Minimal Essential Medium (DMEM)/Ham's F-12 medium supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM), and geneticin (400 μg/ml). The CHO-hKOR cell line was maintained in DMEM supplemented with FBS (10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM), and geneticin (400 μg/ml). Cell cultures were maintained at 37°C in 5% CO_2 humidified air. Binding of [^{35}S]GTPγS to membranes from CHO cells stably expressing the human opioid receptors was conducted according to the published procedure (Ben Haddou et al., 2014). Cell membranes were prepared in Buffer A (20 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.4) as described (Ben Haddou et al., 2014). Cell membranes (5–10 μg) in Buffer A were incubated with 0.05 nM [^{35}S]GTPγS, 10 μM GDP, and various concentrations of test compound in a final volume of 1 ml, for 60 min at 25°C. Nonspecific binding was determined using 10 μM GTPγS, and the basal binding was determined in the absence of test compound. Samples are filtered over Whatman glass GF/B fiber filters and counted as described for binding assays. All experiments were performed in duplicate and repeated at least three times.

Bioassays

Preparations of the myenteric plexus-longitudinal muscle obtained from the small intestine of male guinea-pigs (GPI) and preparations of vasa deferentia of mouse (MVD) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage as described earlier (Lattanzi et al., 2005). Test compounds were evaluated for their ability to inhibit the electrically evoked twitch, and agonist potency was compared with that of the MOR agonist, dermorphin, in GPI, and with the DOR agonist deltorphin I, in MVD. Concentration-response effects were established. All experiments were repeated at least three times.

Antinociception

Antinociception was assessed using the hot-plate assay performed as described (Lattanzi et al., 2005). Hot-plate latencies were determined by placing each mouse on a hot-plate kept at 55 ± 1°C and observing the occurrence of a nociceptive response (licking of a paw or jumping). Each animal served as its own control. Before drug s.c. administration, each animal was tested, and the basal latency to thermal stimulation was recorded. Animals not responding within 3 s were not used. In order to avoid possible tissue damage,

a cut-off time of 12 s was applied. Mice were tested for antinociception after drug administration, and time- and dose-response effect was established. For the antagonism studies, naloxone (1 mg/kg) or naltrindole (3 mg/kg) were s.c. administered 10 min before POMO (2 nmol/kg, s.c.). Nor-BNI (20 mg/kg, s.c.) was administered 24 h before POMO. Antinociception was assessed 20 min after POMO s.c. injection using the hot-plate assay. Doses and pretreatment times of the antagonists were chosen based on pilot studies and previous research (Lattanzi et al., 2002; Erli et al., 2017). Antinociceptive response was expressed as maximum possible effect (%MPE), calculated according to the equation: %MPE = (test latency − basal latency)/(cut-off − basal latency) × 100. Each experimental group included six to eight animals.

Gastrointestinal Transit

The charcoal meal test was used to measure gastrointestinal transit (Broccardo et al., 1998). Mice were fasted for 18 h, with free access to water for the entire study. Animals received 0.25 ml of a suspension of charcoal consisting of 10% (w/v) charcoal suspension in a 5% gum Arabic solution, administered by a gastric tube. Groups of mice were s.c. administered different doses of test drug (morphine: 3900, 6690, and 8000 nmol/kg; 14-OMO: 32, 53, and 90 nmol/kg; POMO: 0.35, 0.70, and 1.6 nmol/kg) or vehicle (saline), 15 min before the charcoal meal, and were sacrificed 15 min later. The stomach and small intestine were separated from the omentum to avoid stretching. The length of the intestine from the pyloric sphincter to the ileocecal junction and the distance traveled by the charcoal meal were measured. The distance traveled by the charcoal meal was expressed as percent of the total length of the small intestine, and the effect was computed as follows: %inhibitory effect = 100 − [(%length traveled after test compound)/(%length traveled after vehicle) × 100]. Each experimental group included eight animals.

Statistical Analysis

Data were analyzed and graphically processed using the GraphPad Prism 5.0 Software (GraphPad Prism Software Inc., San Diego, CA) and are presented as means ± SEM. For *in vitro* assays, inhibitor constant (K_i in nM), potency (EC_{50} or IC_{50} in nM), and efficacy (% stimulation) values were determined from concentration-response curves by nonlinear regression analysis. The K_i values were determined by the method of Cheng and Prusoff (1973). In the [^{35}S]GTPγS binding assays, efficacy was determined relative to the reference full opioid agonists, DAMGO (MOR), DPDPE (DOR), and U69,593 (KOR). The AD_{50} defined as the dose that produced an antinociceptive effect equal to 50% MPE in the hot-plate test, the ED_{50} defined as the dose that produced 50% inhibitory effect in the charcol test, and 95% confidence intervals (95% CI) were calculated from dose-response curves (Tallarida and Murray, 1986). Data were statistically evaluated using one-way ANOVA with Tukey's *post hoc* test for multiple comparisons and unpaired *t*-test for comparisons between two groups, with significance set at $P < 0.05$.

RESULTS

In vitro Pharmacology – Opioid Receptor Binding and Functional Activity

Binding affinity and functional *in vitro* activity of POMO were evaluated at MOR, DOR, and KOR and compared to the profile of 14-OMO. For comparison purposes, the affinity and potency/efficacy data of morphine (Ben Haddou et al., 2014) are also presented. Affinities at opioid receptors were determined in competition binding assays using rat brain (MOR and DOR) and guinea-pig brain (KOR) membrane preparations (Lattanzi et al., 2005). As shown in **Figure 2A**, POMO effectively inhibited in a concentration-dependent manner the binding of selective opioid radioligands to brain membranes. Based on the calculated K_i values, POMO displayed very high affinity in the picomolar range at the MOR (K_i = 0.073 nM), similar to the parent compound 14-OMO ($P > 0.05$, *t*-test). However, POMO had also low K_i values in the subnanomolar range at DOR and KOR, that were significantly lower than the K_i values of 14-OMO ($P < 0.05$, *t*-test), thus indicating a complete loss of MOR selectivity of POMO when compared to 14-OMO, as well as to morphine (**Table 1**).

The opioid agonist *in vitro* activities of POMO were initially assessed on smooth muscle preparations, the GPI and MVD, as well-known widely used bioassays (Leslie, 1987). The GPI is primarily a MOR preparation, even though the ileum also contains KOR. In the MVD, the opioid effects are mostly mediated through the DOR, but MOR and KOR also exist in the tissue. Dermorphin and deltorphin I were used as reference MOR and DOR agonists, respectively. POMO was effective in inhibiting the electrically stimulated twitch in GPI and MVD preparations, with IC_{50} values listed in **Table 2**. In the GPI assay, POMO exhibited potent and full agonist activity at the MOR (IC_{50} = 1.2 nM), with a slight albeit significant increase ($P < 0.05$, *t*-test) than that of 14-OMO. In the MVD preparation, POMO was 1000-fold more potent than 14-OMO as agonist ($P < 0.05$, *t*-test), in line with its enhanced binding affinities at DOR and KOR when compared to 14-OMO. Compared to morphine, POMO was over 250- and 50,000-fold more potent as agonist in the GPI and MVD, respectively (**Table 2**).

In addition to functional bioassays, we assessed the effect of POMO on G protein activation using the ligand-stimulated [^{35}S]GTPγS binding assay with membranes from CHO cells stably expressing the human opioid receptors (Ben Haddou et al., 2014). As shown in **Figure 2B**, POMO produced a concentration-dependent increase in the [^{35}S]GTPγS binding. Agonist potencies (ED_{50}) and efficacies (% stimulation) values are listed in **Table 2**. Stimulation of [^{35}S]GTPγS binding was determined and compared to the effect of prototypical full agonists, DAMGO (MOR), DPDPE (DOR), and U69,593 (KOR). POMO was a highly potent agonist at all three receptors, with full efficacy at MOR and DOR, and partial agonism at the KOR (**Figure 2B** and **Table 2**). The *in vitro* functional activity

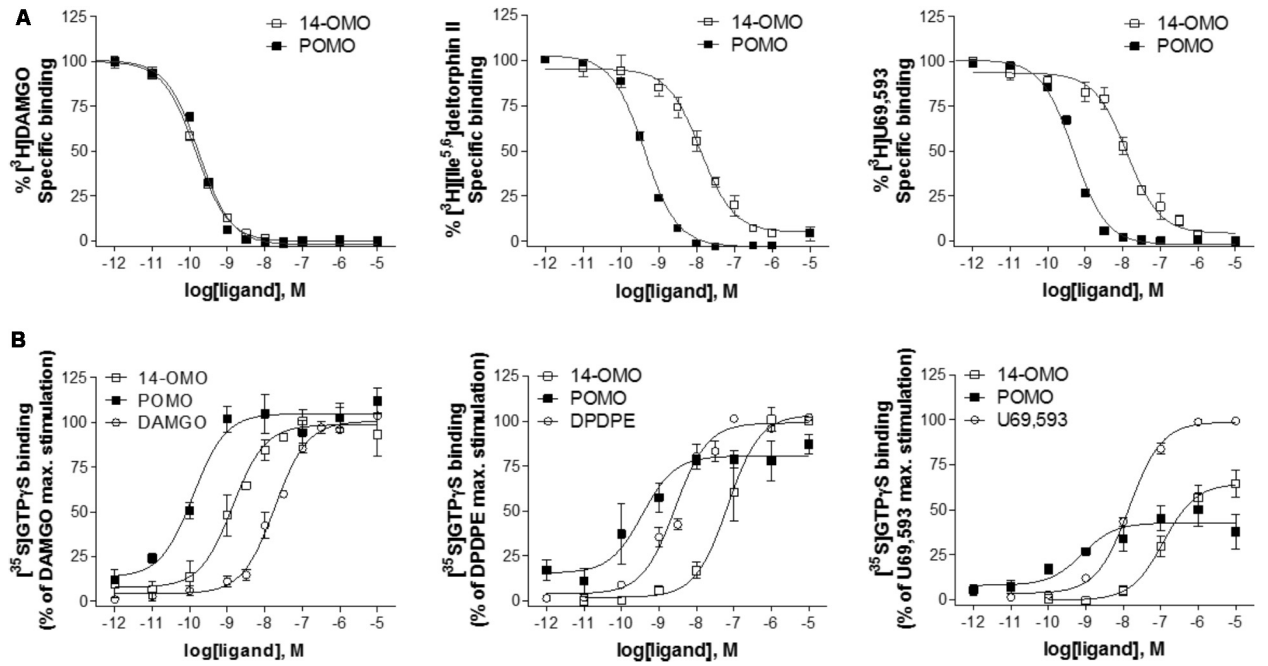

FIGURE 2 | *In vitro* binding and agonist profile of POMO at the opioid receptors. **(A)** Concentration-dependent inhibition by POMO and 14-OMO of [^{3}H]DAMGO (MOR) and [^{3}H][Ile^{5,6}]deltorphin II (DOR) binding using rat brain membranes, and by [^{3}H]U69,593 (KOR) using guinea-pig brain membranes, determined in competition binding assays. **(B)** Concentration-dependent stimulation of [^{35}S]GTPγS binding by POMO and 14-OMO determined in the [^{35}S]GTPγS binding assays using membranes from CHO cells expressing human opioid receptors. Percentage stimulation is presented relative to the maximum simulation of reference agonists DAMGO (MOR), DPDPE (DOR), and U69,593 (KOR). Values are expressed as the mean ± SEM ($n \geq 3$).

was also affected by the substitution of the 14-*O*-methyl group with a 14-*O*-phenylpropyl group, as POMO showed a significant increase in potency than 14-OMO as defined by the higher EC_{50} values (20-fold at MOR, 151-fold at DOR, and 411-fold at KOR) ($P < 0.05$, *t*-test), while retaining the full agonism at MOR/DOR and partial agonism at the KOR (**Table 2**). Subsequently, the functional MOR selectivity was significantly decreased for POMO.

In vivo Pharmacology – Antinociceptive Activity and Gastrointestinal Transit in Mice

POMO was evaluated for antinociceptive activity in a mouse model of acute thermal nociception, the hot-plate assay (Lattanzi et al., 2005). Subcutaneous administration of POMO produced time- and dose-dependent increase in latencies to thermal stimulus, with the peak of antinociceptive response occurring at 20 min (**Figure 3**). Antinociceptive potency as AD_{50} value (and 95% CI) was calculated at the peak of action and compared to 14-OMO and morphine. As shown in **Table 3**, the *in vivo* functional activity was affected by the replacement of the 14-*O*-methyl group with a 14-*O*-phenylpropyl substituent, affording an opioid agonist with more than 70-fold increased antinociceptive potency than 14-OMO. Compared to morphine, POMO was over 9000-fold more effective in producing antinociception in the hot-plate assay in mice.

To determine the relative involvement of the opioid receptor agonist activity in eliciting POMO-induced antinociception, mice were s.c. pretreated with the MOR antagonist naloxone (1 mg/kg), DOR antagonist naltrindole

TABLE 1 | Binding affinities and selectivity of POMO at the opioid receptors.

	Affinity, K_i (nM)			Selectivity	
	MOR	DOR	KOR	DOR/MOR	KOR/MOR
14-OMO[a]	0.10 ± 0.01	4.80 ± 0.22	10.2 ± 2.0	48	102
POMO	0.073 ± 0.007	0.13 ± 0.02***	0.30 ± 0.01**	1.8	4.1
Morphine[a]	6.55 ± 0.74	217 ± 19	113 ± 9	33	17

*Competition binding assays were performed with membranes from rat brain (MOR and DOR) or guinea-pig brain (KOR). [a]Data from Lattanzi et al. (2005). Values are means ± SEM of at least three experiments. **P < 0.01 and ***P < 0.001 vs. 14-OMO (unpaired t-test).*

TABLE 2 | *In vitro* functional activity of POMO at the opioid receptors.

	Bioassay[a]		[^{35}S]GTPγS Binding[b]					
	IC_{50} (nM)		MOR		DOR		KOR	
	GPI	MVD	EC_{50} (nM)	% stim.	EC_{50} (nM)	% stim.	EC_{50} (nM)	% stim.
14-OMO	2.0 ± 0.3[c]	30.5 ± 5.5[c]	1.62 ± 0.48	97 ± 6	43.8 ± 11.7	106 ± 1	144 ± 32	65 ± 7
POMO	1.2 ± 0.21*	0.03 ± 0.0013***	0.082 ± 0.017**	100 ± 8	0.28 ± 0.14**	91 ± 8	0.38 ± 0.13**	39 ± 5
Morphine	311 ± 29[c]	1600 ± 121[c]	34.4 ± 5.1[d]	89 ± 17[d]	668 ± 65[d]	109 ± 14[d]	710 ± 23[d]	76 ± 2[d]
Dermorphin	1.3 ± 0.27	18 ± 0.31						
Deltorphin I	1239 ± 132	0.19 ± 0.03						
DAMGO			14.7 ± 1.9	100				
DPDPE					1.26 ± 0.76	100		
U69,593							16.7 ± 3.0	100

[a]*Functional bioactivity was determined using GPI and MVD preparations.* [b]*[^{35}S]GTPγS binding assays were performed with membranes from CHO stably expressing human opioid receptors. Percentage stimulation (% stim.) is presented relative to the reference full agonists DAMGO (MOR), DPDPE (DOR), or U69,593 (KOR).* [c]*Data from Lattanzi et al. (2005).* [d]*Data from Ben Haddou et al. (2014). Values are means ± SEM of at least three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, vs. 14-OMO (unpaired t-test).*

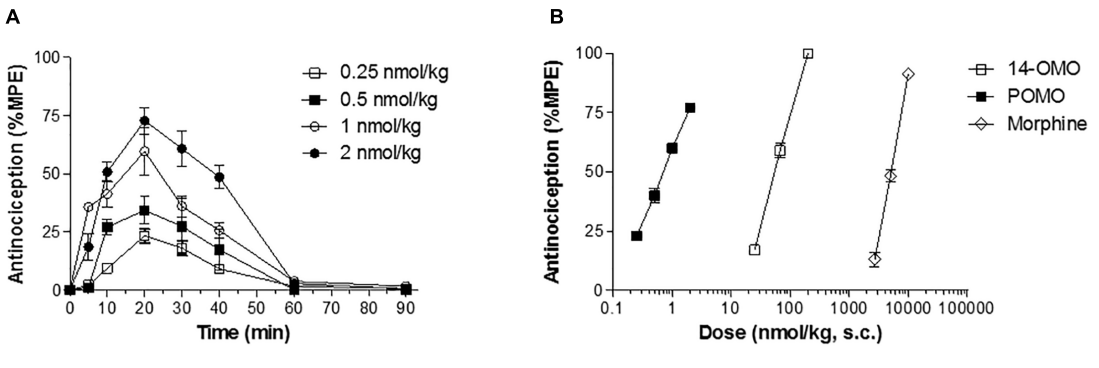

FIGURE 3 | Acute thermal antinociception induced by POMO in the hot-plate assay in mice after s.c. administration. **(A)** Time-dependent antinociceptive effects of POMO. **(B)** Comparison of dose-dependent antinociceptive effects of POMO, 14-OMO, and morphine. Data are shown as mean %MPE ± SEM (n = 6–8 mice per group).

(3 mg/kg), or KOR antagonist nor-BNI (20 mg/kg) prior to POMO s.c. injection, and tested in the hot-plate assay (**Figure 4**). Antinociception induced by the s.c. administration of 2 nmol/kg of POMO was significantly antagonized by naloxone ($P < 0.05$, ANOVA), but not by naltrindole and nor-BNI ($P > 0.05$, ANOVA). Thus, it appears that the activation of the MOR, but not DOR and KOR are responsible for POMO-induced acute thermal antinociception.

One of the most frequent adverse effects of opioid analgesics is constipation, as a consequence of activation of opioid receptors in the gastrointestinal tract (Imam et al., 2018). It is well-recognized that the MOR plays a primary role in the inhibitory control of gastrointestinal motility (Imam et al., 2018). *In vivo* studies were performed with POMO by assessing its effect on gastrointestinal transit in mice after s.c. administration using the charcoal test (Broccardo et al., 1998). The inhibitory effective dose, ED_{50} (and 95% C.I.), was calculated and compared to 14-OMO and morphine (**Table 3**). As expected, morphine effectively slowed transit in a dose-dependent manner, with the highest tested dose completely abolishing transit (**Figure 5**). Similarly, and in agreement with our previous data in the colonic bead expulsion test (Lattanzi et al., 2005), 14-OMO dose-dependently inhibited gastrointestinal motility in mice, with the highest dose producing a 90% inhibition. Although POMO also decreases gastrointestinal transit, its actions reached only a 50% inhibition (**Figure 5**).

TABLE 3 | Antinociceptive activity and gastrointestinal transit inhibition by POMO in mice after s.c. administration.

	Antinociceptive activity[a]	Gastrointestinal transit[b]
	AD_{50} (μg/kg, s.c.) (95% CI)	ED_{50} (μg/kg, s.c.) (95% CI)
14-OMO	53 (48–58)[c]	37 (35–39)
POMO	0.70 (0.63–0.77)	1.70 (0.80–3.58)
Morphine	6690 (4468–9348)[c]	3800 (3400–4330)

[a]*Determined in the hot-plate assay. Antinociceptive dose 50% with 95% confidence intervals (AD_{50}, 95% CI), (n = 6–8 mice per group).* [b]*Determined in the charcoal test. Effective dose 50% with 95% confidence intervals (ED_{50}, 95% CI), (n = 8 mice per group).* [c]*Data from Lattanzi et al. (2005).*

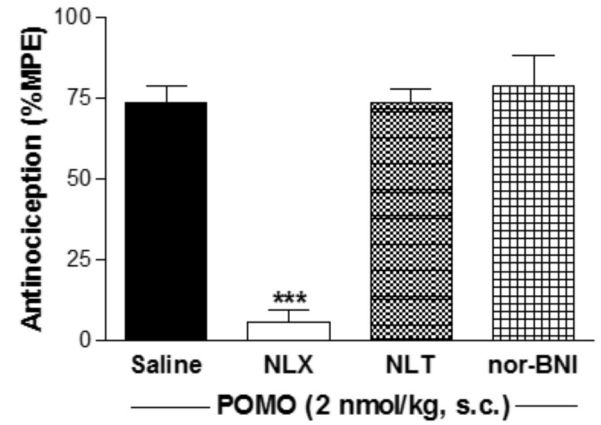

FIGURE 4 | Opioid receptor selectivity of POMO-induced antinociception in the hot-plate assay in mice after s.c. administration. Antinociceptive effect of POMO (2 nmol/kg, s.c.) measured at 20 min was antagonized by pretreatment with naloxone (NLX, 1 mg/kg, s.c., –10 min), but not by naltrindole (NTI, 3 mg/kg, s.c., –10 min) and nor-binaltorphimine (nor-BNI, 20 mg/kg, s.c., –24 h). ***$P < 0.001$ vs. saline-pretreated group (ANOVA with Tukey's *post hoc* test). Data are shown as mean %MPE ± SEM ($n = 6$ mice per group).

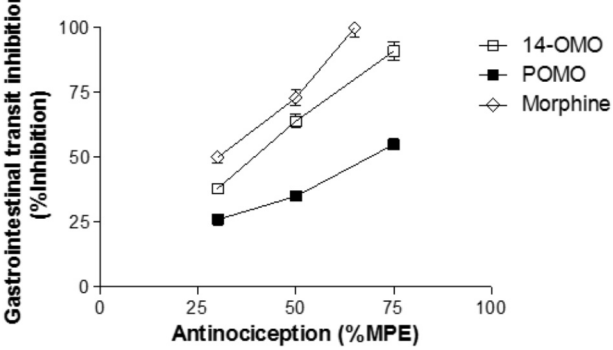

FIGURE 5 | POMO produces antinociception with reduced constipation after s.c. administration in mice. Antinociception (hot-plate assay) vs. gastrointestinal transit inhibition (charcoal test) of POMO, 14-OMO, and morphine. (n = 6–8 mice per group).

DISCUSSION

During the past decades of opioid research, there has been an intensive hunt for an alternative to currently available opioids, which would produce powerful analgesia without the harmful side effects (Bannister et al., 2017; Yekkirala et al., 2017). In this study, we have addressed the exploration of *in vitro* and *in vivo* pharmacological profiles of a new opioid agonist from the class of *N*-methylmorphinan-6-ones, POMO (**Figure 1**). The major finding is that POMO displays potent mixed MOR/DOR/KOR agonism and extraordinarily antinociceptive activity through MOR-mediated mechanisms with considerably reduced propensity for constipation in mice after s.c. administration.

Opioid drug discovery approaches have uncovered that functionalizing position 14 in the morphinan skeleton gives rise to opioid ligands with distinct functional profiles *in vitro* and *in vivo* that are appraised as valuable and potential therapeutics and important research probes (Fürst and Hosztafi, 2008; Lewis and Husbands, 2011; Stavitskaya and Coop, 2011; Spetea and Schmidhammer, 2012; Spetea et al., 2013). Combining *in vitro* ligand binding and functional assays and *in vivo* behavioral approaches, we show that the 14-*O*-phenylpropyl substitution in POMO compared to the 14-*O*-methyl substitution in 14-OMO has a strong influence on the interaction with opioid receptors in terms of receptor binding and activation. The *in vitro* assessment of binding affinities revealed that the introduction of an arylalkoxy group, that is, phenylpropoxy at position 14, maintained the high affinity at the MOR, while markedly increasing affinities at DOR and KOR, hence resulting in a complete loss of MOR selectivity of POMO. These data extend our prior structure-activity relationship (SAR) observations in terms of opioid receptor binding in the series of *N*-methylmorphinan-6-ones when comparing 14-hydroxy and 14-alkoxy analogs (Schmidhammer et al., 1984; Lattanzi et al., 2005; Spetea et al., 2005). Similar to 14-OMO, POMO is characterized as agonist *in vitro* and *in vivo* activity, while exhibiting a distinct functional profile. We showed that *in vitro* functional activity is largely affected by the replacement of the 14-*O*-methyl group with a 14-*O*-phenylpropyl group changing the MOR functionally selective 14-OMO to a potent MOR/DOR full agonist and KOR partial agonist. Notably, POMO exhibited increased affinity and efficacy at the MOR compared to oxymorphone (K_i = 0.97 nM and EC_{50} = 7.89 nM) (Lattanzi et al., 2005; Dumitrascuta et al., 2017) and morphine (K_i = 6.55 nM and EC_{50} = 34.4 nM) (Ben Haddou et al., 2014), two clinically used opioids.

Our findings from behavioral studies using a mouse model of acute thermal nociception establish POMO as an extremely potent opioid agonist *in vivo* exhibiting antinociceptive efficacy (AD_{50} = 0.7 nmol/kg) after s.c. administration in mice. Antinociceptive potency of POMO was found to be more than 70-fold higher than that of 14-OMO, and over 9000-fold when compared to morphine. While introduction of a 14-*O*-methyl group in oxymorphone, affording 14-OMO, caused an increase up to 40-fold in antinociceptive potency (Schmidhammer et al., 1984), the presence of the 14-phenylpropoxy group in POMO resulted in a further substantial increase (>1400-fold) than that reported for oxymorphone in the hot-plate assay in mice after s.c. administration (Dumitrascuta et al., 2017). Thus, substitution of the 14-*O*-methyl group in 14-OMO with a 14-*O*-phenylpropyl substituent in POMO leads to a highly potent and efficacious opioid analgesic. The SAR observations derived in this study from the *in vivo* pharmacological findings on antinociceptive properties are in qualitative agreement with the *in vitro* functional activities of targeted opioid agonists. The current findings support and extend our observations on major alterations of the pharmacological profile upon the introduction of a 14-*O*-phenylpropyl group into the opioid antagonists naloxone and naltrexone (Greiner et al., 2003). Hence, naloxone and naltrexone were converted

into nonselective ligands with very high affinities at all three opioid receptors, and potent antinociceptive agents in mice after s.c. administration as a result of the presence of the 14-*O*-phenylpropyl substituent (Greiner et al., 2003). However, in the present work, we report on a more thorough evaluation on the consequence of the presence of 14-*O*-phenylpropyl group in *N*-methylmorphinan-6-ones including the mechanism of action for analgesic effects, together with first behavioral studies on the inhibition of gastrointestinal transit. Using pharmacological approaches, we demonstrated that POMO-induced antinociception is mediated through the activation of the MOR, and it does not involve DOR and KOR, as naltrindole and nor-BNI, respectively, did not antagonize the acute thermal antinociceptive effect of POMO in the hot-plate assay in mice.

Prescription opioid use has increased rapidly over the past years (Skolnick and Volkow, 2016; Severino et al., 2018) as have related adverse events including constipation, respiratory depression, tolerance, and dependence (Benyamin et al., 2008; Imam et al., 2018). Respiratory depression is of major concern to clinicians due to its potential for producing fatal outcomes and the primary cause of opioid-related overdose mortality (Imam et al., 2018; Severino et al., 2018). Development of analgesic tolerance pose challenges for compliance and is particularly problematic in long-term chronic pain users (Benyamin et al., 2008; Severino et al., 2018). Opioid-induced constipation is one of the most common and most bothersome side effect of opioid analgesics, and can significantly impact the quality of life (Szigethy et al., 2018). The incidence of constipation is reported in 40–95% of opioid treated patients (Imam et al., 2018). In association with constipation, patients develop other gastrointestinal side effects, including vomiting and nausea, which pose major challenges for compliance and continuation of the therapy for chronic pain management (Imam et al., 2018). All three opioid receptors types, MOR, DOR, and KOR, are present in the gastrointestinal tract of humans (Holzer, 2004; Galligan and Akbarali, 2014). However, opioid-induced inhibition of gastrointestinal transit appears to be mainly mediated by the MOR, as MOR agonists predominantly increase gastric emptying time and inhibit gastrointestinal motility that contributes to nausea and vomiting (Herndon et al., 2002; Imam et al., 2018). In this study, we report on the reduced propensity of POMO to produce constipation at antinociceptive doses after s.c. administration in mice. Based on the calculated ratios of ED_{50}(constipation) vs. AD_{50}(antinociception) values of 0.54, 0.67, and 2.43 for morphine, 14-OMO and POMO, respectively, it is evident that morphine and 14-OMO cause inhibition of gastrointestinal motility at subanalgesic doses, while POMO showed a larger therapeutic window. Notably, we established that in the charcoal test, POMO produced fourfold less inhibition of the gastrointestinal transit than 14-OMO and morphine in mice.

Evaluation of pharmacokinetics (PK) is an important aspect in drug discovery and development, specially in understanding the behavior of bioactive molecules and correlation with pharmacological activities (Faller, 2008). The *in silico* determination of the partition coefficient (logP) and distribution coefficient at pH 7.4 ($logD_{7.4}$) of 14-OMO and POMO was made using the software MarvinSketch 18.8 (ChemAxon). The calculated logP (*c*logP) values of 14-OMO and POMO were 1.45 and 3.88, respectively, and the calculated $logD_{7.4}$ (*c*$logD_{7.4}$) values were 0.48 and 2.89, respectively, indicative for their good capability to enter the central nervous system. The *c*logP and *c*$logD_{7.4}$ of morphine are 1.23 and −0.57, respectively. Based on the calculated PK parameters, POMO showed a much higher lipophilicity than 14-OMO and morphine, which may account for its pharmacological effects observed *in vivo*.

Herein, we have shown that POMO was highly potent in inducing acute thermal antinociception, via activation of the MOR. *In vitro*, POMO is a mixed MOR/DOR full agonist, as well as a potent KOR partial agonist. The design of ligands that can act at multiple opioid receptors has emerged as a promising new approach to analgesic drug development to potentially lower side effects and to increase analgesic efficacy, especially in chronic pain conditions (Ananthan, 2006; Kleczkowska et al., 2013; Chan et al., 2017; Günther et al., 2017). All opioid receptors, MOR, DOR, and KOR, are crucial modulators of both nociception and opioid analgesia (Pasternak, 2014; Stein, 2016), and are co-localized in nociceptive sensory neurons (Erbs et al., 2015; Massotte, 2015). Compared to the pain relief triggered upon MOR activation in acute pain conditions, agonism at the DOR alone is relatively ineffective (Gavériaux-Ruff and Kieffer, 2011). However, DOR activation can be therapeutically beneficial in the management of persistent inflammatory pain states (Gavériaux-Ruff et al., 2008; Vanderah, 2010), with synergistic agonism at MOR and DOR increasing the overall analgesic effects (Fujita et al., 2015). Activation of the KOR also leads to effective analgesia, especially in visceral pain models (Kivell and Prisinzano, 2010; Yekkirala et al., 2017). Besides, there is less abuse potential, fewer gastrointestinal-related complications and reduced respiratory depression for DOR and KOR agonists compared to MOR agonists (Benyamin et al., 2008; Pasternak and Pan, 2013; Imam et al., 2018). Numerous biochemical and pharmacological studies and studies with genetically modified mice have provided evidence on the modulatory interactions between opioid receptor types, and the existence of MOR/DOR, DOR/KOR, and MOR/KOR heterodimers is recognized (Fujita et al., 2015; Massotte, 2015), and nowadays targeted for the development of bivalent ligands (Ananthan, 2006; Kleczkowska et al., 2013; Fujita et al., 2015; Massotte, 2015; Chan et al., 2017; Günther et al., 2017). On this basis, the activity profile established in this study for POMO as a ligand that can simultaneously bind and activate multiple opioid receptors is of major relevance.

These results provide valuable insights on the SAR in the *N*-methylmorphinan-6-ones class of opioids, by broadening the current understanding of the impact of different substituents at position 14 on ligand-receptor binding, receptor activation and link between antinociception and side effects (i.e., constipation). Future studies remain to analyze in more detail pathway-dependent agonist efficacy and signaling (i.e., biased agonism), effectiveness in models of chronic pain and other opioid typical-side effects. Thus, position 14 in the morphinan scaffold

represents a feasible site for tuning functional *in vitro* and *in vivo* activities toward finding effective and safer opioid analgesics.

AUTHOR CONTRIBUTIONS

HS, LN, and MS participated in research design. RL, SR, and MS conducted experiments and performed data analysis. HS provided compounds. RL, HS, LN, and MS wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Albert-Vartanian, A., Boyd, M. R., Hall, A. L., Morgado, S. J., Nguyen, E., Nguyen, V. P., et al. (2016). Will peripherally restricted kappa-opioid receptor agonists (pKORAs) relieve pain with less opioid adverse effects and abuse potential? *J. Clin. Pharm. Ther.* 41, 371–382. doi: 10.1111/jcpt.12404

Aldrich, J. V., and McLaughlin, J. P. (2012). Opioid peptides: potential for drug development. *Drug Discov. Today Technol.* 9, e23–e31. doi: 10.1016/j.ddtec.2011.07.007

Ananthan, S. (2006). Opioid ligands with mixed mu/delta opioid receptor interactions: an emerging approach to novel analgesics. *AAPS J.* 8, E118–E125. doi: 10.1208/aapsj080114

Bannister, K., Kucharczyk, M., and Dickenson, A. H. (2017). Hopes for the future of pain control. *Pain Ther.* 6, 117–128. doi: 10.1007/s40122-017-0073-6

Ben Haddou, T., Béni, S., Hosztafi, S., Malfacini, D., Calo, G., Schmidhammer, H., et al. (2014). Pharmacological investigations of N-substituent variation in morphine and oxymorphone: opioid receptor binding, signaling and antinociceptive activity. *PLoS One* 9:e99231. doi: 10.1371/journal.pone.0099231

Benyamin, R., Trescot, A. M., Datta, S., Buenaventura, R., Adlaka, R., Sehgal, N., et al. (2008). Opioid complications and side effects. *Pain Phys.* 11(Suppl. 2), S105–S120.

Bradford, M. (1976). A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Broccardo, M., Improta, G., and Tabacco, A. (1998). Central effect of SNC80, a selective and systemically active delta-opioid receptor agonist, on gastrointestinal propulsion in the mouse. *Eur. J. Pharmacol.* 342, 247–251. doi: 10.1016/S0014-2999(97)01470-2

Chan, H. C. S., McCarthy, D., Li, J., Palczewski, K., and Yuan, S. (2017). Designing safer analgesics via μ-opioid receptor pathways. *Trends Pharmacol. Sci.* 38, 1016–1037. doi: 10.1016/j.tips.2017.08.004

Cheng, Y., and Prusoff, W. H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22, 3099–3108. doi: 10.1016/0006-2952(73)90196-2

Del Vecchio, G., Spahn, V., and Stein, C. (2017). Novel opioid analgesics and side effects. *ACS Chem. Neurosci.* 8, 1638–1640. doi: 10.1021/acschemneuro.7b00195

Dumitrascuta, M., Ben Haddou, T., Guerrieri, E., Noha, S. M., Schläfer, L., Schmidhammer, H., et al. (2017). Synthesis, pharmacology, and molecular docking studies on 6-desoxo-*N*-methylmorphinans as potent μ-opioid receptor agonists. *J. Med. Chem.* 60, 9407–9412. doi: 10.1021/acs.jmedchem.7b01363

Erbs, E., Faget, L., Scherrer, G., Matifas, A., Filliol, D., Vonesch, J. L., et al. (2015). A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* 220, 677–702. doi: 10.1007/s00429-014-0717-9

Erli, F., Guerrieri, E., Ben Haddou, T., Lantero, A., Mairegger, M., Schmidhammer, H., et al. (2017). Highly potent and selective new diphenethylamines interacting with the κ-opioid receptor: synthesis, pharmacology, and structure-activity relationships. *J. Med. Chem.* 60, 7579–7590. doi: 10.1021/acs.jmedchem.7b00981

Erspamer, V., Melchiorri, P., Falconieri-Erspamer, G., Negri, L., Corsi, R., Severini, C., et al. (1989). Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for delta opioid binding sites. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5188–5192. doi: 10.1073/pnas.86.13.5188

Faller, B. (2008). "Physicochemical profiling in early drug discovery: new challenges at the age of high-throughput screen and combinatorial chemistry," in *Chemistry and Molecular Aspects of Drug Design and Action*, eds E. A. Rekka and P. N. Kourounakis (Boca Raton, FL: CRC Press), 303–312. doi: 10.1201/9781420008272.ch22

Filizola, M. (2018). Insights from molecular dynamics simulations to exploit new trends for the development of improved opioid drugs. *Neurosci. Lett.* doi: 10.1016/j.neulet.2018.02.037 [Epub ahead of print].

Fujita, W., Gomes, I., and Devi, L. A. (2015). Heteromers of μ-δ opioid receptors: new pharmacology and novel therapeutic possibilities. *Br. J. Pharmacol.* 172, 375–387. doi: 10.1111/bph.12663

Fürst, S., and Hosztafi, S. (2008). The chemical and pharmacological importance of morphine analogues. *Acta Physiol. Hung.* 95, 3–44. doi: 10.1556/APhysiol.95.2008.1.1

Galligan, J. J., and Akbarali, H. I. (2014). Molecular physiology of enteric opioid receptors. *Am. J. Gastroenterol.* 2, 17–21. doi: 10.1038/ajgsup.2014.5

Gavériaux-Ruff, C., Karchewski, L. A., Hever, X., Matifas, A., and Kieffer, B. L. (2008). Inflammatory pain is enhanced in delta opioid receptor-knockout mice. *Eur. J. Neurosci.* 27, 2558–2567. doi: 10.1111/j.1460-9568.2008.06223.x

Gavériaux-Ruff, C., and Kieffer, B. L. (2011). Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches. *Behav. Pharmacol.* 22, 405–414. doi: 10.1097/FBP.0b013e32834a1f2

Greiner, E., Spetea, M., Krassnig, R., Schüllner, F., Aceto, M., Harris, L. S., et al. (2003). Synthesis and biological evaluation of 14-alkoxymorphinans. 18. N-substituted 14-phenylpropyloxymorphinan-6-ones with unanticipated agonist properties: extending the scope of common structure-activity relationships. *J. Med. Chem.* 46, 1758–1763. doi: 10.1021/jm021118o

Günther, T., Dasgupta, P., Mann, A., Miess, E., Kliewer, A., Fritzwanker, S., et al. (2017). Targeting multiple opioid receptors - improved analgesics with reduced side effects? *Br. J. Pharmacol.* 175, 2857–2868. doi: 10.1111/bph.13809

Herndon, C. M., Jackson, K. C. II, and Hallin, P. A. (2002). Management of opioid-induced gastrointestinal effects in patients receiving palliative care. *Pharmacotherapy* 22, 240–250. doi: 10.1592/phco.22.3.240.33552

Holzer, P. (2004). Opioids and opioid receptors in the enteric nervous system: from a problem in opioid analgesia to a possible new prokinetic therapy in humans. *Neurosci. Lett.* 361, 192–195. doi: 10.1016/j.neulet.2003.12.004

Imam, M. Z., Kuo, A., Ghassabian, S., and Smith, M. T. (2018). Progress in understanding mechanisms of opioid-induced gastrointestinal adverse effects and respiratory depression. *Neuropharmacology* 131, 238–255. doi: 10.1016/j.neuropharm.2017.12.032

Kieffer, B. L., and Evans, C. J. (2009). Opioid receptors: from binding sites to visible molecules in vivo. *Neuropharmacology* 56(Suppl. 1), 205–212. doi: 10.1016/j.neuropharm.2008.07.033

Kivell, B., and Prisinzano, T. E. (2010). Kappa opioids and the modulation of pain. *Psychopharmacology* 210, 109–119. doi: 10.1007/s00213-010-1819-6

Kleczkowska, P., Lipkowski, A. W., Tourwé, D., and Ballet, S. (2013). Hybrid opioid/non-opioid ligands in pain research. *Curr. Pharm. Des.* 19, 7435–7450. doi: 10.2174/13816128194214010516564 6

Lattanzi, R., Negri, L., Schmidhammer, H., and Giannini, E. (2002). Antinociceptive activity of a novel buprenorphine analogue. *Life Sci.* 70, 2177–2185. doi: 10.1016/S0024-3205(01)01553-3

Lattanzi, R., Spetea, M., Schüllner, F., Rief, S. B., Krassnig, R., Negri, L., et al. (2005). Synthesis and biological evaluation of 14-alkoxymorphinans. 22. Influence of

the 14-alkoxy group and the substitution in position 5 in 14-alkoxymorphinan-6-ones on *in vitro* and *in vivo* activities. *J. Med. Chem.* 48, 3372–3378. doi: 10.1021/jm040894o

Leslie, F. M. (1987). Methods used for the study of opioid receptors. *Pharmacol. Rev.* 39, 197–249.

Lewis, J. W., and Husbands, S. M. (2011). "14-Amino-4,5-epoxymorphinan derivatives and their pharmacological actions," in *Chemistry of Opioids. Topics in Current Chemistry*, Vol. 299, ed. H. Nagase (Berlin: Springer), 93–119. doi: 10.1007/128_2010_89

Livingston, K. E., and Traynor, J. R. (2018). Allostery at opioid receptors: modulation with small molecule ligands. *Br. J. Pharmacol.* 175, 2846–2856. doi: 10.1111/bph.13823

Madariaga-Mazón, A., Marmolejo-Valencia, A. F., Li, Y., Toll, L., Houghten, R. A., and Martinez-Mayorga, K. (2017). Mu-Opioid receptor biased ligands: a safer and painless discovery of analgesics? *Drug Discov. Today* 22, 1719–1729. doi: 10.1016/j.drudis.2017.07.002

Massotte, D. (2015). In vivo opioid receptor heteromerization: where do we stand? *Br. J. Pharmacol.* 172, 420–434. doi: 10.1111/bph.12702

Miller, L. R., and Cano, A. (2009). Comorbid chronic pain and depression: who is at risk? *J. Pain* 10, 619–627. doi: 10.1016/j.jpain.2008.12.007

Negri, L., Erspamer, G. F., Severini, C., Potenza, R. L., Melchiorri, P., and Erspamer, V. (1992). Dermorphin-related peptides from the skin of Phyllomedusa bicolor and their amidated analogs activate two mu opioid receptor subtypes that modulate antinociception and catalepsy in the rat. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7203–7207. doi: 10.1073/pnas.89.15.7203

Nicholson, B., and Verma, S. (2004). Comorbidities in chronic neuropathic pain. *Pain Med.* 5(Suppl. 1), S9–S27. doi: 10.1111/j.1526-4637.2004.04019.x

Pasternak, G. W. (2014). Opiate pharmacology and relief of pain. *J. Clin. Oncol.* 32, 6655–6661. doi: 10.1200/JCO.2013.53

Pasternak, G. W., and Pan, Y. X. (2013). Mu opioids and their receptors: evolution of a concept. *Pharmacol. Rev.* 65, 1257–1317. doi: 10.1124/pr.112.007138

Pergolizzi, J. V. Jr., Raffa, R. B., Taylor, R. Jr., and Vacalis, S. (2018). Abuse-deterrent opioids: an update on current approaches and considerations. *Curr. Med. Res. Opin.* 34, 711–723. doi: 10.1080/03007995.2017.1419171

Schmid, C. L., Kennedy, N. M., Ross, N. C., Lovell, K. M., Yue, Z., Morgenweck, J., et al. (2017). Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell* 171, 1165–1175. doi: 10.1016/j.cell.2017.10.035

Schmidhammer, H., Aeppli, L., Atwell, L., Fritsch, F., Jacobson, A. E., Nebuchla, M., et al. (1984). Synthesis and biological evaluation of 14-alkoxymorphinans. 1. Highly potent opioid agonists in the series of (-)-14-methoxy-N-methylmorphinan-6-ones. *J. Med. Chem.* 27, 1575–1579. doi: 10.1021/jm00378a009

Severino, A. L., Shadfar, A., Hakimian, J. K., Crane, O., Singh, G., Heinzerling, K., et al. (2018). Pain therapy guided by purpose and perspective in light of the opioid epidemic. *Front. Psychiatry* 9:119. doi: 10.3389/fpsyt.2018.00119

Shang, Y., and Filizola, M. (2015). Opioid receptors: structural and mechanistic insights into pharmacology and signaling. *Eur. J. Pharmacol.* 763(Pt B), 206–213. doi: 10.1016/j.ejphar.2015.05.012

Skolnick, P., and Volkow, N. D. (2016). Re-energizing the development of pain therapeutics in light of the opioid epidemic. *Neuron* 92, 294–297. doi: 10.1016/j.neuron.2016.09.051

Spetea, M., Bohotin, C. R., Asim, M. F., Stübegger, K., and Schmidhammer, H. (2010). *In vitro* and *in vivo* pharmacological profile of the 5-benzyl analogue of 14-methoxymetopon, a novel μ opioid analgesic with reduced propensity to alter motor function. *Eur. J. Pharm. Sci.* 41, 125–135. doi: 10.1016/j.ejps.2010.05.018

Spetea, M., Faheem, A. M., Wolber, G., and Schmidhammer, H. (2013). The μ opioid receptor and ligands acting at the μ opioid receptor, as therapeutics and potential therapeutics. *Curr. Pharm. Des.* 19, 7415–7434. doi: 10.2174/13816128113199990362

Spetea, M., Greiner, E., Aceto, M. D., Harris, L. S., Coop, A., and Schmidhammer, H. (2005). Effect of a 6-cyano substituent in 14-oxygenated *N*-methylmorphinans on opioid receptor binding and antinociceptive potency. *J. Med. Chem.* 48, 5052–5055. doi: 10.1021/jm0580205

Spetea, M., and Schmidhammer, H. (2012). Recent advances in the development of 14-alkoxy substituted morphinans as potent and safer opioid analgesics. *Curr. Med. Chem.* 19, 2442–2457. doi: 10.2174/092986712800269308

Spetea, M., Schüllner, F., Moisa, R. C., Berzetei-Gurske, I. P., Schraml, B., Dörfler, C., et al. (2004). Synthesis and biological evaluation of 14-alkoxymorphinans. 21. Novel 4-alkoxy and 14-phenylpropoxy derivatives of the mu opioid receptor antagonist cyprodime. *J. Med. Chem.* 47, 3242–3247. doi: 10.1021/jm031126k

Stavitskaya, L., and Coop, A. (2011). Most recent developments and modifications of 14-alkylamino and 14-alkoxy-4,5-epoxymorphinan derivatives. *Mini Rev. Med. Chem.* 11, 1002–1008. doi: 10.2174/138955711797247752

Stein, C. (2016). Opioid receptors. *Annu. Rev. Med.* 67, 433–451. doi: 10.1146/annurev-med-062613-093100

Stein, C., and Machelska, H. (2011). Modulation of peripheral sensory neurons by the immune system: implications for pain therapy. *Pharmacol. Rev.* 63, 860–881. doi: 10.1124/pr.110.003145

Szigethy, E., Knisely, M., and Drossman, D. (2018). Opioid misuse in gastroenterology and non-opioid management of abdominal pain. *Nat. Rev. Gastroenterol. Hepatol.* 15, 168–180. doi: 10.1038/nrgastro.2017.141

Tallarida, R. J., and Murray, R. B. (1986). *Manual of Pharmacological Calculation*, 2nd Edn. New York, NY: Springer-Verlag. doi: 10.1007/978-1-4612-4974-0

Tsang, A., Von Korff, M., Lee, S., Alonso, J., Karam, E., Angermeyer, M. C., et al. (2008). Common chronic pain conditions in developed and developing countries: gender and age differences and comorbidity with depression-anxiety disorders. *J. Pain* 9, 883–891. doi: 10.1016/j.jpain.2008.05.005

Vanderah, T. W. (2010). Delta and kappa opioid receptors as suitable drug targets for pain. *Clin. J. Pain* 26(Suppl. 10), S10–S15. doi: 10.1097/AJP.0b013e3181c49e3a

Yekkirala, A. S., Roberson, D. P., Bean, B. P., and Woolf, C. J. (2017). Breaking barriers to novel analgesic drug development. *Nat. Rev. Drug Discov.* 16, 545–564. doi: 10.1038/nrd.2017.87

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Non-medical Cannabis Self-Exposure as a Dimensional Predictor of Opioid Dependence Diagnosis: A Propensity Score Matched Analysis

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Background: The impact of increasing non-medical cannabis use on vulnerability to develop opioid use disorders has received considerable attention, with contrasting findings. A dimensional analysis of self-exposure to cannabis and other drugs, in individuals with and without opioid dependence (OD) diagnoses, may clarify this issue.

Objective: To examine the age of onset of maximal self-exposure to cannabis, alcohol, cocaine, and heroin, in volunteers diagnosed with OD, using a rapidly administered instrument (the KMSK scales). To then determine whether maximal self-exposure to cannabis, alcohol, and cocaine is a dimensional predictor of odds of OD diagnoses.

Methods: This outpatient observational study examined maximal self-exposure to these drugs, in volunteers diagnosed with DSM-IV OD or other drug diagnoses, and normal volunteers. In order to focus more directly on opioid dependence diagnosis as the outcome, volunteers who had cocaine dependence diagnoses were excluded. Male and female adults of diverse ethnicity were consecutively ascertained from the community, and from local drug treatment programs, in 2002–2013 ($n = 574$, of whom $n = 94$ had OD diagnoses). The age of onset of maximal self-exposure of these drugs was examined. After propensity score matching for age at ascertainment, gender, and ethnicity, a multiple logistic regression examined how increasing self-exposure to non-medical cannabis, alcohol and cocaine affected odds of OD diagnoses.

Results: Volunteers with OD diagnoses had the onset of heaviest use of cannabis in the approximate transition between adolescence and adulthood (mean age = 18.9 years), and onset of heaviest use of alcohol soon thereafter (mean age = 20.1 years). Onset of heaviest use of heroin and cocaine was detected later in the lifespan (mean ages = 24.7 and 25.3 years, respectively). After propensity score matching for demographic variables, we found that the maximal self-exposure to cannabis and cocaine, but not to alcohol, was greater in volunteers with OD diagnoses, than in those without this diagnosis. Also, a multiple logistic regression detected that increasing

self-exposure to cannabis and cocaine, but not alcohol, was a positive predictor of OD diagnosis.

Conclusions/Importance: Increasing self-exposure to non-medical cannabis, as measured with a rapid dimensional instrument, was a predictor of greater odds of opioid dependence diagnosis, in propensity score-matched samples.

Keywords: opioid, cocaine, cannabis, heroin, alcohol, dimensional, exposure, adolescence

INTRODUCTION

Addictions to heroin or illicitly used prescription opioids (short-acting MOP-r agonists) cause major morbidity and mortality (1, 2), and there is considerable poly-drug use in persons with these diseases (3–6). Use of other substances, especially cannabis and alcohol, often precedes non-medical use of MOP-r agonists. The impact of non-medical cannabis use with respect to vulnerability to develop an opioid use disorder remains under study (7). This has been examined primarily with categorical classifications of cannabis use, such as "any use" vs. "no use" or presence vs. absence of a diagnosed cannabis use disorder. For example, epidemiological studies have shown that any cannabis use is associated with a later increase in odds of non-medical use of opioids and other drugs (8, 9). Another recent report from the NESARC longitudinal study found that any use of cannabis at the "wave 1" time point (2001–2002), was a positive predictor of both non-medical prescription opioid use, and opioid use disorder at the "wave 2" time point (2004–2005) (10).

In this study, we focus on dimensional aspects of drug self-exposure and their relationship to an opioid dependence diagnosis (OD). Dimensional measures are those that characterize a behavioral or biological variable along some form of a continuum. Specifically, we examined the ages of onset of heaviest use of different drugs in volunteers with opioid dependence diagnosis, as well as the level of maximal self-exposure. Dimensional aspects of substance use disorders (SUDs) are receiving recent attention, both for examination of disease progression and for the examination of mechanistic and genetic features (11–13).

Intriguingly, some studies have found that state-wide availability of medical cannabis has resulted in decreases in age-adjusted opioid overdose mortality (14), and other apparent protective effects (15). Experimental studies in humans do not detect a protective effect of the main psychoactive component of cannabis (the CB-1 partial agonist Δ9-tetra-hydro-cannabinol; Δ9-THC) on MOP-r agonist-induced respiratory depression, which is the underlying cause of overdose mortality (16). Also, cannabis smoking produced a small but significant increase in the abuse potential of a MOP-r agonist, in a recent laboratory study (17). Studies on the effectiveness of Δ9-THC in decreasing severity of withdrawal from MOP-r agonists have yielded mixed results, possibly due to different methods used (18, 19).

Some preclinical data show that exposure to Δ9-THC in adolescence can increase vulnerability to the addiction-related effects of MOP-r agonists in adulthood (20–22). Some, but not all, preclinical studies suggest that greater exposure to a CB1-r agonist could cause neurobiological effects that increase subsequent vulnerability to addictive-like effects of MOP-r agonists (23–25).

At least two major theories have been proposed to account for the sequence of first use of drugs, and also for specific patterns of poly-drug exposure in persons with specific SUD. Two of these major theories have been termed the "gateway theory" and the "shared vulnerability theory," and their relative impact remains an area of controversy (6, 26–28). An examination of dimensional, as opposed to categorical, aspects of drug self-exposure could also provide a framework to further understand the aforementioned phenomena (11, 29).

Given the changes in cannabis availability and use, and the ongoing epidemic of opioid use disorders, this controversy is of current importance (30, 31). It has been suggested that dimensional data at the individual level would be of value to address this issue (32–34). However, few studies have examined dimensionally, how exposure to non-medical cannabis and other drugs can affect odds of a clinically diagnosed opioid use disorder, at the individual level (10, 35–37). Furthermore, in most studies where such data was examined, the instruments used are not suitable for general clinical or preventive practice, due primarily to their length. In this study, we therefore examined dimensionally how different amounts of self-exposure to major drugs of abuse including non-medical cannabis and alcohol, affected odds of developing an opioid dependence diagnosis, using a relatively rapid and simple instrument (38, 39).

MATERIALS AND METHODS

This was an observational study, with consecutively ascertained adult volunteers who were examined in an outpatient research hospital setting, in the New York City area. This cohort was originally recruited and ascertained as part of genetic association studies of SUD (40–42).

Volunteers

The main outcome under examination was the presence or absence of a DSM-IV opioid dependence (OD) diagnosis. Many

Abbreviations: 95%CI, 95% Confidence interval; Δ9-THC, delta9-tetrahydrocannabinol; CB1-r, Cannabinoid-1 receptor; IQR, Inter-quartile range; KMSK scale, Kreek-McHugh-Schluger-Kellogg scale for maximal self-exposure to specific drugs; MOP-r, mu-opioid receptors; N.S., Non-significant; OD, opioid dependence diagnosis (DSM-IV criteria); OUD, Opioid use disorder (DSM-5 criteria); ROC curve, Receiver operating characteristic curve; SUD, Substance use disorders.

of the volunteers with SUD also had other diagnoses in addition to OD, but the presence of a cocaine dependence diagnosis was an exclusion criterion for this study. However, volunteers with the relatively less severe DSM-IV diagnosis of cocaine *abuse* were not excluded. Volunteers were ascertained sequentially from a number of addictive disease treatment clinics in the greater New York City area, and from the local community in the same area.

Recruitment, Inclusion and Exclusion Criteria

This study was carried out in accordance with the recommendations for Human Subjects Policies and Guidance of the National Institutes of Health. The protocol was approved by the Rockefeller University Hospital Institutional Review Board (IRB). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Male and female volunteers (≥18 years of age) were recruited through IRB-approved posted notices and newspaper advertisements in the community. Volunteers were required to be competent to understand study procedures and understand and sign the IRB-approved informed consent in English. The presence of uncontrolled schizophrenia or other psychotic signs during the interview were exclusion criteria. In order to focus more directly on the impact of cannabis or alcohol exposure on the odds of OD diagnoses, we excluded from this study volunteers who had a cocaine dependence diagnosis. Volunteers who had used cocaine, but did not meet the DSM-IV diagnostic criteria for cocaine dependence diagnosis were not excluded.

Persons were excluded from the normal volunteer category if they had any lifetime drug abuse or dependence diagnosis by DSM-IV criteria, or any of the following: (a) any instance of drinking to a level of intoxication during the previous 30 days, (b) any use of illicit drugs including opiates, cocaine, and amphetamines during the 30 days prior to ascertainment, (c) if they had used cannabis on more than 12 days during the 30 days prior to ascertainment, (d) had used illicit drugs (with the exception of cannabis) for at least three times a week for a period of at least 1 month, in their lifetime (40). This therefore allowed for examination of a range of normative self-exposure to cannabis and alcohol, also in the normal volunteers.

The three diagnostic groups in this study are volunteers with opioid dependence (OD), volunteers with drug diagnoses except OD, and normal volunteers. These groups are described in further detail in **Table 1**. In further analyses, the latter two groups were combined into an overall "not OD" group, for analyses of patterns of self-exposure to specific drugs.

TABLE 1 | Description of diagnostic groups (DSM-IV criteria).

	Volunteers with opioid dependence (OD) diagnosis	Volunteers with drug diagnoses, except OD	Normal volunteers
Description	Volunteers with opioid dependence diagnosis, as well as other drug diagnoses (if applicable)	Volunteers with any drug diagnoses, *except* OD	Volunteers without any drug diagnoses
	Cocaine dependence diagnosis was an overall exclusion criterion for this study		

Instruments Administered During Clinical Interviews

All ascertainments were completed during a standardized private face-to-face interview with a licensed trained clinician (e.g., M.D., D.O., Ph.D. Psychologist, Nurse Practitioner or Registered Nurse).Volunteers underwent the SCID I/P structured interview (Version 2.0; DSM-IV criteria) (43), and received the KMSK questionnaires for maximum self-exposure to cannabis, alcohol, heroin, and cocaine (38) (see below).

KMSK Scales for Maximal Self-Exposure to Specific Drugs ("KMSK Score")

The KMSK scales for cannabis, alcohol, heroin, and cocaine provide ordinal measures of maximal self-exposure, thus focusing on the period in the volunteer's life when use was the heaviest. For each drug, the scales start at a minimum "0" score, which denotes that the volunteer has not had any lifetime exposure to the drug (i.e., no use). The scores then increase in integers up to a maximum (13 for heroin and alcohol, 14 for cannabis, and 16 for cocaine) (see **Table 2**). The KMSK score for each drug is the composite sum of responses on three items: (a) frequency of maximal use (e.g., in times per day or per week), (b) duration of pattern of maximal use (e.g., in months or years) and (c) amount used in one day or sitting (e.g., number of alcoholic drinks or cannabis joints) (38). A separate KMSK scale is also used to characterize illicit use of prescription opioids, but was not analyzed in this study (heroin was the predominant MOP-r agonist used in this cohort). Concurrent validity of KMSK scores with the respective DSM-IV dependence diagnoses has been examined, and yielded optimal "cutpoint" scores for sensitivity and specificity (38, 39).

The KMSK scales have been used to characterize drug exposure in patients with medical and psychiatric conditions (44–46). The scales can be rapidly administered within a clinical interview (e.g., ≤5 min per drug). Each KMSK form also records age of first use, and age of onset of heaviest use (in whole years; the latter was studied herein). The four KMSK forms used in this study (i.e., for cannabis, alcohol, cocaine, and heroin), are provided in the Supplementary Materials. The full text of the scales for these and other drugs can be freely accessed: http://lab.rockefeller.edu/kreek/assets/file/KMSKquestionnaire.pdf.

Statistical Analyses

Missing Data

If there were missing data for specific comparisons for a volunteer, the data for that volunteer was removed from analysis. The cannabis KMSK scale and the age-related items were implemented while cohort ascertainment was in progress. Therefore these items, especially for cannabis, were not available for the complete cohort.

TABLE 2 | KMSK scales for maximal self-exposure to specific drugs[a,b].

Drugs	Sub-scores			KMSK score range: (sum of sub-scores)	Optimal cutpoints (males and females combined)[h]
	Frequency of use[e]	Duration of pattern[f]	Amount used in a sitting or a day[g]		
	Range: never used➔multiple daily use	*Range: <6 months>➔1year*	*Range: (see below)*		
Cannabis[c]	0➔6	0➔3	0➔5 *None➔>5 joints*	0–14	10
Alcohol[c]	0➔5	0➔3	0➔5 *None➔>10 drinks*	0–13	10
Heroin[d]	0➔4	0➔3	0➔6 *None➔>10 doses/bags*	0–13	6
Cocaine[d]	0➔7	0➔3	0➔6 *$0➔>$100* *(also converted from grams, rocks or vials)*	0–16	9

[a] *Ordinal integer scales; quantifying drug self-exposure at the time in the volunteer's life when use is heaviest.*
[b] *Scales for alcohol, heroin, and cocaine were published initially("KMSK-1") (38). Scales for cannabis were developed and used subsequently ("KMSK-2") (39).*
[c] *If the Frequency sub-score ≤2, the Duration sub-score is assigned a "0" value.*
[d] *If the Frequency sub-score ≤1, the Duration sub-score is assigned a "0" value.*
[e] *Questionnaire text reads: "At the time in your life when you were using the most [drug], were you using it."*
[f] *Questionnaire text reads: "How long did this pattern of [drug] use last?"*
[g] *Questionnaire text reads: "During this time when you were using the most, how much [drug] at a sitting [or day] would you typically use?"*
[h] *Optimal cutpoints for concurrent validity in males and females combined, for the respective DSM-IV dependence diagnosis (unpublished data).*

Univariate Analyses

Univariate analyses were carried out with GraphPad Prism software. Demographic variables (age at ascertainment, gender, and ethnicity) and KMSK scores were analyzed non-parametrically (Mann–Whitney *U*-tests or χ^2 analyses, Kruskal–Wallis or Friedman's ANOVAs, and Dunn's *post-hoc* tests).

Propensity Score Matching

As shown in **Table 3**, the overall cohort had a total of $n = 574$ volunteers, of whom $n = 94$ had OD diagnoses. Of the volunteers with OD diagnoses, $n = 89$ had all KMSK scores available, and were used in the propensity score matching procedure. Propensity score matching (47), as implemented in the "MatchIt" package in R software, was applied using a 1:1 "nearest neighbor" algorithm, to minimize heterogeneity in the above demographic variables between volunteers with OD, vs. all the other volunteers. Therefore, the latter comparison group contained the volunteers with drug diagnoses except OD, and also normal volunteers (see **Table 3B**).

Multiple Logistic Regression After Propensity Score Matching, Examining Cannabis, Alcohol and Cocaine KMSK Scores as Dimensional Predictors of Opioid Dependence Diagnosis

A multiple logistic regression was performed with Statistica (TIBCO) software. The predicted outcome was the presence of opioid dependence diagnosis (binary). There were $n = 89$ volunteers with and $n = 89$ without OD diagnoses in this sample, after the propensity score matching procedure, described above.

Alpha Level for Rejection of Null Hypotheses

For all analyses, the alpha level was $p \leq 0.05$.

RESULTS

Sample Demographics

Sample Demographics are in **Table 3**. In order to provide a complete description of the cohort, **Table 3A** presents data for volunteers with OD diagnoses, volunteers with drug diagnoses except OD, and normal volunteers. **Table 3B** presents the same data, but the latter two groups are combined, as this is the design used in the propensity score matching procedure. See also **Table 5**, for demographic data in the two groups after execution of the propensity score matching procedure.

Age at Ascertainment

Mean age at ascertainment was greater for volunteers with OD diagnoses, and also for volunteers with drug diagnoses except OD, vs. normal volunteers (**Table 3A**).

Gender

A χ^2 analysis of gender was significant, with a greater proportion of males among volunteers with OD diagnoses, or drug diagnoses except OD, vs. normal volunteers (**Table 3A**).

Ethnicity

A χ^2 analysis of ethnicity was also significant, with a relatively greater proportion of African Americans in the normal volunteer group, and a relatively greater proportion of Caucasians and Hispanics in the OD group (**Table 3A**).

Missing Data

Of the 94 volunteers with OD diagnosis (see **Table 3A**), five were removed from further analysis, due to missing data (remaining $n = 89$). Therefore, the propensity score matching procedure used these $n = 89$ volunteers with OD diagnoses as the reference

TABLE 3 | Demographics (volunteers sequentially ascertained 4/4/02-8/1/13)[a].

(A) Comparison of volunteers with opioid dependence (OD) diagnoses, volunteers with drug diagnoses except OD, and normal volunteers.

Demographics	Total *n*= 574	Volunteers with OD diagnosis[b]		Volunteers with drug diagnoses, except OD[C]		Normal volunteers[C] (NV)		Kruskal-Wallis statistic or χ^2 [df]; *p* value	
		n = 94		*n* = 187		*n* = 293			
Mean age at ascertainment(SEM)		41.17	(1.23)	39.7	(0.86)	33.4	(0.70)	59.34	<0.0001
Gender	Male	65	69.1%	124	66.3%	132	45.1%	28.91 [2]	<0.0001
	Female	29	30.1%	63	33.7%	161	55.0%		
Ethnicity	African-American	25	26.6%	78	41.7%	127	43.3%	22.12 [6]	0.0012
	Caucasian	31	33.0%	59	31.6%	81	27.7%		
	Hispanic	32	34.0%	31	16.6%	47	16.0%		
	Other	6	6.4%	19	10.2%	38	13.0%		

(B) Comparison of volunteers with OD diagnosis vs. all volunteers without OD (i.e., combining volunteers with drug diagnoses except OD, and normal volunteers).

Demographics	Total *n* = 574	Volunteers with OD diagnosis[b]		All volunteers without OD diagnosis		*U* or χ^2 [df]; *p*-value	
		n = 94		*n* = 480			
Mean age at ascertainment (SEM)		41.17	(1.23)	35.81	(0.5)	16,566	< 0.0001
Gender	Male	65	69.1%	256	53.3%	7.98 [1]	0.0047
	Female	29	30.1%	224	46.7%		
Ethnicity	African-American	25	26.6%	205	42.7%	20.62 [3]	0.0001
	Caucasian	31	33.0%	140	29.2%		
	Hispanic	32	34.0%	78	16.3%		
	Other	6	6.4%	57	11.9%		

[a] *Cocaine dependence diagnosis was an exclusion criterion for this study. See* ***Table 1*** *for further description of diagnostic groups.*
[b] *The same data from volunteers with OD diagnosis are presented in* ***(A,B)****.*
[c] *This group combines the two right-most columns in* ***(A)****. The two groups presented in* ***(B)*** *are used as the input data for the propensity score matching procedure (see* ***Table 5****).*

TABLE 4 | Ages of onset of heaviest use of specific drugs, in volunteers with opioid dependence diagnosis (data available for each of the drugs from $n = 47$).

Drug	Age of onset of heaviest use mean [95%CI][a]
Cannabis	18.9 [16.6–21.1][b]
Alcohol	20.1 [18.1–22.3][c]
Heroin	24.7 [21.9–27.5]
Cocaine	25.3 [22.6–27.9]

[a] *Friedman's ANOVA* $F_{(4)} = 29.22$; $p < 0.0001$.
[b] *Dunn's post-hoc tests: cannabis<heroin; cannabis<cocaine.*
[c] *Dunn's post-hoc tests: alcohol<heroin; alcohol<cocaine.*

group (see below and **Table 5**). Of the 187 volunteers with a drug diagnosis except OD, 13 were removed due to missing data (remaining $n = 174$). Also, of the 293 normal volunteers, 12 were similarly removed due to missing data (remaining $n = 281$).

Ages of Onset of Heaviest Use of Different Drugs, in Volunteers With Opioid Dependence Diagnosis

The mean age of onset of heaviest use of cannabis, alcohol, cocaine and heroin are presented in **Table 4**, for volunteers with opioid dependence diagnosis, for whom all these data were available. A Friedman's ANOVA examining these data was significant [$F_{(4)} = 29.22$; $p < 0.0001$]. Dunn's *post-hoc* tests show that the age of onset of heaviest use of cannabis use was earlier than that for heroin or cocaine. Likewise, age of onset of heaviest use of alcohol was earlier than that for heroin or cocaine. Ages of onset of heaviest use did not differ between cannabis and alcohol, or between heroin and cocaine.

Propensity Score Matching Procedure for Demographic Variables

As shown in **Table 3B**, there were demographic differences between the group with OD diagnoses and the group without OD diagnoses (the latter group being the combination of volunteers with drug diagnoses except OD, and normal volunteers). The goal of the propensity score matching procedure was to minimize the impact of the demographic differences. As is common in propensity score matching procedures, we initially utilized a multiple logistic regression to examine the demographic variables (age at ascertainment, gender, and ethnicity) as predictors of the OD diagnosis outcome. Propensity scores were then generated for each volunteer in the whole cohort, as the predicted values from this regression. These propensity scores were then entered in a matching algorithm as described in the section Materials and Methods. This algorithm selected $n = 89$ volunteers without OD diagnoses, to match the reference group of $n = 89$ volunteers with OD diagnoses. This matching procedure was effective,

TABLE 5 | Demographics after the propensity score matching procedure (see **Table 3B** for data prior to the matching procedure).

Demographics	Total $n = 178$	Volunteers with OD diagnosis		Volunteers without OD diagnosis		U or χ^2 [df]; p-value	
		$n = 89$[a]		$n = 89$			
Mean age at ascertainment (SEM)		41.37	(1.30)	41.60	(1.25)	3,894	N.S. $p = 0.85$
Gender	Male	61	68.5%	56	62.9%	0.985 [1]	N.S. $p = 0.80$
	Female	28	31.5%	33	37.1%		
Ethnicity	African-American	25	28.1%	20	22.5%	0.624 [3]	N.S. $p = 0.43$
	Caucasian	27	30.3%	31	34.8%		
	Hispanic	31	34.8%	33	37.1%		
	Other	6	6.7%	5	5.6%		

[a]*As mentioned in text, data from 5 of the n = 94 volunteers with OD diagnoses (as described in* ***Table 3****) had to be excluded from the matching procedure, due to missing data (thus having a remaining group of n = 89 volunteers with OD diagnoses).*

as confirmed by the lack of significant differences in gender, ethnicity and age at ascertainment, for the two groups (**Table 5**).

Maximal Self-Exposure to Cannabis, Alcohol, and Cocaine Compared in Volunteers With and Without Opioid Dependence Diagnosis, After Propensity Score Matching

Volunteers with OD had significantly greater KMSK scores for cannabis and cocaine, compared to propensity score—matched volunteers without OD (**Figure 1**). Alcohol KMSK scores did not differ significantly between these two groups. As expected, volunteers with an OD diagnosis had significantly greater heroin KMSK scores, compared to volunteers without this diagnosis (not shown) (38).

Multiple Logistic Regression Examining Cannabis, Alcohol, and Cocaine KMSK Scores as Predictors of Opioid Dependence Diagnosis, After Propensity Score Matching

This multiple logistic regression was carried out after propensity score matching for the demographic variables, as indicated above. A Wald test for a global null hypothesis was significant [$\chi^2_{(df=3)} = 25.05$; $p < 0.0001$], showing that the coefficients for the predictor variables were significantly different from 0. A Hosmer–Lemeshow test was non-significant, suggesting no evidence of lack of fit. Cannabis and cocaine KMSK scores were each detected as significant positive predictors of odds of OD diagnosis (**Figure 2**). By contrast, alcohol KMSK scores were not a significant predictor. Odds ratios are presented per point in each KMSK scale (score ranges in the scales are described in **Table 2**).

DISCUSSION

The impact of non-medical cannabis and alcohol use on vulnerability to develop an opioid use disorder, and to recover therefrom, has received considerable recent attention (3, 14, 15, 48). This is an area of current public health importance, given evolving trends in cannabis status across jurisdictions, the ongoing epidemic of opioid use disorders (37, 49), and the increase in prevalence of alcohol use disorders (50). However, few studies have examined dimensionally how exposure to several major drugs, especially non-medical cannabis and alcohol, impacts odds of opioid dependence diagnosis (6, 10, 35, 36, 51, 52).

Ages of Onset of Heaviest Use of Each Drug in Volunteers With Opioid Dependence Diagnosis

We found the ages of onset of heaviest use of both cannabis and alcohol preceded the onset of heaviest use of heroin, in volunteers with OD diagnosis. The ages of onset of heaviest use of cannabis and alcohol did not differ from each other, and occurred in the period of transition from adolescence to adulthood (27, 53). In this group of volunteers with an OD diagnosis, age of onset of heaviest use of cocaine occurred at a similar age as that for heroin. This overall pattern has some similarity to those previously reported (6, 53), but focuses more directly here on the ages of onset of maximal use, rather than on first use. In the context of this study, the aforementioned data provided a rationale for examining cannabis and alcohol KMSK scores as dimensional predictors of OD diagnosis. We also opted to include cocaine KMSK scores as a predictor in the multiple regression below, in order to control for differing levels of exposure to this drug that could occur in volunteers with OD diagnoses (even after exclusion of volunteers with DSM-IV cocaine dependence diagnoses).

Demographic Variables and Rationale for Propensity Score Matching

We detected significant differences in major demographic variables (age at ascertainment, gender, and ethnicity) between the different diagnostic groups. As mentioned above, this was a study of consecutive volunteers responding to advertisements in the community and in drug treatment programs, from a large ethnically diverse urban area. This may have therefore affected some of the demographic parameters of the sample. For example,

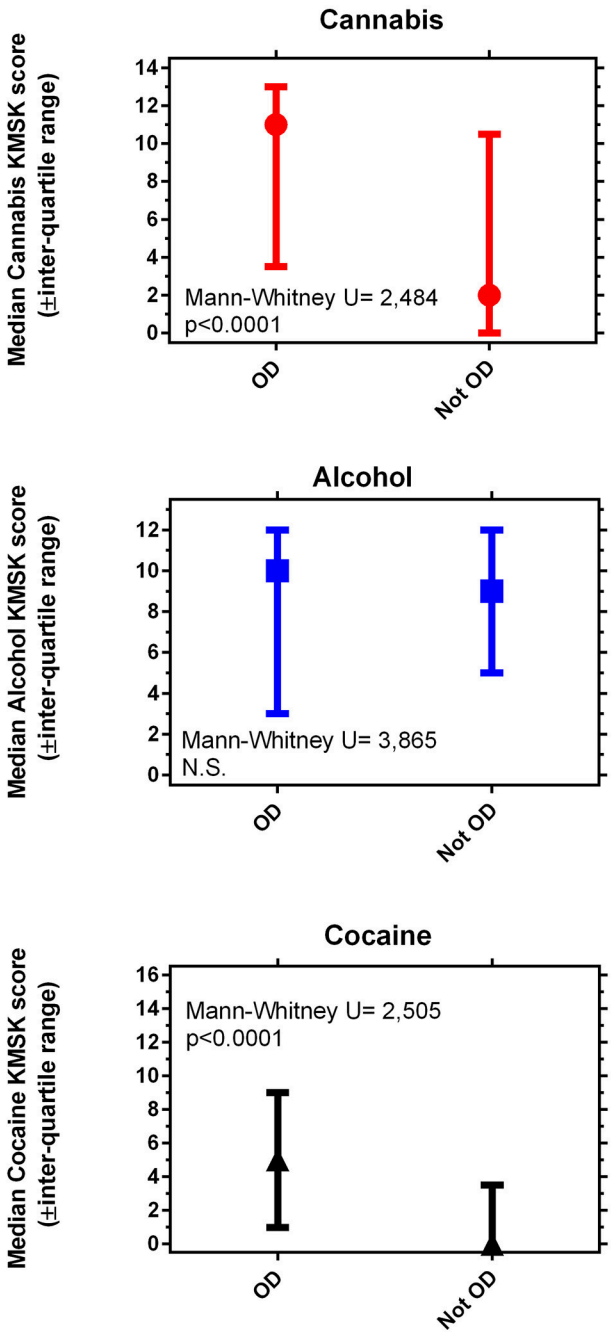

FIGURE 1 | Maximal self-exposure (KMSK scores) to non-medical cannabis **(A)**, alcohol **(B),** and cocaine **(C)**, upper, middle and lower panels, respectively, in volunteers with and without opioid dependence diagnoses ("OD" and "not OD," respectively). The "not OD" group is the combination of volunteers with drug diagnoses except OD, and normal volunteers. The data represent two groups of $n = 89$ each, after the propensity score matching procedure (see **Table 5**).

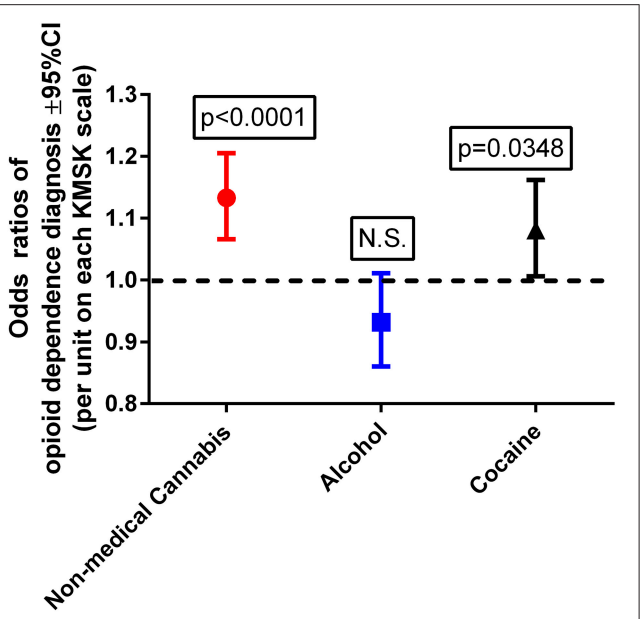

FIGURE 2 | Odds ratios of opioid dependence diagnosis, with cannabis, alcohol, and cocaine KMSK scores as dimensional predictors. The ordinate is the odds ratio (±95%CL), calculated for a 1-unit score increment in each of the KMSK scales (see **Table 2**). The multiple logistic regression was calculated after the propensity score matching procedure (two groups, $n = 89$ each; see **Table 5**).

epidemiological studies show that the prevalence of specific SUD can differ based on major demographic factors, including gender (54, 55). We therefore elected to carry out a propensity score matching procedure for age at ascertainment, gender, and ethnicity, prior to further analysis of maximal self-exposure to specific drugs. This matching procedure was effective in yielding groups with and without OD diagnoses, which did not differ significantly with respect to the aforementioned demographic variables.

Maximal Self-Exposure to Cannabis, Alcohol, and Cocaine, in Volunteers With and Without Opioid Dependence Diagnoses, After Propensity Score Matching

We found that both cannabis and cocaine KMSK scores were significantly greater in volunteers with OD diagnoses, vs. those without this diagnosis. Of note, the median cannabis KMSK score of volunteers with an OD diagnosis was relatively high, denoting heavy self-exposure to cannabis in this clinical group (based on a prior concurrent validity analysis with the DSM-IV cannabis dependence diagnosis (39) and unpublished data). Alcohol KMSK scores did not differ between volunteers with and without OD diagnosis, and a broad range of alcohol scores was observed in the two propensity score-matched groups. We note that cocaine KMSK scores were significantly greater in volunteers with OD than those without this diagnosis, even though a cocaine dependence diagnosis was an exclusion criterion for this study. As expected, the median cocaine KMSK score in the volunteers with OD was lower than the previously determined optimal

"cutpoint" for the cocaine dependence diagnosis (38), due to the aforementioned exclusion criterion. However, the median cocaine KMSK score was even lower in the volunteers without OD (at least 50% of this group reported no lifetime cocaine use).

Multiple Logistic Regression With Cannabis, Alcohol, and Cocaine KMSK Scores as Predictors, After Propensity Score Matching

After propensity score matching for age at ascertainment, gender, and ethnicity, cannabis and cocaine KMSK scores were each positive predictors of odds of an OD diagnosis. The odds in this regression were generated per 1-unit change on each KMSK scale. Therefore, it can be observed that any use of cannabis (i.e., cannabis KMSK score ≥ 1) is a predictor of increased odds of OD diagnosis (i.e., odds ratio $= 1.13$ per point in the cannabis KMSK scale). This study also shows that the odds of an OD diagnosis increase gradually with greater cannabis self-exposure scores. As mentioned above, recent epidemiological data from the NESARC study show that any use of cannabis at the "wave 1" time point (in 2001–2002) was a predictor of greater odds of opioid use disorder at the "wave 2" time point (in 2004–2005) (10). Other studies based on NESARC show that a dimensional measure of cannabis use (i.e., a defined frequency of use in the past year) at "wave 1" was a positive predictor of several SUDs (3), but opioid use disorder was not presented as a specific outcome in that study. Overall, several studies have examined primarily categorical measures of cannabis use as predictors of initiation of other drug use (56, 57). This study therefore provides the first rapid dimensional analysis which detects that self-exposure to non-medical cannabis is a positive predictor of odds of OD diagnosis, in a propensity score-matched sample.

Increasing cocaine self-exposure was also detected as a positive predictor of OD diagnosis, even though volunteers with cocaine dependence diagnoses were excluded from study. Therefore, even relatively smaller amounts of cocaine self-exposure are also associated with an increase in odds of OD diagnoses (further discussed below, in the "Limitations and Design Considerations" section). By contrast, alcohol KMSK scores were not a significant predictor of odds of an OD diagnosis. This study adds to the available literature on different aspects of alcohol use that may be related to opioid use disorders (9). Overall, it can be hypothesized that pharmacological or downstream neurobiological effects of cannabis, but not alcohol, can result in greater later vulnerability to opioid use disorders. An alternative interpretation, in the context of the "common liability" theory (27), is that there is a common pre-existing liability between cannabis and opioid exposure, and that alcohol does not share this liability to the same extent.

In preclinical studies, peri-adolescent exposure to Δ9-THC produces long-lasting neurobiological changes to MOP-r and dopaminergic systems, which mediate direct and indirect effects of MOP-r agonists (20, 22, 58–60). There is also evidence that some of the behavioral and downstream neurobiological effects of Δ9-THC are partially shared with MOP-r systems (25, 61). Preclinical studies show that the amount and pattern of exposure to specific drugs of abuse are critical in the emergence of underlying neurobiological changes and of addiction-like behaviors (62–65). Overall, substantial non-medical cannabis exposure in adolescence and early adulthood may result in long-lasting disruption in these and other systems, and thus result in increased vulnerability to the later development of opioid use disorders.

Limitations and Design Considerations

In this study, volunteers had to recall and report aspects of their drug exposure history. The possibility that recall bias may have affected these data cannot be excluded with this type of design (66), which is very common in studies of SUD (6, 35). Recalling the age(s) at which heaviest use of a specific drug occurred is also a demand of this scale. Studies with larger cohorts, different sampling methods, as well as longitudinal studies, could be used to further extend these findings. These volunteers were ascertained prior to the passage of the relevant medical marijuana statutes for this community (31). Therefore, these findings are not necessarily relevant to the impact of medically sanctioned cannabis. Studies with later birth cohorts could also investigate possible changes to the age trajectory of exposure to different drugs, due to environmental factors (1, 67–69).

We opted here to focus on the opioid dependence diagnosis as an outcome, and to exclude volunteers who had a cocaine dependence diagnosis. This allowed us to examine more directly the impact of cannabis and alcohol self-exposure on opioid dependence diagnosis as a clinical outcome. It is known that persons with dual severe opioid and cocaine use disorder diagnoses can have a different clinical course from those with only the former diagnosis (5, 70, 71). We observed that the volunteers with OD diagnoses still had significantly greater cocaine KMSK scores than the volunteers without this diagnosis. This is not surprising, as cocaine use is relatively common in persons who use heroin (6), and can occur even in the absence of a diagnosed cocaine dependence diagnosis. As mentioned in the Methods, volunteers with cocaine *abuse* diagnoses were not excluded from study. Therefore, we included cocaine KMSK scores in the multiple logistic regression, primarily to control for the level of cocaine self-exposure.

We elected to examine two propensity score matched groups: (a) volunteers with OD, and a comparison group: (b) volunteers without OD. The latter group thus included volunteers with drug diagnoses except OD, and normal volunteers. This allowed us to have a propensity score-matched comparison group with a broad range of KMSK scores for the drugs of interest, of value for a more robust dimensional analysis (11, 38, 72).

Propensity score matching studies have become relatively frequent, and have potential strengths and limitations (3, 73). For further examination of the conclusions, we also carried out an overall multiple logistic regression with OD diagnosis as the outcome, controlling for age at ascertainment, gender and ethnicity, but without propensity score matching (i.e., including data from all volunteers in the cohort). In this overall regression, the same KMSK scores were detected as positive predictors, as in the regression in the propensity score matched groups (not shown). Therefore, the results reported above with respect

to cannabis, cocaine and alcohol KMSK scores as dimensional predictors of OD diagnosis are not likely to be an artifact of the propensity score matching procedure.

CONCLUSIONS

We detected that increasing self-exposure to non-medical cannabis was a positive predictor of odds of an OD diagnosis. We also determined that the level of maximal alcohol self-exposure *per se* was not a predictor of the OD diagnosis outcome. This is one of the few individual-level examinations in which self-exposure to cannabis and alcohol are both examined dimensionally, as predictors of a diagnosed opioid use disorder. Some recent state-wide and epidemiological studies have reported that the legalized status of medical cannabis is associated with decreases in population-wide opioid overdoses and other measures of opioid-related morbidity (14, 15). Other studies have reported divergent findings on the influence of cannabis use on treatment outcomes in opioid-dependent volunteers, possibly due to different methods used (48, 74). Non-medical cannabis use has been associated with increased probability of aberrant opioid-taking behaviors in pain patients (75), and it has also been reported recently that some persons substitute cannabis for other substances, including prescription opioids for non-medical use (76). The use of categorical vs. dimensional measures of drug use has also been suggested as a possible reason for the apparent discrepancies in this area (48). Future studies could determine whether increasing non-medical cannabis exposure, especially in adolescence and young adulthood, can result in neuro-behavioral changes that underlie greater vulnerability to opioid use disorders.

AUTHOR CONTRIBUTIONS

EB, AM, SB, CC, and MK were involved in data collection and organization. All authors were involved in study conceptualization. EB, CC, and JC were involved in data analysis and presentation.

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REFERENCES

1. CDC. *Injury Prevention & Control: Prescription Drug Overdose: Centers for Disease Control and Prevention* (2015). Available online at: http://www.cdc.gov/DrugOverdose/
2. Han B, Compton WM, Jones CM, Cai R. Nonmedical prescription opioid use and use disorders among adults aged 18 through 64 years in the united states, 2003-2013. *JAMA* (2015) 314:1468–78. doi: 10.1001/jama.2015.11859
3. Blanco C, Hasin DS, Wall MM, Florez-Salamanca L, Hoertel N, Wang S, et al. Cannabis use and risk of psychiatric disorders: prospective evidence from a US National Longitudinal Study. *JAMA Psychiatry* (2016) 73:388–95. doi: 10.1001/jamapsychiatry.2015.3229
4. Lopez-Quintero C, Anthony JC. Drug use disorders in the polydrug context: new epidemiological evidence from a foodborne outbreak approach. *Ann N Y Acad Sci.* (2015) 1349:119–26. doi: 10.1111/nyas.12868
5. Leeman RF, Sun Q, Bogart D, Beseler CL, Sofuoglu M. Comparisons of cocaine-only, opioid-only, and users of both substances in the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). *Subst Use Misuse* (2016) 51:553–64. doi: 10.3109/10826084.2015.1122063
6. Woodcock EA, Lundahl LH, Stoltman JJ, Greenwald MK. Progression to regular heroin use: examination of patterns, predictors, and consequences. *Addict Behav.* (2015) 45:287–93. doi: 10.1016/j.addbeh.2015.02.014
7. Shmulewitz D, Greene ER, Hasin D. Commonalities and differences across substance use disorders: phenomenological and epidemiological aspects. *Alcohol Clin Exp Res.* (2015) 39:1878–900. doi: 10.1111/acer.12838
8. Hasin DS, Kerridge BT, Saha TD, Huang B, Pickering R, Smith SM, et al. Prevalence and correlates of DSM-5 cannabis use disorder, 2012-2013: findings from the national epidemiologic survey on alcohol and related conditions-III. *Am J Psychiatry* (2016) 173:588–99. doi: 10.1176/appi.ajp.2015.15070907
9. Arterberry BJ, Horbal SR, Buu A, Lin HC. The effects of alcohol, cannabis, and cigarette use on the initiation, reinitiation and persistence of non-medical use of opioids, sedatives, and tranquilizers in adults. *Drug Alcohol Depend.* (2016) 159:86–92. doi: 10.1016/j.drugalcdep.2015.11.029
10. Olfson M, Wall MM, Liu SM, Blanco C. Cannabis use and risk of prescription opioid use disorder in the United States. *Am J Psychiatry* (2018) 175:47–53. doi: 10.1176/appi.ajp.2017.17040413
11. Kwako LE, Bickel WK, Goldman D. Addiction biomarkers: dimensional approaches to understanding addiction. *Trends Mol Med.* (2018) 24:121–8. doi: 10.1016/j.molmed.2017.12.007
12. Sloan ME, Gowin JL, Yan J, Schwandt ML, Spagnolo PA, Sun H, et al. Severity of alcohol dependence is associated with the fatty acid amide hydrolase Pro129Thr missense variant. *Addict Biol.* (2017) 23:474–84. doi: 10.1111/adb.12491
13. Shmulewitz D, Keyes K, Beseler C, Aharonovich E, Aivadyan C, Spivak B, et al. The dimensionality of alcohol use disorders: results from Israel. *Drug Alcohol Depend.* (2010) 111:146–54. doi: 10.1016/j.drugalcdep.2010.04.002
14. Bachhuber MA, Saloner B, Cunningham CO, Barry CL. Medical cannabis laws and opioid analgesic overdose mortality in the United States, 1999-2010. *JAMA Intern Med.* (2014) 174:1668–73. doi: 10.1001/jamainternmed.2014.4005
15. Kim JH, Santaella-Tenorio J, Mauro C, Wrobel J, Cerda M, Keyes KM, et al. State medical marijuana laws and the prevalence of opioids detected among fatally injured drivers. *Am J Public Health* (2016) 106:2032–7. doi: 10.2105/ajph.2016.303426
16. Johnstone RE, Lief PL, Kulp RA, Smith TC. Combination of Δ9-tetrahydrocannabinol with oxymorphone or pentobarbital: Effects on ventilatory control and cardiovascular dynamics. *Anesthesiology* (1975) 42:674–84.
17. Cooper ZD, Bedi G, Ramesh D, Balter R, Comer SD, Haney M. Impact of co-administration of oxycodone and smoked cannabis on analgesia and abuse liability. *Neuropsychopharmacology* (2018). doi: 10.1038/s41386-018-0011-2
18. Bisaga A, Sullivan MA, Glass A, Mishlen K, Pavlicova M, Haney M, et al. The effects of dronabinol during detoxification and the initiation of treatment with extended release naltrexone. *Drug Alcohol Depend.* (2015) 154:38–45. doi: 10.1016/j.drugalcdep.2015.05.013
19. Epstein DH, Preston KL. No evidence for reduction of opioid-withdrawal symptoms by cannabis smoking during a methadone dose taper. *Am J Addict.* (2015) 24:323–8. doi: 10.1111/ajad.12183

20. Ellgren M, Artmann A, Tkalych O, Gupta A, Hansen HS, Hansen SH, et al. Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. *Eur Neuropsychopharmacol.* (2008) 18:826–34. doi: 10.1016/j.euroneuro.2008.06.009
21. Stopponi S, Soverchia L, Ubaldi M, Cippitelli A, Serpelloni G, Ciccocioppo R. Chronic THC during adolescence increases the vulnerability to stress-induced relapse to heroin seeking in adult rats. *Eur Neuropsychopharmacol.* (2014) 24:1037–45. doi: 10.1016/j.euroneuro.2013.12.012
22. Cadoni C, Simola N, Espa E, Fenu S, Di Chiara G. Strain dependence of adolescent Cannabis influence on heroin reward and mesolimbic dopamine transmission in adult Lewis and Fischer 344 rats. *Addict Biol.* (2015) 20:132–42. doi: 10.1111/adb.12085
23. Mori T, Shibasaki M, Abe M, Udagawa Y, Suzuki T. Effects of dronabinol on morphine-induced dopamine-related behavioral effects in animals. *Synapse* (2012) 66:931–7. doi: 10.1002/syn.21586
24. Solinas M, Panlilio LV, Tanda G, Makriyannis A, Matthews SA, Goldberg SR. Cannabinoid agonists but not inhibitors of endogenous cannabinoid transport or metabolism enhance the reinforcing efficacy of heroin in rats. *Neuropsychopharmacology* (2005) 30:2046–57. doi: 10.1038/sj.npp.1300754
25. Le Merrer J, Befort K, Gardon O, Filliol D, Darcq E, Dembele D, et al. Protracted abstinence from distinct drugs of abuse shows regulation of a common gene network. *Addict Biol.* (2012) 17:1–12. doi: 10.1111/j.1369-1600.2011.00365.x
26. Kandel DB, Yamaguchi K, Chen K. Stages of progression in drug involvement from adolescence to adulthood: further evidence for the gateway theory. *J Stud Alcohol* (1992) 53:447–57.
27. Vanyukov MM, Tarter RE, Kirillova GP, Kirisci L, Reynolds MD, Kreek MJ, et al. Common liability to addiction and "gateway hypothesis": theoretical, empirical and evolutionary perspective. *Drug Alcohol Depend.* (2012) 123(Suppl. 1):S3–17. doi: 10.1016/j.drugalcdep.2011.12.018
28. Secades-Villa R, Garcia-Rodriguez O, Jin CJ, Wang S, Blanco C. Probability and predictors of the cannabis gateway effect: a national study. *Int J Drug Policy* (2015) 26:135–42. doi: 10.1016/j.drugpo.2014.07.011
29. Silins E, Horwood LJ, Patton GC, Fergusson DM, Olsson CA, Hutchinson DM, et al. Young adult sequelae of adolescent cannabis use: an integrative analysis. *Lancet Psychiatry* (2014) 1:286–93. doi: 10.1016/s2215-0366(14)70307-4
30. Hall W, West R, Marsden J, Humphreys K, Neale J, Petry N. It is premature to expand access to medicinal cannabis in hopes of solving the US opioid crisis. *Addiction* (2018) 113:987–8. doi: 10.1111/add.14139
31. Carliner H, Brown QL, Sarvet AL, Hasin DS. Cannabis use, attitudes, and legal status in the U.S.: a review. *Prev Med.* (2017) 104:13–23. doi: 10.1016/j.ypmed.2017.07.008
32. Finney JW, Humphreys K, Harris AS. What ecologic analyses cannot tell us about medical marijuana legalization and opioid pain medication mortality. *JAMA Intern Med.* (2015) 175:655–6. doi: 10.1001/jamainternmed.2014.8006
33. Wilkinson ST, Yarnell S, Radhakrishnan R, Ball SA, D'Souza DC. Marijuana Legalization: impact on physicians and public health. *Annu Rev Med.* (2016) 67:453–66. doi: 10.1146/annurev-med-050214-013454
34. Volkow ND, Baler RD, Compton WM, Weiss SR. Adverse health effects of marijuana use. *N Engl J Med.* (2014) 370:2219–27. doi: 10.1056/NEJMra1402309
35. Sartor CE, Kranzler HR, Gelernter J. Rate of progression from first use to dependence on cocaine or opioids: a cross-substance examination of associated demographic, psychiatric, and childhood risk factors. *Addict Behav.* (2014) 39:473–9. doi: 10.1016/j.addbeh.2013.10.021
36. Beseler CL, Hasin DS. Cannabis dimensionality: dependence, abuse and consumption. *Addict Behav.* (2010) 35:961–9. doi: 10.1016/j.addbeh.2010.06.011
37. Hill KP. Cannabis use and risk for substance use disorders and mood or anxiety disorders. *JAMA* (2017) 317:1070–1. doi: 10.1001/jama.2016.19706
38. Kellogg SH, McHugh PF, Bell K, Schluger JH, Schluger RP, LaForge KS, et al. The Kreek-McHugh-Schluger-Kellogg scale: a new, rapid method for quantifying substance abuse and its possible applications. *Drug Alcohol Depend.* (2003) 69:137–50. doi: 10.1016/S0376-8716(02)00308-3
39. Tang YL, Khoury L, Bradley B, Gillespie CF, Ressler KJ, Cubells JF. Substance use disorders assessed using the Kreek-McHugh-Schluger-Kellogg (KMSK) scale in an urban low-income and predominantly African American sample of primary care patients. *Am J Addict.* (2011) 20:292-9. doi: 10.1111/j.1521-0391.2011.00121.x
40. Bond C, LaForge KS, Tian M, Melia D, Zhang S, Borg L, et al. Single-nucleotide polymorphism in the human mu opioid receptor gene alters beta-endorphin binding and activity: possible implications for opiate addiction. *Proc Natl Acad Sci USA.* (1998) 95:9608–13.
41. Levran O, Peles E, Randesi M, Correa da Rosa J, Ott J, Rotrosen J, et al. Synaptic plasticity and signal transduction gene polymorphisms and vulnerability to drug addictions in populations of European or African Ancestry. *CNS Neurosci Ther.* (2015). doi: 10.1111/cns.12450.
42. Levran O, Peles E, Randesi M, Correa da Rosa J, Ott J, Rotrosen J, et al. Susceptibility loci for heroin and cocaine addiction in the serotonergic and adrenergic pathways in populations of different ancestry. *Pharmacogenomics* (2015) 16:1329–42. doi: 10.2217/pgs.15.86.
43. First MB, Spitzer RL, Gibbon M, Williams JBW. *Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Patient Edition (SCID-I/P; version 2.0).* Biometrics Research Department; New York State Psychiatric Institute (2002).
44. Jackson CB, Varon J, Ho A, Marks KM, Talal AH, Kreek MJ. Identification of substance use and dependence among patients with viral hepatitis. *Dig Liver Dis.* (2010) 42:650–6. doi: 10.1016/j.dld.2010.03.002
45. Vassileva J, Ahn WY, Weber KM, Busemeyer JR, Stout JC, Gonzalez R, et al. Computational modeling reveals distinct effects of HIV and history of drug use on decision-making processes in women. *PLoS ONE* (2013) 8:e68962. doi: 10.1371/journal.pone.0068962
46. Khoury L, Tang YL, Bradley B, Cubells JF, Ressler KJ. Substance use, childhood traumatic experience, and posttraumatic stress disorder in an urban civilian population. *Depress Anxiety* (2010) 27:1077-86. doi: 10.1002/da.20751.
47. Austin PC. An introduction to propensity score methods for reducing the effects of confounding in observational studies. *Mult Behav Res.* (2011) 46:399–424. doi: 10.1080/00273171.2011.568786
48. Zielinski L, Bhatt M, Sanger N, Plater C, Worster A, Varenbut M, et al. Association between cannabis use and methadone maintenance treatment outcomes: an investigation into sex differences. *Biol Sex Differ.* (2017) 8:8. doi: 10.1186/s13293-017-0130-1
49. CDC. *Understanding the Epidemic* (2016). Available online at: https://www.cdc.gov/drugoverdose/epidemic/index.html
50. Grant BF, Chou S, Saha TD, Pickering RP, Kerridge BT, Ruan WJ, et al. Prevalence of 12-month alcohol use, high-risk drinking, and DSM-IV alcohol use disorder in the united states, 2001-2002 to 2012-2013: results from the national epidemiologic survey on alcohol and related conditions. *JAMA Psychiatry* (2017) 74:911–23. doi: 10.1001/jamapsychiatry.2017.2161
51. Hernandez-Avila CA, Rounsaville BJ, Kranzler HR. Opioid-, cannabis- and alcohol-dependent women show more rapid progression to substance abuse treatment. *Drug Alcohol Depend.* (2004) 74:265–72
52. Chen CY, Storr CL, Anthony JC. Early-onset drug use and risk for drug dependence problems. *Addict Behav.* (2009) 34:319–22. doi: 10.1016/j.addbeh.2008.10.021
53. Cleveland HH, Wiebe RP. Understanding the association between adolescent marijuana use and later serious drug use: gateway effect or developmental trajectory? *Dev Psychopathol.* (2008) 20:615–32. doi: 10.1017/s0954579408000308
54. Kerridge BT, Pickering R, Chou P, Saha TD, Hasin DS. DSM-5 cannabis use disorder in the National Epidemiologic Survey on Alcohol and Related Conditions-III: gender-specific profiles. *Addict Behav.* (2017) 76:52–60. doi: 10.1016/j.addbeh.2017.07.012
55. Hasin DS, Grant BF. The National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) Waves 1 and 2: review and summary of findings. *Soc Psychiatry Psychiatr Epidemiol.* (2015) 50:1609–40. doi: 10.1007/s00127-015-1088-0
56. Mayet A, Legleye S, Falissard B, Chau N. Cannabis use stages as predictors of subsequent initiation with other illicit drugs among French adolescents: Use of a multi-state model. *Addict Behav.* (2012) 37:160–6. doi: 10.1016/j.addbeh.2011.09.012
57. Fergusson DM, Horwood LJ. Does cannabis use encourage other forms of illicit drug use? *Addiction* (2000) 95:505–20. doi: 10.1046/j.1360-0443.2000.9545053.x

58. Bloomfield MA, Ashok AH, Volkow ND, Howes OD. The effects of Delta9-tetrahydrocannabinol on the dopamine system. *Nature* (2016) 539:369–77. doi: 10.1038/nature20153
59. Gorelick DA, Kim YK, Bencherif B, Boyd SJ, Nelson R, Copersino M, et al. Imaging brain mu-opioid receptors in abstinent cocaine users: time course and relation to cocaine craving. *Biol Psychiatry* (2005) 57:1573–82. doi: 10.1016/j.biopsych.2005.02.026
60. Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA*. (1988) 85:5274–8.
61. Ghozland S, Matthes HW, Simonin F, Filliol D, Kieffer BL, Maldonado R. Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. *J Neurosci.* (2002) 22:1146-54. doi: 10.1523/JNEUROSCI.22-03-01146.2002
62. Panlilio LV, Justinova Z. preclinical studies of cannabinoid reward, treatments for cannabis use disorder, and addiction-related effects of cannabinoid exposure. *Neuropsychopharmacology* (2017) 43:116–41. doi: 10.1038/npp.2017.193
63. Kruzich PJ, Chen AC, Unterwald EM, Kreek MJ. Subject-regulated dosing alters morphine self-administration behavior and morphine-stimulated [35S]GTPgammaS binding. *Synapse* (2003) 47:243–9. doi: 10.1002/syn.10173
64. Unterwald EM, Kreek MJ, Cuntapay M. The frequency of cocaine administration impacts cocaine-induced receptor alterations. *Brain Res.* (2001) 900:103–9. doi: 10.1016/S0006-8993(01)02269-7
65. Szutorisz H, Hurd YL. High times for cannabis: epigenetic imprint and its legacy on brain and behavior. *Neurosci Biobehav Rev.* (2017) 85:93–101. doi: 10.1016/j.neubiorev.2017.05.011
66. Birrell L, Newton NC, Teesson M, Slade T. Early onset mood disorders and first alcohol use in the general population. *J Affect Disord.* (2016) 200:243–9. doi: 10.1016/j.jad.2016.04.032
67. Paulozzi LJ. Prescription drug overdoses: a review. *J Safety Res.* (2012) 43:283–9. doi: 10.1016/j.jsr.2012.08.009
68. Banerjee G, Edelman EJ, Barry DT, Becker WC, Cerda M, Crystal S, et al. Non-medical use of prescription opioids is associated with heroin initiation among US veterans: a prospective cohort study. *Addiction* (2016) 111:2021–31. doi: 10.1111/add.13491
69. Wu LT, Woody GE, Yang C, Mannelli P, Blazer DG. Differences in onset and abuse/dependence episodes between prescription opioids and heroin: results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Subst Abuse Rehabil.* (2011) 2011:77–88. doi: 10.2147/sar.s18969
70. Borg L, Broe DM, Ho A, Kreek MJ. Cocaine abuse sharply reduced in an effective methadone maintenance program. *J Addict Dis.* (1999) 18:63–75. doi: 10.1300/J069v18n04_06
71. Bandettini Di Poggio A, Fornai F, Paparelli A, Pacini M, Perugi G, Maremmani I. Comparison between heroin and heroin-cocaine polyabusers: a psychopathological study. *Ann N Y Acad Sci.* (2006) 1074:438–45. doi: 10.1196/annals.1369.044
72. Cuthbert BN. The RDoC framework: facilitating transition from ICD/DSM to dimensional approaches that integrate neuroscience and psychopathology. *World Psychiatry* (2014) 13:28–35. doi: 10.1002/wps.20087
73. Okoli GN, Sanders RD, Myles P. Demystifying propensity scores. *BJA: Br J Anaest.* (2014) 112:13–5. doi: 10.1093/bja/aet290
74. Hill KP, Bennett HE, Griffin ML, Connery HS, Fitzmaurice GM, Subramaniam G, et al. Association of cannabis use with opioid outcomes among opioid-dependent youth. *Drug Alcohol Depend* (2013) 132:342–5. doi: 10.1016/j.drugalcdep.2013.02.030
75. Reisfield GM, Wasan AD, Jamison RN. The prevalence and significance of cannabis use in patients prescribed chronic opioid therapy: a review of the extant literature. *Pain Med.* (2009) 10:1434–41. doi: 10.1111/j.1526-4637.2009.00726.x
76. Corroon JM Jr, Mischley LK, Sexton M. Cannabis as a substitute for prescription drugs - a cross-sectional study. *J Pain Res.* (2017) 10:989–98. doi: 10.2147/jpr.s134330

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Oxycodone Self-Administration Induces Alterations in Expression of Integrin, Semaphorin and Ephrin Genes in the Mouse Striatum

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Oxycodone is one a commonly used medication for pain, and is also a widely abused prescription opioid, like other short-acting MOPr agonists. Neurochemical and structural adaptations in brain following chronic MOPr-agonist administration are thought to underlie pathogenesis and persistence of opiate addiction. Many axon guidance molecules, such as integrins, semaphorins, and ephrins may contribute to oxycodone-induced neuroadaptations through alterations in axon-target connections and synaptogenesis, that may be implicated in the behaviors associated with opiate addiction. However, little is known about this important area. The aim of this study is to investigate alterations in expression of selected integrin, semaphorin, ephrins, netrin, and slit genes in the nucleus accumbens (NAc) and caudate putamen (CPu) of mice following extended 14-day oxycodone self-administration (SA), using RNAseq.

Methods: Total RNA from the NAc and CPu were isolated from adult male C57BL/6J mice within 1 h after the last session of oxycodone in a 14-day self-administration paradigm (4h/day, 0.25 mg/kg/infusion, FR1) or from yoked saline controls. Gene expressions were examined using RNA sequencing (RNA-Seq) technology. RNA-Seq libraries were prepared using Illumina's TruSeq® Stranded Total RNA LT kit. The reads were aligned to the mouse reference genome (version mm10) using STAR. DESeq2 was applied to the counts of protein coding genes to estimate the fold change between the treatment groups. False Discovery Rate (FDR) $q < 0.1$ were used to select genes that have a significant expression change. For selection of a subset of genes related to axon guidance pathway, REACTOME was used.

Results: Among 38 known genes of the integrin, semaphorin, and ephrin gene families, RNA-seq data revealed up-regulation of six genes in the NAc: heterodimer receptor, integrins *Itgal*, *Itgb2,* and *Itgam*, and its ligand semaphorin *Sema7a*, two semaphorin receptors, plexins *Plxnd1* and *Plxdc1*. There was down-regulation of eight genes in this region: two integrin genes *Itga3* and *Itgb8*, semaphorins *Sema3c, Sema4g, Sema6a, Sema6d*, semaphorin receptor neuropilin *Nrp2*, and ephrin receptor *Epha3.* In the CPu, there were five differentially expressed axon guidance genes: up-regulation of three

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Yuferov V, Zhang Y, Liang Y, Zhao C, Randesi M and Kreek MJ (2018) Oxycodone Self-Administration Induces Alterations in Expression of Integrin, Semaphorin and Ephrin Genes in the Mouse Striatum. Front. Psychiatry 9:257. doi: 10.3389/fpsyt.2018.00257

integrin genes, *Itgal, Itgb2, Itga1*, and down-regulation of *Itga9* and ephrin *Efna3* were thus observed**.** No significant alterations in expression of Netrin-1 or Slit were observed.

Conclusion: We provide evidence for alterations in the expression of selective axon guidance genes in adult mouse brain following chronic self-administration of oxycodone. Further examination of oxycodone-induced changes in the expression of these specific axon guidance molecules and integrin genes in relation to behavior may provide new insights into development of addiction to oxycodone.

Keywords: oxycodone, RNA seq, axon guidance genes, cell type enrichment, integrins

INTRODUCTION

Oxycodone is one of the most commonly use medications for pain, and like many short-acting MOPr agonists, it also has abuse potential. The neuroadaptations in specific brain regions following chronic opioid administration occur at the neurochemical and structural levels, and may underlie opioid use disorders. Like other drugs of abuse, opioids have the ability to cause neuroplasticity by altering morphology of dendrites and spines, which are the primary sites of excitatory synapses in brain regions involved in incentive motivation, reward, and learning (1). A decrease in the complexity of dendritic branching and number of dendritic spines on neurons specifically located in the nucleus accumbens (NAc) and cortex of rats was found following morphine self-administration (2, 3). Molecular mechanisms that underlie drug-induced structural alterations are still not fully understood. However, there are pharmacological and genetics evidence that the axon guidance genes may contribute to these morphological alterations (4, 5).

Researchers have identified five families of canonical guidance proteins: semaphorins, ephrins, slits, netrins, and integrins (4, 6). Proteins of the axon guidance gene family may contribute to oxycodone-induced neuroadaptations, through alterations in axon-target connections and synaptogenesis, and these may be implicated in the behaviors associated with opioid use disorders. Originally, integrins, semaphorins, ephrins, slits, and netrins with their cognate receptors were found to be implicated in establishing functional neural circuits, cell migration, and synapse formation during development (5). However, axon guidance molecules are also expressed in adult brain, and may contribute to alterations in neural circuit regulation, throughout axon pruning, synaptogenesis, dendrite, and spine morphogenesis. Several studies showed an implication of axon guidance proteins in neuroadaptation following drugs of abuse administration, and response to brain injury (7–12).

EPH receptor tyrosine kinases are divided into two classes, EPHA receptors (A1–A8, A10) and EPHB receptors (B1–B4, B6), based on their binding affinities for ligands ephrin-A (A1–A5) or ephrin-B (B1–B3) ligands (13). Eph receptors and ephrins mediate bidirectional signaling, being both membrane proteins. In general, cellular response to the Eph receptor activation is local actin fiber depolymerization, which results in rapid cytoskeletal collapse and loss of focal adhesions, leading to cell detachment. EphB1 receptor and ligand ephrin-B2 ligand are expressed in the midbrain dopaminergic neurons. Activation of the EphB1 inhibits the growth of neurites and induces the cell loss of substantia nigra, but not ventral tegmental, dopaminergic neurons (14). The same study showed that ephrin-B2 expression is upregulated by cocaine or amphetamine in the striatum of adult mice, suggesting that ephrin-B2/EphB1 interaction may play a role in drug-induced plasticity. Of interest, Liu et al showed that escalating morphine treatment up-regulates expression of EphB1 in the mouse spinal cord, and that EphB2 blocker (EphB2-Fc) attenuated most of naloxone-precipitated morphine withdrawal signs (15).

Twenty semaphorins fall into five classes, semaphorins 3–7 (16). Class 3 semaphorins are secreted proteins, classes 4–6 are transmembrane proteins, and semaphorin 7A is linked to the plasma membrane via a glyco-phosphatidylinositol (GPI) anchor. Most of the effects of semaphorins are mediated by plexins, a group of nine transmembrane receptors that can be subdivided into four classes, plexins A–D, and two neuropilin receptors, Nrp1 and Nrp2. The semaphorin–plexin system is involved in multiple functions during development and in the adult organism, particularly in the nervous system, the immune system and during angiogenesis. Deregulation of semaphorin expression was documented in many pathological conditions such as ischemia, degenerative diseases, multiple sclerosis (5, 16). Semaphorins have also been shown to have immune regulatory functions in B and T cells. *In vitro*, Sema4d enhanced B cell survival, and play a role in monocytes and macrophages migration. Recently, Sema7A was identified as an effector molecule in T-cell-mediated inflammation through an integrin-mediated mechanism (17).

Integrins are a large family of receptors for components of the extracellular matrix (ECM). Integrins are obligatory αβ-heterodimers that undergo large conformational changes in their extracellular domains in response to signaling events inside cells. In mammals, there are 18 different α-subunits and 8 different β-subunits, which together generate 24 distinct integrin αβ-heterodimer receptor combinations (18). Intracellularly, integrins link to the actin cytoskeleton via adaptor proteins, such as talin and vinculin, and engage second messenger signaling cascades through several kinases such as Srk, ILK, FAK, and PI3K. Given the diversity of integrin effects, regional differences in receptor expression could be involved in activity-dependent synaptic plasticity, including activity-dependent neural circuit adaptation, and in turn its dysfunctions might

promote neurological disorders (19). In relation to drug addiction-like behavior, inhibition of integrin-linked kinase (ILK) in the rat NAc core blocked the induction of cocaine psychomotor sensitization, and prevented cocaine-induced increase in dendritic density and dendritic spine numbers (20). It has been shown that the basal levels of the integrin beta-1 (Itgb1) were elevated after chronic cocaine administration (11). Alpha and beta integrins are receptors for semaphorin 7A, and mediate its function (4). This supports potential involvement of integrins in the chronic cocaine-induced behaviors. However, there are no reports regarding an involvement of integrins in the opioid-induced pathology in humans or animal models.

Netrins play various important roles in the correct wiring of the nervous system during development (21). To date, several netrins have been described: netrin-1, netrin-3, netrin-4 and G-netrins. The most studied netrin-1 induces axon outgrowth via one of its receptors DCC (Deleted in Colorectal Cancer) in several types of neurons. Recent morphological analyses suggested a possibility of a shift in the function of netrin-1 in cortical axons during development, from promotion of outgrowth to promotion of branch formation (22). Function-blocking experiments suggested that DCC may contribute not only to axon outgrowth but branching. There is no information on modulation of opioid-induced behaviors by netrin-1 or its receptor DCC expression. Of interest, *in vitro* treatment of neurons of dorsal root ganglion (DRG) with netrin-1 stimulates translation of the kappa opioid receptor (KOR), which activates its downstream target the focal adhesion kinase (FAK) (23).

Another class of axon guidance proteins is Robo receptor and its ligand Slit. The Slit/Robo pair not only functions in axon guidance in development but also in diverse processes in the CNS, like cell migration, axonal branching, axonal targeting or cell differentiation (24). In most vertebrates there are 3 Robo receptors expressed in CNS cells, Robo-1, Robo-2, and Robo-3. Three Slit genes have been identified in mammals, Slit 1-Slit-3. Full-length Slits can be cleaved by proteases generating shorter functional N-terminal isoforms (Slit1-N, Slit2-N, and Slit3-N). In relation to drug of abuse area, several studies showed their involvement in regulation of dopaminergic neurons (25, 26). For example, Slit-2 inhibits growth of tyrosine-ir positive (TH+) axons in primary cultures of the rat ventral midbrain. Similarly, Slit-2 reduced the number and length of TH+ axons in explants from the ventral midbrain tissue of mouse brain (26). However, little is known about the effect of MOPr agonist self-administration on these important guidance molecules (10).

Nonmedical use and misuse of prescription opioids, including oxycodone is an increasing public health problem (27, 28). We hypothesize that specific representatives of the axon guidance gene family are implicated in development of neurobiological adaptation that occurs during chronic oxycodone self-administration. The aim of this study is to identify alterations in expression of specific axon guidance genes in the nucleus accumbens and caudate putamen of mice, following chronic oxycodone self-administration using RNA-seq technology.

METHODS

Animals and Oxycodone Self-Administration Procedure

Male adult (11 weeks old) C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, ME. Animal care and experimental procedures were conducted according to the Guide for the Care and Use of laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences 2016). Animals had free access to food and water in a light (12:12 h) reverse cycle, lights on at 7:00 p.m. and off at 7:00 a.m. Mice were handled prior to surgery. Catheter implantation for drug self-administration was carried out after acclimation of animals for 7 days. The experimental protocol used was approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

Oxycodone Self-Administration

Details of surgery and catheter implantation, and subsequent the procedure of oxycodone self-administration (SA) in mice has been described previously (29). Briefly, the self-administration experiments were carried out in chamber ENV-307W (21.6, 17.8, and 12.7 cm; Med Associates, St Albans, VT). A 4-h self-administration session was conducted once a day for 14 consecutive days with oxycodone, $n = 6$ (0.25 mg/kg/infusion) or yoked saline controls ($n = 6$).

RNA Extraction

Mice were sacrificed within 1 h after the last session of oxycodone self-administration, by exposure to CO_2. The brain tissues of 12 mice (6 oxycodone and 6 yoked saline controls) were used for the RNA-seq study. Total RNA was isolated from the nucleus accumbens (NAc) and caudate putamen (CPu) using the miRNeasy Kit (Qiagen, Valencia, CA). Agilent 6000 RNA Nano Chips were used to examine the integrity of RNA in samples.

RNA-seq Library Preparation and Sequencing

RNA-seq library preparation and sequencing of samples isolated from the CPu was performed by LC Sciences (Houston, TX), whereas RNA-seq library preparation and sequencing of RNA isolated from NAc was performed by the Genomic Resource Center at the Rockefeller University. Both RNA-seq libraries were prepared using Illumina's TruSeq® Stranded Total RNA LT kit following the manufacturer's protocol. Libraries were validated using Agilent Tape Station High-Sensitivity RNA kits and normalized. Libraries were multiplexed, four samples per lane, and sequenced. An Illumina HiSeq 2500 apparatus was used to obtain 100 bp single end reads for samples from the nucleus accumbens, whereas Illumina HiSeq 2000 was used to obtain 100 bp paired-end reads for samples from the CPu. All samples were analyzed exactly as described previously (30). Briefly, the reads were aligned to the mouse reference genome (version mm10) using STAR (31) aligner with default parameters. The alignment results were evaluated through RNA-SeQC (32). Aligned reads were then summarized through featureCounts (33) with the gene

model from Ensemble (Mus_musculus GRCm38.75.gtf) at gene level.

Samples were normalized through a set of housekeeping genes *Ppia*, *Gusb,* and *Gapdh* as described previously (30). Principal Component Analysis (PCA) was then applied to normalized counts of all the samples by brain region, to detect outlier samples. In the CPu, data from one saline control, and from one oxycodone-treated animals were thus excluded from downstream analysis. In the NAc, data from one oxycodone-treated animal was excluded from the downstream analysis.

Axon Guidance Gene Selection

REACTOME (linked to KEGG) was used to identify 416 genes in the NAc and 445 genes in CPu in the axon guidance canonical pathway (stable Identifier R-MMU-422475.1). Statistical significance and fold change of oxycodone-induced alterations in expression of 32 axon guidance-related genes (integrins, semaphorins and ephrins, and their receptors) were extracted from the total list of differentially expressed genes in the RNA-seq data for the NAc and CPu in this study (Supplement Tables 1s, 2s, respectively).

Analyses of Cell-Type Specific Enrichment of Integrin, Semaphorin and Ephrin Gene Transcripts

To examine whether DE axon guidance genes may produce their effect in cell type specific manner, we have studied potential enrichment in transcripts of the axon guidance genes in specific cell types such as astrocytes, neurons, microglial and endothelial cells. Publicly available RNA-Seq transcriptome data (34) were downloaded from GEO (GSE52564). For each gene of integrin, semaphorin and ephrin gene families, that had a significant change (FDR < 0.1) in either NAc or CPu, expression fold change of each cell type was calculated as described recently (34) (**Table 2**)**.** For example, in astrocytes: FC = gene X's expression in astrocytes/average gene X's expression in all non-astrocyte cell types.

STATISTICS

RNA-seq Differential Gene Expression Analysis

We used **DESeq2** (https://doi.org/10.1186/s13059-014-0550-8) (35), a method for differential analysis of RNA-seq data to estimate fold-change and significance testing. Specifically, DESeq2 estimate gene-wise log fold of changes (LFCs) between conditions from the standard Generalized Linear Model (GLM) fits to obtain maximum-likelihood estimates, and then fit a zero-centered normal distribution to the observed distribution of Maximum-likehood Estimate (MLE) over all genes. This distribution is used as a prior on LFC in a second round of GLM fits, and the maximum a posteriory (MAP) estimates are kept as final estimates of LFC. For significance testing, DESeq2 uses a Wald test: the shrunken estimate of LFC is divided by its standard error, resulting in a z-statistic, which is compared to a standard normal distribution. The Wald test P values from the subset of genes that pass an independent filtering step, are adjusted for multiple testing using the procedure of Benjamini and Hochberg (36).

DESeq2 was applied to the normalized counts to estimate the fold-change between the samples from mice that had self-administered oxycodone versus those from yoked saline controls, using negative binomial distribution. An adjusted *p*-value of less than 0.1 (FDR < 0.1) and fold change $\geq$15% were used to select genes that have a significant expression change.

RESULTS

Extended Access Oxycodone Self-Administration

Oxycodone self-administration behavior in the animals in this sample was reported in our previous publication (30). Our previous studies of oxycodone dose-response effects showed that a dose of 0.25 mg//kg per infusion lead to escalation of oxycodone over the sessions (29). Animals showed a robust escalation of daily oxycodone intake across 14 consecutive daily sessions. A two-way ANOVA for drug condition (oxycodone or saline) $\times$ session (days 1–14), showed a significant main effect of drug condition [$F_{(1,\ 252)} = 672.6$, $p < 0.0001$] and a significant main effect of session [$F_{(13,\ 252)} = 2.359, p < 0.01$]. The average amount of oxycodone self-administered for each mouse increased from 3 mg/kg on the 1st session to 7.5 mg/kg on the last sessions. Oxycodone self-administering mice showed a much greater frequency of response on the active versus inactive holes. In contrast, yoked-saline control mice showed lower responding, and stayed stable across 14 daily sessions.

Oxycodone-Induced Alterations in Expression of Integrin, Semaphorin and Ephrin Genes

Nucleus Accumbens (NAc)

Among 38 integrin, semaphorin and ephrin genes selected for further analyses, we found significant alterations in 14 genes in the NAc at fold change $\geq$15%, with a false discovery rate (FDR) of $<$0.1, of which six were up-regulated and 8 were down-regulated (**Table 1A**). Among up-regulated genes, there were integrin receptors *Itgal* and *Itgb2*, and their ligand semaphorin *Sema7a*. There was also up-regulation of semaphorin receptors *Plxdc1* and *Plxnd1,* with concomitant lower levels in expression of their cognate ligands *Sema3c, Sema4g, Sem6a,* and *Sema6d.* In contrast to elevated expression of the receptor *Plxd1*, the receptor neuropilin *Nrp2* was down-regulated. Two integrin genes, *Itga3* and *Itgb8* had lower level expression in the oxycodone treatment group, compared to the saline group, and only one gene from the ephrin receptor family, *Epha3* was down-regulated in this group.

Caudate-Putamen (CPu)

As observed above in the NAc, in this region there was strong up-regulation of three integrin genes, *Itgal, Itgb2,* and *Itga1* (**Table 1B**). Among down-regulated genes, there were integrin *Itga9* and the ephrin receptor ligand, *Efna3*.

TABLE 1 | Axon gudance and integrin genes with differential expression in the mouse nucleus accumbens and caudate putamen following 14 days of oxycodone self-administration (FDR < 0.1, FC > 15%).

Gene symbol	Fold change	Direction	*p*-value	FDR	Gene name
A. NUCLEUS ACCUBBENS					
Itgal	2.54	↑	0.00000	0.0000	Integrin alpha L
Itgb2	1.69	↑	0.00006	0.0068	Integrin beta 2
Sema7a	1.40	↑	0.00637	0.0910	Semaphorin 7a
Plxdc1	1.32	↑	0.00102	0.0464	Plexin dc1
Plxnd1	1.48	↑	0.00376	0.0693	Plexin d1
Itgam	1.29	↑	0.00519	0.0852	Integrin alpha M
Epha3	0.77	↓	0.00298	0.0620	Ephrin receptor a3
Sema3c	0.77	↓	0.00223	0.0618	Semaphorin 3c
Sema4g	0.75	↓	0.00482	0.0821	Semaphorin 4g
Sema6a	0.82	↓	0.00782	0.1050	Semaphorin 6a
Sema6d	0.84	↓	0.06142	0.0583	Semaphorin 6d
Nrp2	0.61	↓	0.00165	0.0583	Neuropilin 2
Itga3	0.77	↓	0.00244	0.0620	Integrin a3
Itgb8	0.86	↓	0.00583	0.0861	Integrin b8
Gene symbol	**Fold change**	**Direction**	***p*-value**	**FDR**	**Entrez gene name**
B. CAUDATE PUTAMEN					
Itgal	2.53	↑	0.00000	0.0002	Integrin alpha L
Itgb2	2.14	↑	0.00003	0.0041	Integrin beta 2
Itga1	1.48	↑	0.00060	0.0323	Integrin alpha 1
Itga9	0.72	↓	0.00004	0.0042	Integrin alpha 9
Efna3	0.71	↓	0.00185	0.0747	Ephrin A3

Cell Type Specific Enrichment Analysis of Integrin, Semaphorin, and Ephrin Gene Transcripts

Recently, an RNA-sequencing transcriptome and splicing database has been reported for purified representative populations of neurons, astrocytes, oligodendrocyte precursor cells, newly formed oligodendrocytes, myelinating oligodendrocytes, microglia, and endothelial cells from mouse cerebral cortex (34). This database provided a platform for analyzing and comparing transcription profiles for various cell classes in the brain herein. We have used this publicly available database to perform analyses of enrichment in expression levels of axon guidance genes that would be expected in astrocytes, neurons, microglial and endothelial cells. The analyses have revealed an enrichment of integrins *Itgal*, *Itgb2, Itgam*, and receptors *Plxdc1* and *Plxdc2* expression in microglia cells (**Table 2**). Similar analysis showed that ephrin *Efna3*, and its receptor *Epha3*, and semaphorins *Sema3c* and *Sema4g* are abundoned in neurons. Expression levels of semaphorins *Sema7a* and *Sema3c*, semaphorin receptor *Nrp2,* and integrin *Itga1* were enriched in endothelial cells. Astrocytes were enriched with semaphorin *Sema6d.*

DISCUSSION

In this study, we have for the first time RNA-seq technology applied to examine differential gene expression of the axon guidance gene family and integrins, in the NAc and CPu of adult male mice, following 14-day extended-access self-administration of oxycodone, compared with the yoked saline controls. This self-administration regimen resulted in substantial daily intake and escalation of oxycodone across sessions (37).

We have found substantial difference in number of the differentially expressed axon guidance genes in response to the chronic oxycodone self-administration, 14 genes in the NAc, and five genes in the CPu. This difference between these two regions could be due to, in part, difference in neuroanatomical structure and functions of ventral and dorsal striatum in the development of dependence to drugs of abuse, with the NAc implemented in drug rewards, and CPu in drug-induced habituation behavior (38). Earlier studies showed that in contrast to NAc, injections of morphine to CPu did not produce a condition place preference (39). This could be also a result of intrinsic differences in the dopamine fiber responsiveness that innervate these specific regions of the striatum as well as in dopamine transporters in regulation of drug-induced dopamine release. For example, repeated morphine administration at a dose of 1.0 mg/kg (s.c.) increased synaptic dopamine concentrations preferentially in the rat NAc, not in CPu (40, 41). After 15 days of withdrawal after 3 days of morphine treatment, challenge with 1 mg/kg (s.c.) morphine failed to significantly modify extracellular DA in the CPu of sensitized as well as in control rats (41).

We have found oxycodone-induced alterations in expression mainly in three axon guidance gene families such as integrins, semaphorins, and ephrins. These systems may contribute

TABLE 2 | Cell type enrichment of integrin, semaphorin, and ephrin.

Transcripts (log2 of Fold Enrichment)				
Gene	**Astrocytes (A)**	**Neurons (N)**	**Microglia (MGL)**	**Endothelia (Endo)**
Itgal	−2.235	−2.235	**2.956**[a]	−1.419
Itga1	−2.179	−4.461	−5.737	**6.439**
Itga3	−2.095	**2.048**	−4.006	**1.321**
Itga9	−5.707	−3.4	**2.081**	−2.568
Itgal.1	−2.235	−2.235	**2.956**	−1.419
Itgam	−8.218	−7.842	**5.905**	−8.752
Itgb2	−7.874	−6.023	**6.834**	−5.103
Itgb2.1	−7.874	−6.023	**6.834**	−5.103
Itgb8	**0.978**	−1.988	−6.774	−6.7
Nrp2	−2.55	0.197	0.802	**1.935**
Plxdc1	−4.265	0.264	**2.822**	0.494
Plxdc2	−1.234	−1.446	**3.347**	−2.651
Plxnd1	−3.509	1.016	−0.667	**3.113**
Sema3c	−3.559	**1.725**	−5.425	**3.192**
Sema4g	−0.77	**2.445**	−0.153	−4.345
Sema6a	**0.642**	−0.977	−7.241	−0.245
Sema6d	**2.287**	−1.622	−3.912	0.376
Sema7a	−3.782	−2.209	−3.772	**4.542**
Efna3	−2.091	**4.598**	−2.668	−2.987
Epha3	−2.207	**4.406**	−3.799	−3.525

[a] *Example of calculation of a fold enrichment of a gene in a specific cell type: Log2 [expression of X gene in astroglia divided by average expression of X gene in non-astrocyte cell types (N+MGL+Endo)]. Bolded numbers show the highest enrichment of X gene transcripts in a specific cell type. Positive numbers indicate a higher abundance of gene X expression in a particularly cell type, compared with its expression in other cells.*

to oxycodone-induced neuroadaptations through alterations in axon-target connections and synaptogenesis and may be implicated in the behavioral and neurobiological adaptations occurring in opioid use disorders. No significant oxycodone-induced alterations in expression of Netrin-1 or Slit were observed. We found here that integrins Itgal and Itgb2 have the greatest increase in expression in both the NAc and CPu immediately after chronic oxycodone SA**.** However, oxycodone-induced increase of their potential ligand semaphorin Sema7a was observed only in the NAc, but not in the CPu. In the adult brain, many integrins are present at high levels at synapses. The sequence arginine-glycine-aspartic acid RGD (42) was identified as a general integrin-binding motif. Application of soluble GRGDS (Gly-Arg-Gly-Asp-Ser) peptides completely abolished the mu opioid receptor agonist DAMGO inhibitory effect on cyclic AMP (cAMP) accumulation in bradykinin-primed trigeminal ganglia neurons (43, 44). This suggests that activation of specific integrins at focal adhesions may modulate the mu opioid receptor signaling by altering interactions with G proteins. Also, RGD peptides, or anti-integrin antibodies block N-methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic currents in hippocampal neurons (45), suggesting that RGD-binding integrins are important in neurotransmission.

We have found only one gene from the ephrin receptor family, Epha3, which was down-regulated in the NAc of oxycodone-treated mice. In other studies, similar down regulation in the expression of ephrin genes in the rat NAc was observed at 24 h after secession of heroin self-administration (6 h/day for 5 days) (12). In contrast, cocaine treatment increased expression of many ephrin and ephrin receptor in the rat and mouse striatum and hippocampus (7, 14) and in striatum of nonhuman primates (46). Alterations in expression of the Eph/ephrin genes have also been linked to neuropathology ranging from inhibition of neural repair after traumatic injury and stroke to neurodegenerative diseases (47, 48). Ephrin receptors and their ligands are implicated in dendritic spine morphology throughout an interaction with integrins. For instance, activation of the receptor EphA4 by ephrinA3 inhibits activity of integrin Itgb1 and downstream signaling, and leads to decreases spine length and density (49). Transgenic overexpression of ephrinA3 in astrocytes reduces glutamate transporter levels and elevates extracellular glutamate concentrations (50). In contrast, loss of ephrin-A3 raises glutamate transporter currents in astrocytes. Functionally, EphA4 and ephrinA3 modulate transporter glutamate currents in astrocytes.

Previously, microarray studies of morphine-treated mice showed alterations in expression of genes related to the semaphorin pathway in the NAc such as Sema3f, Sema4b, sema6c, Sema6d, Sema7a, and Plxna3 (10) and Sema6a (44). Heroin self-administration induced downregulation of Sema5a, Sema6c and receptor Plxnb1 in the rat NAc (12). Consistent with these earlier reports, in this study we have found oxycodone-induced downregulation of Sema3c, Sema4g, Sema6a, Sema6d, and upregulation Sema7a in the mouse NAc. Our results are also consistent with alterations in the expression of semaphorins and their receptors in post-mortem brain of patients chronically exposed to alcohol or cocaine (51), particularly upregulation of *SEMA7A* and plexin *PLXDC1*, and down-regulation *SEMA4B* in hippocampus. Alterations in expression of semaphorins and their receptors were documented in many pathological conditions such as ischemia, degenerative diseases, multiple sclerosis (5).

Early studies showed that chronic morphine modulate both adaptive and innate immune systems, as well as activate neuroinflammation (52). Several studies showed that this effect of morphine on immune system is mediated by the central MOPr**,** and can be antagonized by naltrexone (53, 54). Several studies showed that microglia, astrocytes, oligodendrocytes, and endothelial cells actively respond to opioids by producing an inflammatory immune response (55, 56). Recently, we have reported mRNA levels of numerous genes related to the inflammation and immune functions changed as a result of oxycodone self-administration, in the CPu and NAc (30). We have found oxycodone-induced upregulation in the NAc of many glial- and immune cell-specific genes, such as the chemokine receptor Ccr5, chemokine Ccl 12, toll-like receptor Tlr7, interleukin Il1b, interleukin-17 receptor, antigen CD14, antigen CD163, complement component 1 C1qc, interferon regulatory factor Irf1, and others. Astrocytes and microglia

release several neuroactive molecules, such as glutamate, D-serine, ATP, GABA, TNFa, that can actively regulate many aspects of neuronal function, including neurotransmitter release, gene regulation, dendritic morphology, and synaptic connectivity (57).

Semaphorins were shown to be involved in initiation of the immune response in brain (58), and alterations in their expression may be related to the oxycodone-induced changes in inflammation/immune gene expression found in this study. Sema7A has been identified as an effector molecule in T-cell-mediated inflammation through an integrin-mediated mechanism, reviewed in (17). Of interest, many immune proteins have been found in healthy, uninfected nervous system, and they may participate in regulation of neuronal functions, including neurotransmitter release, dendritic morphology, and synaptic transmission (59–61).

Several studies proposed axon guidance molecules for contact interaction of neuronal and glial cells (13). The oxycodone-induced differential expression of integrins, semaphorins, and ephrins in ventral and dorsal regions of the mouse striatum implies that they likely participate in the altered communication occurring between neurons and glial cells. Therefore, an identification of cell type specificity of axon guidance gene expression would help in understanding of their specific roles in drug-induced alterations in neuronal activity. We did not perform a cell sorting procedure of brain tissues in our experiments. However, our bioinformatics analysis of oxycodone-induced differentially expressed axon guidance genes showed that their mRNA enrichment varied among neuronal, astrocyte, microglial and endothelial cells, supporting their pleiotropic functions in adaptation to chronic oxycodone treatment.

Since gene expression changes were examined immediately after 14 consecutive days of chronic oxycodone self-administration, it is also not clear whether the changes in axon guidance gene expression are long-lasting, which may play an important role in drug-induced adaptation. The exact molecular mechanisms of regulation of neurotransmission (e.g., dopaminergic, GABAergic, serotoninergic) by different axon guidance molecules remain unknown. However, it is known that axon guidance molecules are involved in glutamatergic transmission and long term potentiation (60). Therefore, oxycodone induced alterations in axon guidance gene expression may be relevant to neuronal plasticity which may occur in addictive-like state (1, 62). This is the first RNAseq study on the impact of a chronic period of oxycodone self-administration, a widely abused prescription drug on expression of axon guidance genes in the mouse brain.

In conclusion, we have found alterations in expression of specific axon guidance genes in the mouse striatum following chronic oxycodone self-administration. Although, their exact functions in drug taking or drug seeking behaviors are not known, these proteins are promising targets for further studies and development of treatment of oxycodone addiction.

AUTHOR CONTRIBUTIONS

YZ and MK designed the experiment. YZ and CZ performed experiments. YL, MR, and VY analyzed data, and drafted the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Russo SJ, Dietz DM, Dumitriu D, Morrison JH, Malenka RC, Nestler EJ. The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. *Trends Neurosci.* (2010) **33:**267–76. doi: 10.1016/j.tins.2010.02.002
2. Robinson TE, Gorny G, Savage VR, Kolb B. Widespread but regionally specific effects of experimenter- versus self-administered morphine on dendritic spines in the nucleus accumbens, hippocampus, and neocortex of adult rats. *Synapse* (2002) **46:**271–9. doi: 10.1002/syn.10146
3. Ballesteros-Yanez I, Ambrosio E, Benavides-Piccione R, Perez J, Torres I, Miguens M, et al. The effects of morphine self-administration on cortical pyramidal cell structure in addiction-prone lewis rats. *Cereb Cortex* (2007) **17:**238–49. doi: 10.1093/cercor/bhj142
4. Pasterkamp RJ. Getting neural circuits into shape with semaphorins. *Nat Rev Neurosci.* (2012) **13:**605–18. doi: 10.1038/nrn3302
5. Van Battum EY, Brignani S, Pasterkamp RJ. Axon guidance proteins in neurological disorders. *Lancet Neurol.* (2015) **14:**532–46. doi: 10.1016/S1474-4422(14)70257-1
6. Pasterkamp RJ, Kolodkin AL. SnapShot: axon guidance. *Cell* (2013) **153:**494e1–2. doi: 10.1016/j.cell.2013.03.031
7. Bahi A, Dreyer JL. Cocaine-induced expression changes of axon guidance molecules in the adult rat brain. *Mol Cell Neurosci.* (2005) **28:**275–91. doi: 10.1016/j.mcn.2004.09.011
8. Xiao D, Miller GM, Jassen A, Westmoreland SV, Pauley D, Madras BK. Ephrin/Eph receptor expression in brain of adult nonhuman primates: implications for neuroadaptation. *Brain Res.* (2006) **1067:**67–77. doi: 10.1016/j.brainres.2005.10.073
9. Jassen AK, Yang H, Miller GM, Calder E, Madras BK. Receptor regulation of gene expression of axon guidance molecules: implications for adaptation. *Mol Pharmacol.* (2006) **70:**71–7. doi: 10.1124/mol.105.021998
10. Grice DE, Reenila I, Mannisto PT, Brooks AI, Smith GG, Golden GT, et al. Transcriptional profiling of C57 and DBA strains of mice in the absence and presence of morphine. *BMC Genomics* (2007) **8:**76. doi: 10.1186/1471-2164-8-76
11. Wiggins AT, Pacchioni AM, Kalivas PW. Integrin expression is altered after acute and chronic cocaine. *Neurosci Lett.* (2009) **450:**321–3. doi: 10.1016/j.neulet.2008.12.006

12. Imperio CG, McFalls AJ, Colechio EM, Masser DR, Vrana KE, Grigson PS, et al. Assessment of individual differences in the rat nucleus accumbens transcriptome following taste-heroin extended access. *Brain Res Bull.* (2016) **123:**71–80. doi: 10.1016/j.brainresbull.2015.12.005
13. Murai KK, Pasquale EB. Eph receptors and ephrins in neuron-astrocyte communication at synapses. *Glia* (2011) **59:**1567–78. doi: 10.1002/glia.21226
14. Yue Y, Widmer DA, Halladay AK, Cerretti DP, Wagner GC, Dreyer JL, et al. Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. *J Neurosci.* (1999) **19:**2090–101. doi: 10.1523/JNEUROSCI.19-06-02090.1999
15. Liu WT, Li HC, Song XS, Huang ZJ, Song XJ. EphB receptor signaling in mouse spinal cord contributes to physical dependence on morphine. *FASEB J.* (2009) **23:**90–8. doi: 10.1096/fj.08-114462
16. Worzfeld T, Offermanns S. Semaphorins and plexins as therapeutic targets. *Nat Rev Drug Discov*. (2014) **13:**603–21. doi: 10.1038/nrd4337
17. Roth L, Koncina E, Satkauskas S, Cremel G, Aunis D, Bagnard D. The many faces of semaphorins: from development to pathology. *Cell Mol Life Sci.* (2009) **66:**649–66. doi: 10.1007/s00018-008-8518-z
18. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* (2002) **110:**673–87. doi: 10.1016/S0092-8674(02)00971-6
19. Park YK, Goda Y. Integrins in synapse regulation. *Nat Rev Neurosci*. (2016) **17:**745–56. doi: 10.1038/nrn.2016.138
20. Chen Q, Xiong X, Lee TH, Liu Y, Wetsel WC, Zhang X. Neural plasticity and addiction: integrin-linked kinase and cocaine behavioral sensitization. *J Neurochem* (2008) **107:**679–89. doi: 10.1111/j.1471-4159.2008.05619.x
21. Barallobre MJ, Pascual M, Del Rio JA, Soriano E. The Netrin family of guidance factors: emphasis on Netrin-1 signalling. *Brain Res Brain Res Rev.* (2005) **49:**22–47. doi: 10.1016/j.brainresrev.2004.11.003
22. Matsumoto H, Nagashima M. Shift in the function of netrin-1 from axon outgrowth to axon branching in developing cerebral cortical neurons. *BMC Neurosci.* (2017) **18:**74. doi: 10.1186/s12868-017-0392-x
23. Tsai NP, Bi J, Loh HH, Wei LN. Netrin-1 signaling regulates de novo protein synthesis of kappa opioid receptor by facilitating polysomal partition of its mRNA. *J Neurosci.* (2006) **26:**9743–9. doi: 10.1523/JNEUROSCI.3014-06.2006
24. Chedotal A. Slits and their receptors. *Adv Exp Med Biol.* (2007) **621:**65–80. doi: 10.1007/978-0-387-76715-4_5
25. Cornide-Petronio ME, Barreiro-Iglesias A. Role of slit and robo proteins in the development of dopaminergic neurons. *Dev Neurosci.* (2013) **35:**285–92. doi: 10.1159/000351023
26. Dugan JP, Stratton A, Riley HP, Farmer WT, Mastick GS. Midbrain dopaminergic axons are guided longitudinally through the diencephalon by Slit/Robo signals. *Mol Cell Neurosci*. (2011) **46:**347–56. doi: 10.1016/j.mcn.2010.11.003
27. Comer SD, Sullivan MA, Whittington RA, Vosburg SK, Kowalczyk WJ. Abuse liability of prescription opioids compared to heroin in morphine-maintained heroin abusers. *Neuropsychopharmacology* (2008) **33:**1179–91. doi: 10.1038/sj.npp.1301479
28. Paulozzi LJ, Strickler GK, Kreiner PW, Koris CM. Centers for disease c, and prevention, controlled substance prescribing patterns–prescription behavior surveillance system, eight states, 2013. *MMWR Surveill Summ*. (2015) **64:**1–14. doi: 10.15585/mmwr.ss6409a1
29. Zhang Y, Picetti R, Butelman ER, Schlussman SD, Ho A, Kreek MJ. Behavioral and neurochemical changes induced by oxycodone differ between adolescent and adult mice. *Neuropsychopharmacology* (2009) **34:**912–22. doi: 10.1038/npp.2008.134
30. Zhang Y, Liang Y, Levran O, Randesi M, Yuferov V, Zhao C, et al. Alterations of expression of inflammation/immune-related genes in the dorsal and ventral striatum of adult C57BL/6J mice following chronic oxycodone self-administration: a RNA sequencing study. *Psychopharmacology* (2017) **234:**2259–75. doi: 10.1007/s00213-017-4657-y
31. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* (2013) **29:**15–21. doi: 10.1093/bioinformatics/bts635
32. DeLuca DS, Levin JZ, Sivachenko A, Fennell T, Nazaire MD, Williams C, et al. RNA-SeQC: RNA-seq metrics for quality control and process optimization. *Bioinformatics* (2012) **28:**1530–2. doi: 10.1093/bioinformatics/bts196
33. Liao Y, Smyth GK, Shi W. Featurecounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* (2014) **30:**923–30. doi: 10.1093/bioinformatics/btt656
34. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci.* (2014) **34:**11929–47. doi: 10.1523/JNEUROSCI.1860-14.2014
35. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) **15:**550. doi: 10.1186/s13059-014-0550-8
36. Benjamini Y, Hochberg Y. Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J R Stat Soc Ser B Methodol.* (1995) **57:**289–300.
37. Zhang Y, Mayer-Blackwell B, Schlussman SD, Randesi M, Butelman ER, Ho A, et al. Extended access oxycodone self-administration and neurotransmitter receptor gene expression in the dorsal striatum of adult C57BL/6J mice. *Psychopharmacology* (2014) **231:**1277–87. doi: 10.1007/s00213-013-3306-3
38. Everitt BJ, Robbins TW. From the ventral to the dorsal striatum: devolving views of their roles in drug addiction. *Neurosci Biobehav Rev*. (2013) **37:**1946–54. doi: 10.1016/j.neubiorev.2013.02.010
39. Olmstead MC, Franklin KB. The development of a conditioned place preference to morphine: effects of microinjections into various CNS sites. *Behav Neurosci.* (1997) **111:**1324–34. doi: 10.1037/0735-7044.111.6.1324
40. Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA*. (1988) **85:**5274–8. doi: 10.1073/pnas.85.14.5274
41. Cadoni C, Di Chiara G. Reciprocal changes in dopamine responsiveness in the nucleus accumbens shell and core and in the dorsal caudate-putamen in rats sensitized to morphine. *Neuroscience* (1999) **90:**447–55. doi: 10.1016/S0306-4522(98)00466-7
42. Brudzynski SM, Gibson B, Silkstone M, Burgdorf J, Kroes RA, Moskal JR, et al. Motor and locomotor responses to systemic amphetamine in three lines of selectively bred Long-Evans rats. *Pharmacol Biochem Behav* (2011) **100:**119–24. doi: 10.1016/j.pbb.2011.08.006
43. Berg KA, Zardeneta G, Hargreaves KM, Clarke WP, Milam SB. Integrins regulate opioid receptor signaling in trigeminal ganglion neurons. *Neuroscience* (2007) **144:**889–97. doi: 10.1016/j.neuroscience.2006.10.033
44. Tapocik JD, Luu TV, Mayo CL, Wang BD, Doyle E, Lee AD, et al. Neuroplasticity, axonal guidance and micro-RNA genes are associated with morphine self-administration behavior. *Addict Biol* (2013) **18:**480–95. doi: 10.1111/j.1369-1600.2012.00470.x
45. Chavis P, Westbrook G. Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. *Nature* (2001) **411:**317–21. doi: 10.1038/35077101
46. Saka E, Goodrich C, Harlan P, Madras BK, Graybiel AM. Repetitive behaviors in monkeys are linked to specific striatal activation patterns. *J Neurosci* (2004) **24:**7557–65. doi: 10.1523/JNEUROSCI.1072-04.2004
47. Boyd AW, Bartlett PF, Lackmann M. Therapeutic targeting of EPH receptors and their ligands. *Nat Rev Drug Discov* (2014) **13:**39–62. doi: 10.1038/nrd4175
48. Barquilla A, Pasquale EB. Eph receptors and ephrins: therapeutic opportunities. *Annu Rev Pharmacol Toxicol* (2015) **55:**465–87. doi: 10.1146/annurev-pharmtox-011112-140226
49. Klein R. Bidirectional modulation of synaptic functions by Eph/ephrin signaling. *Nat Neurosci* (2009) **12:**15–20. doi: 10.1038/nn.2231
50. Carmona MA, Murai KK, Wang L, Roberts AJ, Pasquale EB. Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport. *Proc Natl Acad Sci USA*. (2009) **106:**12524–9. doi: 10.1073/pnas.0903328106
51. Zhou Z, Yuan Q, Mash DC, Goldman D. Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. *Proc Natl Acad Sci USA*. (2011) **108:**6626–31. doi: 10.1073/pnas.1018514108
52. Wang X, Loram LC, Ramos K, de Jesus AJ, Thomas J, Cheng K, et al. Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc Natl Acad Sci USA*. (2012) **109:**6325–30. doi: 10.1073/pnas.1200130109
53. Hernandez MC, Flores LR, Bayer BM. Immunosuppression by morphine is mediated by central pathways. *J Pharmacol Exp Ther.* (1993) **267:**1336–41.
54. Lysle DT, Coussons ME, Watts VJ, Bennett EH, Dykstra LA. Morphine-induced alterations of immune status: dose dependency, compartment

specificity and antagonism by naltrexone. *J Pharmacol Exp Ther*. (1993) **265:**1071–8.
55. Jacobsen JH, Hutchinson MR, Mustafa S. Drug addiction: targeting dynamic neuroimmune receptor interactions as a potential therapeutic strategy. *Curr Opin Pharmacol*. (2016) **26:**131–7. doi: 10.1016/j.coph.2015.10.010
56. Lacagnina MJ, Rivera PD, Bilbo SD. Glial and neuroimmune mechanisms as critical modulators of drug use and abuse. *Neuropsychopharmacology* (2017) **42:**156–77. doi: 10.1038/npp.2016.121
57. Eroglu C, Barres BA. Regulation of synaptic connectivity by glia. *Nature* (2010) **468:**223–31. doi: 10.1038/nature09612
58. Suzuki K, Kumanogoh A, Kikutani H. Semaphorins and their receptors in immune cell interactions. *Nat Immunol*. (2008) **9:**17–23. doi: 10.1038/ni1553
59. Boulanger LM. Immune proteins in brain development and synaptic plasticity. *Neuron* (2009) **64:**93–109. doi: 10.1016/j.neuron.2009.09.001
60. Filosa A, Paixao S, Honsek SD, Carmona MA, Becker L, Feddersen B, et al. Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport. *Nat Neurosci*. (2009) **12:**1285–92. doi: 10.1038/nn.2394
61. Paixao S, Klein R. Neuron-astrocyte communication and synaptic plasticity. *Curr Opin Neurobiol*. (2010) **20:**466–73. doi: 10.1016/j.conb.2010.04.008
62. Rich MT, Torregrossa MM. Molecular and synaptic mechanisms regulating drug-associated memories: Towards a bidirectional treatment strategy. *Brain Res Bull*. (2017). doi: 10.1016/j.brainresbull.2017.09.003. [Epub ahead of print].

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REVIEW
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Enkephalin as a Pivotal Player in Neuroadaptations Related to Psychostimulant Addiction

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Enkephalin expression is high in mesocorticolimbic areas associated with psychostimulant-induced behavioral and neurobiological effects, and may also modulate local neurotransmission in this circuit network. Psychostimulant drugs, like amphetamine and cocaine, significantly increase the content of enkephalin in these brain structures, but we do not yet understand the specific significance of this drug-induced adaptation. In this review, we summarize the neurochemical and molecular mechanism of psychostimulant-induced enkephalin activation in mesocorticolimbic brain areas, and the contribution of this opioid peptide in the pivotal neuroadaptations and long-term behavioral changes underlying psychostimulant addiction. There is evidence suggesting that adaptive changes in enkephalin content in the mesocorticolimbic circuit, induced by acute and chronic psychostimulant administration, may represent a key initial step in the long-term behavioral and neuronal plasticity induced by these drugs.

Keywords: enkephalin, cocaine, amphetamine, neuroadaptations, addiction, opioid antagonists

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INTRODUCTION

Psychostimulant addiction is a severe worldwide health problem. The most challenging aspects in its treatment are compulsive drug use and relapse. Currently, there are no effective pharmacotherapies for this disorder. New therapeutic approaches are required based on understanding the neurobiology of drug addiction. Numerous lines of research suggest that exposure to psychostimulant drugs causes neurochemical and molecular adaptations that explain the stability of the behavioral disorders characterizing the addictive state (1, 2). Attention has focused on how the mesocorticolimbic dopamine circuit is affected by drugs of abuse, and particularly on the role of glutamate and dopamine neurotransmission in determining the neuroplastic changes related to psychostimulant addiction (3–6). At molecular level, it has been shown that activation of glutamate and dopamine neurotransmission after repeated psychostimulant administrations, affects intracellular signaling cascades (7–9), alters the expression of membrane receptors (10, 11) and changes gene expression within the neural circuits (12, 13), which leads to sensitization of the drug's behavioral effects (14) and other behavioral alterations observed in addiction, like intense drug craving and relapse (15).

Enkephalin, an opioid peptide derived from proenkephalin (PENK), is widely expressed in the mesocorticolimbic circuit (16) and interacts with glutamate and dopamine in the brain reward structures related to psychostimulant-induced effects. Both delta-opioid (DOPr) and mu-opioid receptors (MOPr) can be activated by enkephalin, and each has its particular pattern of expression

within the motivational circuit (17). Although several pharmacological and genetic approaches demonstrate a role of both MOPr and DOPr in psychostimulant-induced behavioral effects, the role of the endogenous opioid peptides in this process has not been fully examined. Previous studies from our lab have demonstrated a long-lasting increase in enkephalin levels within the mesocorticolimbic circuit after psychostimulant administration (18, 19). Enkephalin has also been shown to positively modulate dopamine and glutamate neurotransmission within this circuit (20–25). These data indicate that cocaine-induced enkephalin elevation may drive the neuronal plasticity induced by the drug and the long-term behavioral effects of psychostimulant exposure.

ACTIVATION OF THE ENKEPHALIN SYSTEM BY PSYCHOSTIMULANTS IN THE CENTRAL NERVOUS SYSTEM

Mesolimbic dopamine activity is directly affected by psychostimulants (26). These drugs bind to monoamine transporters and block reuptake mechanisms (cocaine), or competitively inhibit dopamine uptake and disrupt vesicular storage (amphetamine). The activation of this system is a primary conditioner of their psychomotor stimulant and rewarding effects (27). Acute or chronic administration of psychostimulants also alters, among others, the levels of endogenous opioid peptides, including enkephalin, within areas of the mesocorticolimbic circuit.

Acute cocaine (28, 29) and amphetamine (30–33) elevate PENK mRNA levels in the striatum, and these levels are also increased after chronic cocaine exposure in different dopamine mesolimbic afferents (34–36). Elevated PENK levels were also observed within the caudate putamen on the second day of binge cocaine administration (37). Following chronic cocaine treatment, no changes were observed in the cortex in PENK mRNA levels (38), prefrontal cortex (39, 40), amygdala (41, 42), hypothalamus, pituitary, central gray and cerebellum, nucleus accumbens or caudate putamen (39). However, PENK mRNA levels were significantly elevated during long-term extinction (10 days) of a cocaine self-administration paradigm in the caudate putamen, nucleus accumbens, piriform cortex and olfactory tubercle regions, and decreased in the central amygdale of rats (43). Similarly, sensitized PENK mRNA expression was observed in the nucleus accumbens and/or caudate putamen in response to an amphetamine challenge following acute (44–47) or chronic (48) amphetamine pretreatment in animals after short-term abstinence from the drug.

Furthermore, data from our lab demonstrate an increase in the levels of met-enkephalin in the nucleus accumbens from rats after acute amphetamine (5 mg/kg i.p.) following 4, but not 7 or 21 days after the last drug injection (18, 49, 50). Met-enkephalin elevation was also observed after 4 days withdrawal period from chronic amphetamine (5 × 2 mg/kg i.p) (49). Interestingly, long-lasting sensitization to amphetamine-induced increases in met-enkephalin levels was evidenced in the same brain area following amphetamine challenge (1 mg/kg i.p.) 21 days after the last acute (5 mg/kg i.p.) administration of the drug (18). Similarly, persistent met-enkephalin immunoreactivity was evidenced in the nucleus accumbens from mice treated chronically with cocaine (9 × 15 mg/kg i.p.) after a long-term abstinence from the drug (12 days after last injection). Met-enkephalin immunoreactivity elevations induced by chronic cocaine is not dependent of cocaine challenge administration (7.5 mg/kg i.p., day 21), as this effect on met-enkephalin immunoreactivity was also observed after saline challenge injection (19).

Altogether these data demonstrate that PENK mRNA levels are increased in specific dopaminergic regions following psychostimulant administration, be the injection acute, chronic or remote.

NEUROCHEMICAL AND MOLECULAR MECHANISMS IN PSYCHOSTIMULANT-INDUCED PROENKEPHALIN EXPRESSION

Psychostimulant-induced PENK mRNA expression at striatal level may be the result of multiple neurotransmitter interactions (31, 51, 52). Cocaine and amphetamine stimulate the PENK mRNA expression in striatal neurons (19, 28, 31, 35, 49), which mostly express D2 receptors (53, 54), and also induce prodynorphin and substance P in striatal neurons (31), which mainly express D1 receptors (53, 55). Similarly, the full D1 receptor agonist SKF-82958 induced PENK, prodynorphin and substance P gene expression in both the dorsal and ventral striatum (33). Interestingly, the increase in met-enkephalin induced by amphetamine (50) or PENK mRNA levels stimulated by SKF-82958 in striatal neurons (33) was blocked by the D1 receptor antagonist SCH-23390 (50) and by scopolamine, the muscarinic receptor antagonist (32). Oppositely, the D2 receptor antagonist eticlopride did not affect SKF-82958-induced PENK mRNA expression (33). Similarly, amphetamine-induced met-enkephalin levels was not modified by raclopride, another D2 receptor antagonist (50). Thus, this evidence suggests that D1-mediated induction of PENK may involve trans-synaptic activation of cholinergic neurotransmission. That is, the psychostimulant-induced dopamine elevations stimulates acetylcholine release via a D1-dependent mechanism (56, 57). The acetylcholine released then activates muscarinic M1 receptors (32, 44) and associative signaling pathways in enkephalin-containing neurons thus facilitating PENK mRNA expression (**Figure 1**). Opioid receptors located at striatal level are also involved in psychostimulant-induced PENK mRNA expression. Selective kappa opioid receptor (KOPr) agonists appear to inhibit psychostimulant-induced alterations in PENK mRNA in the striatum (58), and DOPr antagonists significantly decreased amphetamine-induced mRNA PENK expression (45). In contrast to DOPr's inhibitory effects, MOPr antagonists, alone or combined with amphetamine, increase PENK mRNA levels in the dorsal striatum (45). Opioid

receptors thus probably differentially regulate psychostimulant-induced PENK gene expression in the striatum, as a result of the predominantly MOPr expression at D1+ medium spiny neuron vs. D2+ medium spiny neuron and the selective pre-synaptic DOPr location in the local network. Similarly, pre-synaptic KOPr located at striatal dopamine and glutamate nerve terminals could regulates psychostimulant-evoked neurotransmitter release (59) indirectly affecting PENK expression within this brain area.

Glutamate transmission actively regulates PENK gene expression under normal or stimulated conditions (51, 52). However, the precise mechanism by which glutamate participates in psychostimulant-stimulated PENK mRNA expression requires further study. Several reports indicate that cocaine (60, 61) and amphetamine (62–64) administration increases extracellular glutamate levels as well as dopamine levels in the striatum. Also, glutamate tone may be important for amphetamines to stimulate dopamine release from nerve terminals (64–67). Thus, glutamate transmission could also play a role in regulating the stimulant effect of psychostimulants on PENK mRNA expression. There is evidence from our lab that pretreatment with NMDA receptor antagonists attenuates long-lasting amphetamine-induced PENK mRNA expression and met-enkephalin levels in the nucleus accumbens (18). In addition, there is evidence that glutamate transmission mediated by the AMPA receptor is involved in acute amphetamine-induced PENK levels in the striatum (52). Alternatively, elevated glutamate transmission seems to increase acetylcholine release (68, 69), and this induces acetylcholine-sensitive PENK gene expression, possibly through a NMDA receptor mechanism.

The regulation of PENK in the brain is usually preceded by the induction of AP-1, cAMP response element-binding protein (CREB) and c-Fos (70–74). Dopamine D1 receptor stimulation activates these transcription factors and, if dopamine D2 is also activated, there is a synergistic mechanism (75, 76). This initiates a sequence of molecular steps critically involved in psychostimulant-induced behavioral responses. CREB is the primary regulator of transcriptional activity in accumbal projection neurons and is phosphorylated by protein kinases, including the extracellular signaling-regulated kinase (ERK1/2) (77, 78). Glutamate-stimulated CREB phosphorylation in the striatum is attenuated by the ERK1/2 kinase inhibitor, PD98059 (77–79).

Psychostimulant drugs, which increase dopamine, glutamate and PENK content in mesocorticolimbic brain areas, also up-regulate ERK2/CREB phosphorylation (8, 9, 80). Consistent with this, the inhibition of the ERK2/CREB signaling pathway prevents the increase of psychostimulant-induced PENK mRNA expression (47). This strongly indicates that the long-term increase in met-enkephalin levels, induced by psychostimulants in mesocorticolimbic brain structures, is mediated by a dopamine- and glutamate-dependent mechanism, with the activation of dopamine D1 and glutamate NMDA receptors leading to ERK2/CREB signaling pathway activation in the same brain areas.

ROLE OF THE ENKEPHALINERGIC SYSTEM IN PSYCHOSTIMULANT-INDUCED LONG-TERM BEHAVIORAL EFFECTS AND ASSOCIATED NEUROADAPTATIONS

Although enkephalin seems to exert an influence on key areas involved in psychostimulant-induced behavioral effects, the mechanism underlying long-term effects has not yet been fully explained. Pharmacologically, PENK-derived opioid peptides seem to show high affinity for DOPr, but also good affinity for MOPr (17). Furthermore, dopamine release in the nuceleus accumbens appears to be promoted by enkephalin in the ventral tegmental area (20, 81), while MOPr antagonists administered intra-ventral tegmental area cause a decrease in dopamine neurotransmission (82). Pharmacological studies have shown that MOPr and DOPr contribute to increasing dopamine and glutamate release induced by psychostimulants in the nucleus accumbens (83–86). Consistently, pharmacological approaches using MOPr and DOPr antagonists, as well as MOPr knockout mice, demonstrate that the endogenous opioid system is involved in dopamine-related behaviors (87–89). This evidence, together with studies showing that PENK is one of the mediators of the positive reinforcing effects of nicotine, alcohol and marihuana (90–92), suggests that enkephalin may also have a role in psychostimulant-induced behaviors. However, further study is needed to explain the mechanism of its involvement.

Behavioral Sensitization

Repeated intermittent exposure to cocaine steadily increases the locomotor response to the drug (behavioral sensitization) (14), which is mostly coupled to a greater drug-induced dopamine efflux in the nucleus accumbens (93–95). However, a reduction (96) or non-augmentation (97) in the levels of the neurotransmitter in the nucleus accumbens was found simultaneously with this phenomenon. Behavioral sensitization to psychostimulants may well be mediated by converging extracellular signals, which give rise to a number of specific molecular and cellular events, such as activating the ERK/CREB signaling pathway, and enhancing GluR1 AMPA receptor cell surface expression and brain-derived neurotrophic factor/tyrosine kinase B (BDNF/TrkB) receptor signaling within the nucleus accumbens (11, 98). As mentioned previously, the enkephalinergic system increases mesoaccumbal dopamine neurotransmission (25). Likewise, pharmacological studies have demonstrated that MOPr and DOPr receptors contribute to amphetamine (99, 100) and cocaine-induced enhancement of dopamine levels in the nucleus accumbens (84, 86), and there is data of the anatomical selectivity of MOPr receptors within the ventral tegmental area-nucleus accumbens pathway in cocaine-induced reward and locomotor-stimulating effects (101). It has also been proposed that cocaine may cause the release of endogenous opioid peptides. These then activate MOPr within the nucleus accumbens and ventral tegmental area and modulate the drug-induced behavioral effects (102).

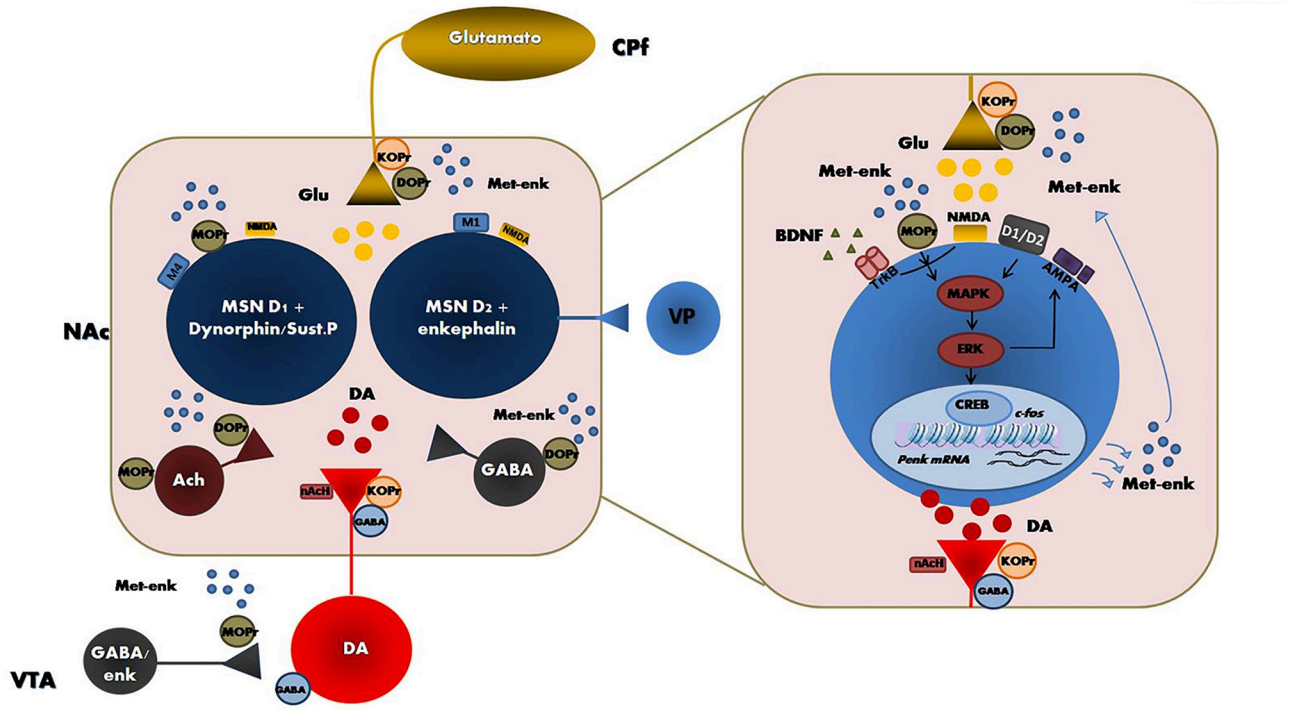

FIGURE 1 | (Left) Principal met-enkephalin target nucleus in the mesocorticolimbic circuit. Distribution of opioid receptors within the circuit network is also shown and reveals the modulation exerted by enkephalin on dopamine and glutamate transmission at this level. **(Right)** Persistent adaptations in the enkephalin content followed by psychostimulant treatment and the subsequent activation of MOPr could result in a feedback mechanism critical for the neuronal plasticity induced by these drugs in the NAc. Enkephalin transmission activation promotes the development of psychostimulant-induced long-term neurochemical and molecular changes in the NAc, such as increases in BDNF/TrkB, phospho-ERK2/CREB signaling activation and GluR1 AMPA cell surface expression. VP, ventral pallidum; VTA, ventral tegmentalarea; PfC, Prefrontal cortex; DA, dopamine; Ach, acetylcholine; Glu, glutamate; met-enk, methionine encephalin; GABA, γ-aminobutyric, M1 and M4: muscarinic acetylcholine receptors type 1 and 4, respectively, acetylcholine nAch, nicotinic acetylcholine receptor.

The role of MOPr and DOPr in the development and expression of psychostimulant sensitization has been shown pharmacologically. It has been reported that naloxone and naltrexone, non-selective opioid receptor antagonists, attenuate the development of sensitization to cocaine in rats (103) and mice (19, 88, 104). Naltrindole, a DOPr antagonist (87) and CTAP (D-Phe-cyc(Cys-Tyr-D-Trp-Arg-Thr-Pen)-Thr-NH2), a selective MOPr antagonist(105), also reduce cocaine-induced sensitization in rats. Similarly, the development (106) and expression (107, 108) of amphetamine-induced behavioral sensitization were reduced following non-selective opioid receptor administration. Additionally, there is evidence of ERK1/2 signaling stimulation induced by MOPr/DOPr activation in the striatum (109). However, there is data showing that acute morphine caused a reduction in ERK 1/2 levels in the nucleus accumbens (110, 111). Interestingly, although chronic morphine, a MOPr agonist, caused a reduction (110) or tolerance to morphine-induced ERK1/2 activation (111), naloxone-precipitated withdrawal in morphine-dependent animals induced a robust stimulation of ERK1/2 in the striatum (109, 112). Together this evidence demonstrates a prominent role for MOPr in the regulation of molecular events, associated not only with psychostimulant induced-behavioral sensitization, but also with the underlying opiate dependence. However, studies using MOPr mice seem to be inconclusive (88) or did not show a significant influence of this receptor in cocaine sensitization (113–115). It is important to note that these behavioral evaluations were performed after short-term cocaine withdrawal [(88): 10 × 15 mg/kg i.p./7days withdrawal; (113): 5 × 20 mg/kg i.p./dose–response experiment; (114): 6 × 15 mg/kg i.p./6 days withdrawal; (115): 20 mg/kg i.p./3 days withdrawal], possibly masking the role of MOPr in long-term behavioral effects induced by cocaine (116). There is also evidence that, after long- but not short-term withdrawal, naloxone blockade is observed of the expression of behavioral sensitization to psychostimulants [(108): amphetamine 1.5 mg/kg i.p./14 days abstinence]. Despite all these studies, and reports demonstrating that met-enkephalin and MOPr have a prominent role in the ventral tegmental area at the initial step of sensitization (101, 117, 118), there is still no explanation in the literature of the influence of enkephalin on the psychostimulant-induced neuronal plasticity underpinning long-term sensitization. Data from our lab demonstrate an essential role of enkephalin in the development of neuroadaptations in the nucleus accumbens leading to cocaine-induced psychomotor

sensitization (19). PENK knockout mice treated chronically with cocaine (9 days x 15 mg/kg) do not become sensitized to cocaine's properties stimulating locomotor activity and dopamine release in the nucleus accumbens 21 days after starting drug treatment. Additionally, the nucleus accumbens and dorsal striatum from PENK knockout mice showed no pivotal neuroadaptations such as the increase in phospho-TrkB receptor, phospho-ERK/CREB and GluR1 AMPA cell surface expression related to sensitized responses to cocaine. Consistent with these observations, full suppression of cocaine-induced behavioral and neuronal plasticity was observed in wild-type animals after naloxone pretreatment (1 mg/kg s.c. 15 min prior to cocaine injections). Reduced activity-dependent BDNF/TrkB signaling within the ventral tegmental area-nucleus accumbens circuit may attenuate the ability of cocaine to induce pathological changes in the nucleus accumbens that promote addiction (119, 120). Related with this, the lack of dopamine sensitization of a cocaine-induced increase in BDNF/TrkB signaling, identified in knockout- and naloxone-pretreated mice, strongly suggests that both enkephalin and BDNF have an important role in dopamine-sensitized behaviors. There is thus considerable evidence that the MOPr/endogenous enkephalin system has a prominent role in the establishment of long-term neuroadaptations within the nucleus accumbens underlying the expression of sensitization to cocaine.

Conditioned Place Preference

Pharmacological evidence clearly demonstrates the role of MOPr and DOPr in the modulation of psychostimulant-induced rewarding properties by studying the development of conditioned place preference (CPP); i.e., acquisition of associative learning between a context and the rewarding effects of a drug. In this sense, the establishment of CPP induced by amphetamine was prevented by the non-selective opioid receptor antagonist naloxone (0.02, 0.2 or 2.0 mg/kg s.c.), administered during the conditioning sessions (121). Similarly, naltrexone implants can attenuate cocaine-induced CPP in rats (122), although high doses of the opioid antagonist were required. This effect could be due to the non-selective opioid receptor antagonism. Naltrindole, a highly selective DOPr antagonist, blocked the acquisition of cocaine and amphetamine-induced-CPP in rats (123, 124), indicating that a selective opioid receptor antagonism can fully attenuate the reinforcing properties of cocaine. Furthermore, several studies have demonstrated that selective MOPr receptor antagonists attenuate psychostimulant-induced CPP. Specifically, systemic pretreatment with the selective MOPr type-1 receptor antagonist naloxonazine (125) and intracerebroventricular administration (i.c.v.) of CTAP paired with peripheral injections of cocaine (105), prevented the development of cocaine-induced CPP. It has also been demonstrated that animals pre-treated with CTAP into the nucleus accumbens core or rostral ventral tegmental area, but not into the caudal ventral tegmental area, caudate putamen or medial nucleus accumbens shell, during cocaine conditioning, showed an attenuation of the establishment of cocaine-induced CPP, demonstrating the involvement of mesolimbic MOPr in cocaine-induced reward (101). Although all this evidence has focused on the role of MOPr and DOPr in the development of psychostimulant-induced CPP, their involvement in the expression of this behavior cannot be ruled out. In line, Gerrits et al. (126) assessed the effect of naloxone (0.01–0.1 mg/kg s.c.) administered prior the conditioning test, demonstrating the role of opioid receptors in the expression of cocaine's motivational effects.

Despite this pharmacological evidence, the data regarding cocaine-induced CPP in MOPr knockout mice seems to be inconsistent. For example, the development of cocaine induced-CPP has been reported to be attenuated (113), unchanged (127) or induced after higher doses of cocaine compared to that used in wild-type littermates (128). The mechanisms that underlie these discrepancies in behavioral effects induced by cocaine in MOPr knockout mice are unknown. One possible explanation involves the different protocols of conditioning and cocaine doses used [(128): 4 days conditioning/5 or 10 mg/kg; (127): 3 days–two conditioning sessions per day/10 mg/kg; (113): 2 days–two conditioning sessions per day/5 or 10 mg/kg]. Another explanation could be the genetic background of the mice [(128): 129/Ola × C57BL F2; (113): congenic C57B F10; (127): hybrid 129SV/C57BL/6 F1] that may influence the differences in the process of acquisition of cocaine-induced CPP.

Although the evidence indirectly indicates a potential role of enkephalin in psychostimulant-induced CPP, its role in this process has not been addressed yet. Moreover, the molecular mechanism that underlies the MOPr/DOPr contribution to psychostimulant-induced CPP and the potential role of enkephalin has not been fully studied. Interestingly, there is data suggesting that morphine (a MOPr agonist)-induced CPP is associated with neuroadaptations similar to that observed following chronic psychostimulant treatment in important brain areas associated with drug addiction and those related to memory consolidation. Augmented phosphorylation levels of the GluR1 AMPAR subunit and ERK/CREB were observed in the hippocampus (129–131) as well as in the nucleus accumbens (132) and ventral tegmental area (130, 133) following morphine-induced conditioned behavior. This, together with data from our lab demonstrating that the PENK gene regulates cocaine-induced long-lasting molecular changes, such as enhancement in dopamine transmission, GluR1 AMPA receptor cell surface expression, ERK/CREB signaling pathway activation and modulation of TrkB/BDNF levels in the nucleus accumbens (19), suggests that enkephalin and the MOPr system may favor neuronal plasticity within the mesolimbic circuit that underlies psychostimulant and opiate-induced CPP. Further genetic (PENK knockout mice) and pharmacological studies need to be carried out to confirm this hypothesis and demonstrate the role of enkephalin in psychostimulant-induced CPP.

Psychostimulant Self-Administration

There is now considerable pharmacological evidence of the important role that MOPr plays in mediating the reinforcing effects of cocaine in a self-administration paradigm. GSK1521498 (0.1, 1, and 3 mg/kg s.c.), a MOPr antagonist, and naltrexone

administered at the same doses and route, reduced cocaine-seeking under a second-order schedule of reinforcement but did not affect cocaine self-administration under a simple fixed-ratio schedule (FR1) (134), indicating modulation of mechanisms regulating cocaine-seeking behavior rather than cocaine reinforcement (135). Additionally, GSK1521498 was more effective than naltrexone in reducing cocaine seeking, possibly because of different opioid receptor subtype selectivity. Similarly, low doses of naltrexone (0.1 mg/kg i.p.) showed no changes in cocaine self-administration (FR2 schedule), but attenuated cocaine- and cue-induced reinstatement of drug-seeking behavior administered 30 min prior to the reinstatement test (136). Consistently, the MOPr irreversible antagonist, beta-funaltrexamine, administered intra-ventral tegmental area or nucleus accumbens, had no effect on cocaine self-administration under a FR1 schedule of reinforcement. In contrast, MOPr blockade in both brain regions did attenuate the response to cocaine under a progressive ratio (PR) schedule, supporting the notion that MOPr within the mesolimbic system is involved in motivation to respond to cocaine (137). Regarding the role of MOPr, the selective MOPr antagonist CTAP (0.3 and 3 μg) administered in the ventral pallidum, but not in the nucleus accumbens or lateral hypothalamus, blocked the reinstatement of drug-seeking in rats that extinguished from cocaine self-administration (138). Given the GABA/enkephalin projection from the nucleus accumbens to the ventral pallidum, chronic cocaine may result in enkephalin release in this brain area, activating MOPr and eliciting cocaine relapse.

Data regarding the role of DOPr in mediating the rewarding effects of cocaine are conflicting. Naltrindole (0.03–3.0 mg/kg i.p. prior to self-administration session) did not alter the intake of cocaine (FR2 schedule of reinforcement) or the re-acquisition of cocaine self-administration (139). Similarly, a selective DOPr type-2 antagonist (administered i.c.v.) has been reported to have a slight effect on cocaine self-administration (FR1 schedule) (140). In contrast, there is data demonstrating that naltrindole (10 mg/kg i.p. 15 min prior FR1) reduced cocaine self-administration (141). These discrepancies regarding the role of DOPr in cocaine reinforcement may be due to the different types and doses of DOPr antagonist and cocaine-self-administration protocols. Importantly, none of these studies evaluated a possible role of DOPr within specific brain areas associated with cocaine reinforcement. DAMGO (1–3 ng) and DPDPE (300–3,000 ng), MOPr- and DOPr-selective ligands respectively, as well as β-endorphin (100–1,000 ng) and the enkephalinase inhibitor thiorphan (3–10 μg) microinjected into the nucleus accumbens, are sufficient to reinstate cocaine-seeking behavior in rats following extinction of cocaine self-administration (142). Thus, the stimulation of either accumbal MOPr or DOPr seems to be necessary to precipitate cocaine relapse.

Cocaine self-administration was reduced in MOPr knockout mice (143), suggesting a critical role of this receptor in cocaine reinforcement. In contrast, Gutiérrez-Cuesta et al. (144), found no changes in cocaine self-administration in this genotype. This discrepancy could be explained in the framework of the differences in experimental protocols used regarding cocaine dose and the time of the conditioning sessions, as in the study of Mathon et al. (143), which demonstrated significant differences in this genotype at high cocaine doses in shorter session times. Moreover, cocaine self-administration was reduced in both DOPr knockout and PENK knockout mice (144), mainly when animals were trained in FR3 and PR schedules. These findings suggest that DOPr and PENK are involved in the motivation to obtain cocaine, and the absence of these opioid components engenders an impaired response of cocaine self-administration, mainly when greater effort to obtain a reward is required. In addition, Gutiérrez-Cuesta et al. (144), demonstrated that cue-induced reinstatement of cocaine-seeking behavior was attenuated in both DOPr knockout and MOPr knockout. These data support previous pharmacological studies of Simmons and Self (142) addressing an important role of both receptors within the mesolimbic system in cocaine relapse. Consistent with these data, an enduring MOPr tone has been demonstrated within brain reward structures following extinction of cocaine self-administration (145), indicating that up regulating enkephalin levels may lead to long-lasting adaptations in response to repeated cocaine. Thus, all this evidence indicates that enkephalin, presumably acting on MOPr (although a role of DOPr cannot be ruled out) has a facilitatory influence on cocaine-induced behavioral and neuronal plasticity.

Importantly, the human literature shows encouraging evidence regarding the use of opioid antagonist in the treatment of psychostimulants relapse (146–148). Indeed, naltrexone (50 mg/day) administered in combination with relapse prevention therapy reduced cocaine use in a study of cocaine-addicted patients ($n = 85$). Thus, people receiving the combination of naltrexone (administered throughout 12 weeks) and relapse prevention therapy evidenced significantly reduced cocaine use than participants receiving other treatment combinations such us naltrexone alone or combined with drug counseling therapy (147). The same treatment protocol (naltrexone 50 mg/day during 12 weeks of medication and relapse prevention therapy) reduced amphetamine use as well as craving in amphetamine dependent patients ($n = 55$) (149). Additionally, naltrexone (50 mg/day) reduced the subjective effects of dexamphetamine (30 mg, oral) in amphetamine-dependent people ($n = 20$) (150). In constrast, patients who received oral naltrexone doses (0, 12.5, or 50 mg) before smoked cocaine (0, 12.5, 25, and 50 mg or placebo), or oral amphetamine (0, 10, and 20 mg or placebo) did not show alterations in positive subjective effects in cocaine users ($n = 12$) (146). This evidence suggests that this opioid antagonist did not alter positive subjective ratings after cocaine. Importantly, naltrexone did not alter physiological effects of psychostimulants in terms of cardiovascular function (146), cortisol levels and skin conductance (149, 150). Morever, naltrexone did significantly reduce craving for cocaine and tobacco during cocaine sessions (146) as well as amphetamine craving (149, 150). These data demonstrated that behavioral alterations observed in psychostimulants addiction, such us drug craving could be modulated by the endogenous opioid system.

It is important to address that in these studies, participants do not show evidence of any increase in the intake of other drugs of

abuse during naltrexone protocol therapy to compensate for the reduction in the drug consumption that is being evaluated. On the other hand, these studies were restricted to short periods of naltrexone treatment and long-term effects in these patients are unknown. Thus, future longitudinal studies are required in order to follow patients over prolonged periods of time.

Similar effects on opioid antagonists were observed in patients with cocaine/alcohol comorbidity (148, 151–153) or cocaine/opiate dependence (154).

In summary, several studies show promising results for psychostimulants addiction treatment, suggesting a potential role of naltrexone as an anti-craving therapy for this psychiatric disorder.

CONCLUSIONS AND FUTURE DIRECTIONS

This review emphasizes the important role of endogenous enkephalin during the development of the long-term neurobiological changes underlying psychostimulant addiction. It has been suggested that polymorphisms in genes encoding components of the endogenous opioid system are involved in predisposing to addiction to cocaine and opiates (155). Similarly, it is likely that genetic variations in the endogenous PENK gene (155–158) influence the development of behavioral and neurobiological adaptations in response to psychostimulant exposure, and thus modify vulnerability to psychostimulant addiction. This review also helps to understand how opioid antagonists can be effective in treating psychostimulant addiction (146, 147, 149), supporting their use as therapy for this disorder. Thus, the evidence presented in this review provides a basis for the development of new drug therapies for psychostimulant addiction based on specific modulation of the endogenous PENK system.

AUTHOR CONTRIBUTIONS

BMB wrote the article and performed the research related with the topic of this review; MPA, ASG and FAB contributed with the research related with the topic of this review and the writing of the review; LMC wrote the article, designed the research and provided the funds to perform papers related with the topic of this review.

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REFERENCES

1. Nestler EJ, Aghajanian GK. Molecular and cellular basis of addiction. *Science* (1997) **278** :58–63. doi: 10.1126/science.278.5335.58
2. White FJ, Kalivas PW. Neuroadaptations involved in amphetamine and cocaine addiction. *Drug Alcohol Depend.* (1998) **51**:141–53. doi: 10.1016/S0376-8716(98)00072-6
3. Kalivas PW. Cocaine and amphetamine-like psychostimulants: neurocircuitry and glutamate neuroplasticity. *Dialogues Clin Neurosci.* (2007) **9**:389–397.
4. Pierce RC, Wolf ME. Psychostimulant-induced neuroadaptations in nucleus accumbens AMPA receptor transmission. *Cold Spring Harbor Perspec Med*. (2013) **3**: a012021. doi: 10.1101/cshperspect.a012021
5. Steketee JD, Kalivas PW. Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. *Pharmacol Rev*. (2011) **63**:348–65. doi: 10.1124/pr.109.001933
6. Wolf ME, Sun X, Mangiavacchi S, Chao SZ. Psychomotor stimulants and neuronal plasticity. *Neuropharmacol.* (2004) **47**:61–79. doi: 10.1016/j.neuropharm.2004.07.006
7. Girault JA, Valjent E, Caboche J, Hervé D. ERK2: a logical AND gate critical for drug-induced plasticity? *Curr Opin Pharmacol.* (2007) **7**:77–85. doi: 10.1016/j.coph.2006.08.012
8. Valjent E, Corvol J-C, Pagès C, Besson, M-J, Maldonado R, Caboche J. Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *J Neurosci*. (2000) **20**:8701–9. doi: 10.1523/JNEUROSCI.20-23-08701.2000
9. Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol J-C, et al. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc Natl Acad Sci USA*. (2005) **102**:491–6. doi: 10.1073/pnas.0408305102
10. Boudreau AC, Wolf ME. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J Neurosci*. (2005) **25**:9144–51. doi: 10.1523/JNEUROSCI.2252-05.2005
11. Boudreau AC, Reimers JM, Milovanovic M, Wolf ME. Cell surface AMPA receptors in the rat nucleus accumbens increase during cocaine withdrawal but internalize after cocaine challenge in association with altered activation of mitogen-activated protein kinases. *J Neurosci* (2007) **27**:10621–35. doi: 10.1523/JNEUROSCI.2163-07.2007
12. McGinty JF, Shi XD, Schwendt M, Saylor A. Regulation of psychostimulant-induced signaling and gene expression in the striatum. *J Neurochem*. (2008) **104**:1440–9. doi: 10.1111/j.1471-4159.2008.05240.x
13. Robison AJ, Nestler EJ. Transcriptional and epigenetic mechanisms of addiction. *Nat Rev Neurosci*. (2011) **12**:623–37. doi: 10.1038/nrn3111
14. Robinson TE, Berridge KC. Incentive-sensitization and addiction. *Addiction* (2001) **96**:103–14. doi: 10.1046/j.1360-0443.2001.9611038.x
15. Scofield MD, Heinsbroek JA, Gipson CD, Kupchik YM, Spencer S, Smith ACW, et al. The nucleus accumbens: mechanisms of addiction across drug classes reflect the importance of glutamate homeostasis. *Pharmacol Rev*. (2016) **68**:816–71. doi: 10.1124/pr.116.012484
16. Le Merrer J, Becker JAJ, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev*. (2009) **89**:1379–412. doi: 10.1152/physrev.00005.2009
17. Mansour A, Fox CA, Akil H, and Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci*. (1995) **18**:22–9. doi: 10.1016/0166-2236(95)93946-U
18. Assis MA, Hansen C, Lux-Lantos V, Cancela LM. Sensitization to amphetamine occurs simultaneously at immune level and in met-enkephalin of the nucleus accumbens and spleen: an involved NMDA

glutamatergic mechanism. *Brain Behav Immun*. (2009) **23**:464–73. doi: 10.1016/j.bbi.2009.01.003
19. Mongi-Bragato B, Zamponi E, García-Keller C, Assis MA, Virgolini MB, Mascó DH, et al. Enkephalin is essential for the molecular and behavioral expression of cocaine sensitization. *Addic Biol*. (2016) **21**:326–38. doi: 10.1111/adb.12200
20. Daugé V, Kalivas PW, Duffy T, Roques BP. Effect of inhibiting enkephalin catabolism in the VTA on motor activity and extracellular dopamine. *Brain Res*. (1992) **599**:209–14. doi: 10.1016/0006-8993(92)90393-N
21. Dourmap N, Costentin J. Involvement of glutamate receptors in the striatal enkephalin-induced dopamine release. *Eur J Pharmacol*. (1994) **253**:R9–11. doi: 10.1016/0014-2999(94)90210-0
22. Dourmap N, Michael-Titus A, Costentin J. Local enkephalins tonically modulate dopamine release in the striatum: a microdialysi study. *Brain Res*. (1990) **524**:153–5. doi: 10.1016/0006-8993(90)90505-6
23. Hipólito L, Sánchez-Catalá, M. J., Zanolini I, Polache A, Granero L. Shell/core differences in mu-and delta-opioid receptor modulation of dopamine efflux in nucleus accumbens. *Neuropharmacol*. (2008) **55**:183–9. doi: 10.1016/j.neuropharm.2008.05.012
24. Kalivas PW, Duffy P. Effect of acute and daily neurotensin and enkephalin treatments on extracellular dopamine in the nucleus accumbens. *J Neurosci*. (1990b) **10**:2940–9. doi: 10.1523/JNEUROSCI.10-09-02940.1990
25. Latimer LG, Duffy P, Kalivas PW. Mu opioid receptor involvement in enkephalin activation of dopamine neurons in the ventral tegmental area. *J Pharmacol Exp Ther*. (1987) **241**:328–37.
26. Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Neurobiology* (1988) **85**:5274–8. doi: 10.1073/pnas.85.14.5274
27. Di Chiara G, Bassareo V, Fenu S, De Luca MA, Spina L, Cadoni C, et al. Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology* (2004) **47**:227–41. doi: 10.1016/j.neuropharm.2004.06.032
28. Hurd YL, Herkenham M. Influence of a single injection of cocaine, amphetamine or Gbr-12909 on Messenger-Rna expression of striatal neuropeptides. *Mol Brain Res*. (1992) **16**:97–104. doi: 10.1016/0169-328X(92)90198-K
29. Przewłocka B, Lason W. Adaptive changes in the proenkephalin and D2 dopamine receptor mRNA expression after chronic cocaine in the nucleus accumbens and striatum of the rat. *Eur Neuropsychopharmacol*. (1995) **5**:465–9. doi: 10.1016/0924-977X(95)80005-M
30. Smith AJW, McGinty JF. Acute amphetamine or methamphetamine alters opioid peptide mRNA expression in rat striatum. *Mol Brain Res*. (1994) **21**:359–62. doi: 10.1016/0169-328X(94)90268-2
31. Wang JQ, McGinty JF. D1 and D2 receptor regulation of preproenkephalin and preprodynorphin mRNA in rat striatum following acute injection of amphetamine or methamphetamine. *Synapse* (1996) **22**:114–22. doi: 10.1002/(SICI)1098-2396(199602)22:2<114::AID-SYN4>3.0.CO;2-G
32. Wang JQ, McGinty JF. Intrastriatal injection of a muscarinic receptor agonist and antagonist regulates striatal neuropeptide mRNA expression in normal and amphetamine-treated rats. *Brain Res*. (1997a) **748**:62–70. doi: 10.1016/S0006-8993(96)01244-9
33. Wang JQ, McGinty JF. The Full D1 dopamine receptor agonist SKF-82958 induces neuropeptide mRNA in the normosensitive striatum of rats: regulation of D1/D2 interactions by muscarinic receptors. *J Pharmacol Exp Ther*. (1997b) **281**:972–82.
34. Mathieu-Kia A-M, Besson M-J. Repeated administration of cocaine, nicotine and ethanol: effects on preprodynorphin, preprotachykinin A and preproenkephalin mRNA expression in the dorsal and the ventral striatum of the rat. *Mol Brain Res*. (1998) **54**:141–51. doi: 10.1016/S0169-328X(97)00338-0
35. Steiner H, Gerfen C. Cocaine-induced c-fos messenger RNA is inversely related to dynorphin expression in striatum. *J Neurosci*. (1993) **13**:5066–81. doi: 10.1523/JNEUROSCI.13-12-05066.1993
36. Zhang Y, Schlussman SD, Butelman ER, Ho A, Kreek MJ. Effects of withdrawal from chronic escalating-dose binge cocaine on conditioned place preference to cocaine and striatal preproenkephalin mRNA in C57BL/6J mice. *Neuropharmacology* (2012) **63**:322–9. doi: 10.1016/j.neuropharm.2012.03.021
37. Spangler R, Zhou Y, Maggos CE, Schlussman SD, Ho A, Kreek MJ. Prodynorphin, proenkephalin and κ opioid receptor mRNA responses to acute "binge" cocaine. *Mol Brain Res*. (1997) **44**:139–42.
38. Daunais JB, McGinty JF. Acute and chronic cocaine administration differentially alters striatal opioid and nuclear transcription factor mRNAs. *Synapse* (1994) **18**:35–45. doi: 10.1002/syn.890180106
39. Branch AD, Unterwald EM, Lee SE, Kreek MJ. Quantitation of preproenkephalin mRNA levels in brain regions from male Fischer rats following chronic cocaine treatment using a recently developed solution hybridization assay. *Mol Brain Res*. (1992) **14**:231–8. doi: 10.1016/0169-328X(92)90178-E
40. Bailey A, Yuferov V, Bendor J, Schlussman SD, Zhou Y, Ho A, et al. Immediate withdrawal from chronic "binge" cocaine administration increases μ-opioid receptor mRNA levels in rat frontal cortex. *Mol Brain Res*. (2005) **137**:258–62. doi: 10.1016/j.molbrainres.2005.02.017
41. Turchan J, Maj M, Przewocka B, Przewocki R. Effect of cocaine and amphetamine on biosynthesis of proenkephalin and prodynorphin in some regions of the rat limbic system. *Pol J Pharmacol*. (2002) **54**:367–72.
42. Ziółkowska B, Stefanski R, Mierzejewski P, Zapart G, Kostowski W, Przewłocki R. Contingency does not contribute to the effects of cocaine self-administration on prodynorphin and proenkephalin gene expression in the rat forebrain. *Brain Res*. (2006) **1069**:1–9. doi: 10.1016/j.brainres.2005.11.042
43. Crespo JA, Manzanares J, Oliva JM, Corchero J, Palomo T, Ambrosio E. Extinction of cocaine self-administration produces a differential time-related regulation of proenkephalin gene expression in rat brain. *Neuropsychopharmacology* (2001) **25**:185–94. doi: 10.1016/S0893-133X(01)00221-4
44. Gonzalez-Nicolini V, McGinty JF. Gene expression profile from the striatum of amphetamine-treated rats: a cDNA array and *in situ* hybridization histochemical study. *Gene Expr Patterns* (2002) **1**:193–8. doi: 10.1016/S1567-133X(02)00017-0
45. Gonzalez-Nicolini MV, Berglind W, Cole KS, Keogh CL, Mcginty JF. Local mu and deltaopioid receptors regulate amphetamine- induced behavior and neuropeptide mrna in the striatum. *Neuroscience* (2003) **121**:387–98. doi: 10.1016/S0306-4522(03)00488-3
46. Morales-Mulia M, Panayi F, Lambás-Señas L, Scarna H, Méndez M. Changes in Proenkephalin mRNA expression in forebrain areas after amphetamine-induced behavioural sensitization. *Pharmacol Biochem Behav*. (2007) **87**:232–40. doi: 10.1016/j.pbb.2007.04.019
47. Shi X, McGinty JF. Extracellular signal-regulated mitogen-activated protein kinase inhibitors decrease amphetamine-induced behavior and neuropeptide gene expression in the striatum. *Neuroscience* (2006) **138**:1289–98. doi: 10.1016/j.neuroscience.2005.12.024
48. Wang JQ, McGinty JF. Alterations in striatal zif/268, preprodynorphin and preproenkephalin mRNA expression induced by repeated amphetamine administration in rats. *Brain Res*. (1995) **673**:262–74. doi: 10.1016/0006-8993(94)01422-E
49. Assis MA, Collino C, Figuerola Mde L, Sotomayor C, Cancela LM. Amphetamine triggers an increase in met-enkephalin simultaneously in brain areas and immune cells. *J Neuroimmunol*. (2006) **178**:62–75. doi: 10.1016/j.jneuroim.2006.05.009
50. Assis MA, Valdomero A, García-Keller C, Sotomayor C, Cancela LM. Decrease of lymphoproliferative response by amphetamine is mediated by dopamine from the nucleus accumbens: influence on splenic met-enkephalin levels. *Brain Behav Immun*. (2011) **25**:647–57. doi: 10.1016/j.bbi.2011.01.001
51. Liste I, Rodriguez-Pallares J, Caruncho HJ, Labandeira-Garcia JL. Locomotor-activity-induced changes in striatal levels of preprotachykinin and preproenkephalin mRNA. Regulation by the dopaminergic and glutamatergic systems. *Mol Brain Res*. (1999) **70**:74–83. doi: 10.1016/S0169-328X(99)00140-0
52. Mao L, Wang JQ. Contribution of ionotropic glutamate receptors to acute amphetamine- stimulated preproenkephalin mRNA expression in the rat striatum *in vivo*. *Neurosci Lett*. (2003) **346**:17–20. doi: 10.1016/S0304-3940(03)00542-1
53. Gerfen C, Engber T, Mahan L, Susel Z, Chase T, Monsma F, et al. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* (1990) **250**:1429–32. doi: 10.1126/science.2147780

54. Le Moine C, Normand E, Guitteny AF, Fouquet B, Teoulet R, Bloch B. Dopamine receptor gene expression by enkephalin neurons in rat forebrain (nigrostriatal pathway/D2 receptor/preproenkephalin A/*in situ* hybridization). *Neurobiology* (1990) **87**:230–4.
55. Le Moine C, Bloch B. D1 and D2 dopamine receptor gene expression in the rat striatum: Sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAS in distinct neuronal populations of the dorsal and ventral striatum. *J Com Neurol*. (1995) **355**:418–26. doi: 10.1002/cne.903550308
56. Damsma G, Robertson GS, Tham CS, Fibiger HC. Dopaminergic regulation of striatal acetylcholine release: importance of D1 and N-methyl-D-aspartate receptors. *J Pharmacol Exp Ther*. (1991) **259**:1064–72.
57. Guix T, Hurd YL, Ungerstedt U. Amphetamine enhances extracellular concentrations of dopamine and acetylcholine in dorsolateral striatum and nucleus accumbens of freely moving rats. *Neurosci Lett*. (1992) **138**:137–40. doi: 10.1016/0304-3940(92)90490-X
58. Tzaferis JA, McGinty JF. Kappa opioid receptor stimulation decreases amphetamine-induced behavior and neuropeptide mRNA expression in the striatum. *Mol Brain Res*. (2001) **93**:27–35. doi: 10.1016/S0169-328X(01)00178-4
59. Gray AM, Rawls SM, Shippenberg TS, McGinty JF. The kappa-opioid agonist, U-69593, decreases acute amphetamine-evoked behaviors and calcium-dependent dialysate levels of dopamine and glutamate in the ventral striatum. *J Neurochem.* (1999) **73**:1066–74. doi: 10.1046/j.1471-4159.1999.0731066.x
60. Pierce RC, Bell K, Duffy P, Kalivas PW. Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. *J Neurosci*. (1996) **16**:1550–60. doi: 10.1523/JNEUROSCI.16-04-01550.1996
61. Vanderschuren LJ, Kalivas PW. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology* (2000) **151**:99–120. doi: 10.1007/s002130000493
62. Del Arco A, González-Mora JL, Armas VR, Mora F. Amphetamine increases the extracellular concentration of glutamate in striatum of the awake rat: involvement of high affinity transporter mechanisms. *Neuropharmacology* (1999) **38**:943–54. doi: 10.1016/S0028-3908(99)00043-X
63. Reid MS, Hsu K, Berger SP. Cocaine and amphetamine preferentially stimulate glutamate release in the limbic system: studies on the involvement of dopamine. *Synapse* (1997) **27**:95–105. doi: 10.1002/(SICI)1098-2396(199710)27:2<95::AID-SYN1>3.0.CO;2-6
64. Wolf ME, Xue CJ. Amphetamine and D1 dopamine receptor agonists produce biphasic effects on glutamate efflux in rat ventral tegmental area: modification by repeated amphetamine administration. *J Neurochem*. (1998) **70**:198–209. doi: 10.1046/j.1471-4159.1998.70010198.x
65. Darracq L, Drouin C, Blanc G, Glowinski J, Tassin J-P. Stimulation of metabotropic but not ionotropic glutamatergic receptors in the nucleus accumbens is required for the d-amphetamine-induced release of functional dopamine. *Neuroscience* (2001) **103**:395–403. doi: 10.1016/S0306-4522(00)00578-9
66. Kalivas PW, Duffy P. Dopamine regulation of extracellular glutamate in the nucleus accumbens. *Brain Res*. (1997) **761**:173–7. doi: 10.1016/S0006-8993(97)00464-2
67. Weihmuller FB, O'Dell SJ, Cole BN, Marshall JF. MK-801 attenuates the dopamine-releasing but not the behavioral effects of methamphetamine: an *in vivo* microdialysis study. *Brain Res*. (1991) **549**: 230–5.
68. Bickerdike MJ, Abercrombie ED. Enhanced acetylcholine release in striatum after chronic amphetamine is NMDA-dependent. *Neuroreport* (1999) **10**:77–80. doi: 10.1097/00001756-199901180-00015
69. Knauber J, Kischka U, Roth M, Schmidt WJ, Hennerici M, Fassbender K. Modulation of striatal acetylcholine concentrations by NMDA and the competitive NMDA receptor-antagonist AP-5: an *in vivo* microdialysis study. *J Neural Transm*. (1999) **106**:35–45. doi: 10.1007/s007020050139
70. Bacher B, Wang X, Schulz S, Höllt V. Induction of proenkephalin gene expression in cultured bovine chromaffin cells is dependent on protein synthesis of AP-1 proteins. *J Neurochem*. (1996) **66**:2264–71. doi: 10.1046/j.1471-4159.1996.66062264.x
71. Cole RL, Konradi C, Douglass J, Hyman SE. Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron* (1995) **14**:813–23. doi: 10.1016/0896-6273(95)90225-2
72. Kobierski LA, Wong AE, Srivastava S, Borsook D, Hyman SE. Cyclic AMP-dependent activation of the proenkephalin gene requires phosphorylation of CREB at serine-133 and a src-related kinase. *J Neurochem*. (1999) **73**:129–38. doi: 10.1046/j.1471-4159.1999.0730129.x
73. Konradi C, Cole RL, Heckers S, Hymanls SE. Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *J Neurosci*. (1994) **74**:5623–6634. doi: 10.1523/JNEUROSCI.14-09-05623.1994
74. Monnier D, Loeffler JP. Pituitary adenylate cyclase-activating polypeptide stimulates proenkephalin gene transcription through AP1- and CREB-dependent mechanisms. *DNA Cell Biol*. (1998) **17**:151–9. doi: 10.1089/dna.1998.17.151
75. Cho DI, Quan W, Oak MH, Choi HJ, Lee KY, Kim KM. Functional interaction between dopamine receptor subtypes for the regulation of c-fos expression. *Biochem Biophys Res Commun*. (2007) **357**:1113–8. doi: 10.1016/j.bbrc.2007.04.066
76. Kashihara K, Ishihara T, Akiyama K, Abe K. D1/D2 receptor synergism on CREB DNA-binding activities in the caudate-putamen of rat. *Neurol Res*. (1999) **21**:781–4. doi: 10.1080/01616412.1999.11741014
77. Sgambato V, Pagès C, Rogard M, Besson MJ, Caboche J. Extracellular Signal-Regulated Kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. *J Neurosci*. (1998) **18**:8814–25. doi: 10.1523/JNEUROSCI.18-21-08814.1998
78. Vanhoutte P, Barnier JV, Guibert B, Pagès C, Besson MJ, Hipskind RA, et al. Glutamate induces phosphorylation of Elk-1 and CREB, along with c *-fos* activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. *Mol Cell Biol*. (1999) **19**:136–46. doi: 10.1128/MCB.19.1.136
79. Choe ES, McGinty JF. Cyclic AMP, and mitogen-activated protein kinases are required for glutamate-dependent cyclic AMP response element binding protein and Elk-1 phosphorylation in the dorsal striatum *in vivo*. *J Neurochem*. (2001) **76**:401–12. doi: 10.1046/j.1471-4159.2001.00051.x
80. Carlezon W Jr, Duman R, Nestler E. The many faces of CREB. *Trends Neurosci*. (2005) **28**:436–45. doi: 10.1016/j.tins.2005.06.005
81. Johnson SW, North RA. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci*. (1992) **12**:483–8. doi: 10.1523/JNEUROSCI.12-02-00483.1992
82. Spanagel R, Herz A, Shippenberg TS. Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway (mirodialysis/nucleus accumbens/dopamine release and metabolism/opiate dependence). *Pharmacology* (1992) **89**:2046–50.
83. Rawls SM, McGinty JF. Delta opioid receptors regulate calcium-dependent, amphetamine-evoked glutamate levels in the rat striatum: an *in vivo* microdialysis study. *Brain Res*. (2000) **861**:296–304. doi: 10.1016/S0006-8993(00)02030-8
84. Shippenberg TS, Chefer VI. (2003) Opioid modulation of psychomotor stimulant effects. In: Maldonado R, editor. *Molecular Biology of Drug Addiction*. Totowa, NJ:Humana Press, 107–32.
85. Shippenberg TS, Elmer GI. The neurobiology of opiate reinforcement. *Crit Rev Neurobiol* (1988) **12**:267–303.
86. Van Ree JM, Niesink RJ, Van Wolfswinkel L, Ramsey NF, Kornet MM, Vanderschuren LJ, et al. Endogenous opioids and reward. *Eur J Pharmacol*. (2000) **405**:89–101. doi: 10.1016/S0014-2999(00)00544-6
87. Heidbreder C, Shoaib M, Shippenberg TS. Differential role of 6-opioid receptors in the development and expression of behavioral sensitization to cocaine. *Eur J Pharmacol*. (1996) **298**:207–16. doi: 10.1016/0014-2999(95)00815-2
88. Hummel M, Ansonoff MA, Pintar JE, Unterwald EM. Genetic and pharmacological manipulation of mu opioid receptors in mice reveals a differential effect on behavioral sensitization to cocaine. *Neuroscience* (2004) **125**:211–20. doi: 10.1016/j.neuroscience.2004.01.025
89. Racz I, Schürmann B, Karpushova A, Reuter M, Cichon S, Montag C, et al. The opioid peptides enkephalin and beta-endorphin in alcohol dependence. *Biol Psychiatry* (2008) **64**:989–97. doi: 10.1016/j.biopsych.2008.05.008

90. Berrendero F. Nicotine-induced antinociception, rewarding effects, and physical dependence are decreased in mice lacking the preproenkephalin gene. *J Neurosci*. (2005) **25**:1103–12. doi: 10.1523/JNEUROSCI.3008-04.2005
91. Gianoulakis C. Endogenous opioids and excessive alcohol consumption. *J Psychiatr Neurosci*. (1993) **18**:148–56.
92. Marinelli PW, Bai L, Quirion R, Gianoulakis C. A microdialysis profile of Met-enkephalin release in the rat nucleus accumbens following alcohol administration. *Alcohol Clin Exp Res*. (2005) **29**:1821–8. doi: 10.1097/01.alc.0000183008.62955.2e
93. Kalivas PW, Duffy P. Effect of acute and daily cocaine treatment on extracellular dopamine in the nucleus accumbens. *Synapse* (1990a) **5**:48–58. doi: 10.1002/syn.890050104
94. Kalivas PW, Stewart J. Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain Res Rev*. (1991) **16**:223–44. doi: 10.1016/0165-0173(91)90007-U
95. Kalivas PW, Duffy P. Time course of extracellular dopamine and behavioral sensitization to cocaine. I. Dopamine Axon Terminals. *J Neurosci*. (1993) **13**:266–75. doi: 10.1523/JNEUROSCI.13-01-00266.1993
96. Segal DS, Kuczenski R. Repeated cocaine administration induces behavioral sensitization and corresponding decreased extracellular dopamine responses in caudate and accumbens. *Brain Res*. (1992) **577**:351–5. doi: 10.1016/0006-8993(92)90297-M
97. Hurd YL, Weiss F, Koob GF, And N-E, Ungerstedt U. Cocaine reinforcement and extracellular dopamine overflow in rat nucleus accumbens: an *in vivo* microdialysis study. *Brain Res*. (1989) **498**:199–203. doi: 10.1016/0006-8993(89)90422-8
98. Crooks KR, Kleven DT, Rodriguiz RM, Wetsel WC, McNamara JO. TrkB signaling is required for behavioral sensitization and conditioned place preference induced by a single injection of cocaine. *Neuropharmacology* (2010) **58**:1067–77. doi: 10.1016/j.neuropharm.2010.01.014
99. Bosse KE, Jutkiewicz EM, Gnegy ME, Traynor JR. The selective delta opioid agonist SNC80 enhances amphetamine-mediated efflux of dopamine from rat striatum. *Neuropharmacology* (2008) **55**:755–62. doi: 10.1016/j.neuropharm.2008.06.017
100. Schad CA, Justice JB, Holtzman SG. Differential effects of δ- and μ-Opioid receptor antagonists on the amphetamine-induced increase in extracellular dopamine in striatum and nucleus accumbens. *J Neurochem*. (1996) **67**:2292–9. doi: 10.1046/j.1471-4159.1996.67062292.x
101. Soderman AR, Unterwald EM. Cocaine reward and hyperactivity in the rat: sites of mu opioid receptor modulation. *Neuroscience* (2008) **154**:1506–16. doi: 10.1016/j.neuroscience.2008.04.063
102. Soderman AR, Unterwald EM. Cocaine-induced mu opioid receptor occupancy within the striatum is mediated by dopamine D2 receptors. *Brain Res*. (2009) **1296**:63–71. doi: 10.1016/j.brainres.2009.08.035
103. Sala M, Braida D, Colombo M, Groppetti A, Sacco S, Gori E, et al. Behavioral and biochemical evidence of opioidergic involvement in cocaine sensitization. *J Pharmacol Exp Ther*. (1995) **274**:450–7.
104. Kim HS, Park WK, Jang CG, Oh KW, Kong JY, Oh S, et al. Blockade by naloxone of cocaine-induced hyperactivity, reverse tolerance and conditioned place preference in mice. *Behav Brain Res*. (1997) **85**:37–46. doi: 10.1016/S0166-4328(96)00162-3
105. Schroeder JA, Hummel M, Simpson AD, Sheikh R, Soderman AR, Unterwald EM. A role for mu opioid receptors in cocaine-induced activity, sensitization, and reward in the rat. *Psychopharmacology* (2007) **195**:265–72. doi: 10.1007/s00213-007-0883-z
106. Balcells-Olivero M, Vezina P. Effects of naltrexone on amphetamine-induced locomotion and rearing: acute and repeated injections. *Psychopharmacology* (1997) **131**:230–8. doi: 10.1007/s002130050288
107. Häggkvist J, Björkholm C, Steensland P, Lindholm S, Franck J, Schilström B. Naltrexone attenuates amphetamine-induced locomotor sensitization in the rat. *Addict Biol*. (2011) **16**:20–9. doi: 10.1111/j.1369-1600.2009.00199.x
108. Magendzo K, Bustos G. Expression of amphetamine-induced behavioral sensitization after short-and long-term withdrawal periods: participation of m-and d-opioid receptors. *Neuropsychopharmacology* (2003) **28**:468–77. doi: 10.1038/sj.npp.1300063
109. Asensio VJ, Miralles A, García-Sevilla JA. Stimulation of mitogen-activated protein kinase kinases (MEK1/2) by μ-, δ- and κ-opioid receptor agonists in the rat brain: Regulation by chronic morphine and opioid withdrawal. *Eur J Pharmacol*. (2006) **539**:49–56. doi: 10.1016/j.ejphar.2006.04.001
110. Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, Kaith D Jr, et al. Brain region-specific mechanisms for acute morphine- induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *J Neurosci.* (2003) **23**:8360–9. doi: 10.1523/JNEUROSCI.23-23-08360.2003
111. Muller DL, Unterwald EM. *In vivo* regulation of extracellular signal-regulated protein kinase (ERK) and Protein Kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther*. (2004) **10**:774–82. doi: 10.1124/jpet.104.066548
112. Schulz S, Höllt V. Opioid withdrawal activates MAP kinase in locus coeruleus neurons in morphine-dependent rats *in vivo*. *Eur J Neurosci.* (1998) **10**:1196–201. doi: 10.1046/j.1460-9568.1998.00103.x
113. Hall FS, Goeb M, Li X-F, Sora I, Uhl GR. A-Opioid receptor knockout mice display reduced cocaine conditioned place preference but enhanced sensitization of cocaine-induced locomotion. *Mol Brain Res*. (2004) **121**:123–30. doi: 10.1016/j.molbrainres.2003.10.024
114. Yoo JH, Yang EM, Lee SY, Loh HH, Ho IK, Jang CG. Differential effects of morphine and cocaine on locomotor activity and sensitization in m-opioid receptor knockout mice. *Neurosci Lett*. (2003) **344**:37–40. doi: 10.1016/S0304-3940(03)00410-5
115. Lesscher HM, Hordijk M, Bondar NP, Alekseyenko OV, Burbach JP, van Ree JM, et al. Mu-opioid receptors are not involved in acute cocaine-induced locomotor activity nor in development of cocaine-induced behavioral sensitization in mice. *Neuropsychopharmacology* (2005) **30**:278–85. doi: 10.1038/sj.npp.1300529
116. Yoo JH, Kitchen I, Bailey A. The endogenous opioid system in cocaine addiction: what lessons have opioid peptide and receptor knockout mice taught us? *Br J Pharmacol*. (2012) **166**:1993–2014. doi: 10.1111/j.1476-5381.2012.01952.x
117. DuMars LA, Rodger LD, Kalivas PW. Behavioral cross-sensitization between cocaine and enkephalin in the A 10 dopamine region. *Behav Brain Res*. (1988) **27**:87–91. doi: 10.1016/0166-4328(88)90111-8
118. Kalivas PW, Widerlöv E, Stanley D, Breese G, Prange AJ. Enkephalin action on the mesolimbic system: a dopamine-dependent and a dopamine-independent increase in locomotor activity. *J Pharmacol Exp Ther*. (1983) **227**:229–37.
119. Bahi A, Boyer F, Chandrasekar V, Dreyer J-L. Role of accumbens BDNF and TrkB in cocaine-induced psychomotor sensitization, conditioned-place preference, and reinstatement in rats. *Psychopharmacology* (2008) **199**:169–82. doi: 10.1007/s00213-008-1164-1
120. Lobo MK, Iii HEC, Chaudhury D, Friedman AK, Sun H, Damez-werno D, et al. Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. *Science* (2011) **330**:385–90. doi: 10.1126/science.1188472
121. Trujillo KA, Belluzzi JD, Stein L. Naloxone blockade of amphetamine place preference conditioning. *Psychopharmacology* (1991) **104**:265–74. doi: 10.1007/BF02244190
122. Mitchem LD, Kruschel CK, Dallman E, Anders KA, Czapiga M, Panos JJ, et al. The effects of the naltrexone implant on rodent social interactions and cocaine-induced conditioned place preference. *Pharmacol Biochem Behav*. (1999) **62**:97–102. doi: 10.1016/S0091-3057(98)00150-6
123. Menkens K, Bilsky EJ, Wild KD, Portoghese PS, Reid LD, Porreca F. Cocaine place preference is blocked by the 6-opioid receptor antagonist, naltrindole. *Eur J Pharmacol*. (1992) **219**:345–6. doi: 10.1016/0014-2999(92)90319-Y
124. Suzuki T, Mori T, Tsuji M, Misawa M, Nagase H. The role of δ-opioid receptor subtypes in cocaine- and methamphetamine-induced place preferences. *Life Sci*. (1994) **55**:PL339–4. doi: 10.1016/0024-3205(94)00774-8
125. Rademacher DJ, Steinpreis RE. Effects of the selective mu(1)-opioid receptor antagonist, naloxonazine, on cocaine-induced conditioned place preference and locomotor behavior in rats. *Neurosci Lett*. (2002) **332**:159–62. doi: 10.1016/S0304-3940(02)00950-3
126. Gerrits MA, Patkina N, Zvartau EE, van Ree JM. Opioid blockade attenuates acquisition and expression of cocaine-induced place preference conditioning in rats.*Psychopharmacology* (1995) **119**:92–8. doi: 10.1007/BF02246059
127. Contarino A, Picetti R, Matthes HW, Koob GF, Kieffer BL, Gold LH. Lack of reward and locomotor stimulation induced by heroin in

A-opioid receptor-deficient mice. *Eur J Pharmacol*. (2002) **446**:103–9. doi: 10.1016/S0014-2999(02)01812-5

128. Becker A, Grecksch G, Kraus J, Loh H, Schroeder H, Höllt V. Rewarding effects of ethanol and cocaine in μ opioid receptor-deficient mice. *Naunyn Schmiedebergs Arch Pharmacol*. (2002) **365**:296–302. doi: 10.1007/s00210-002-0533-2
129. Billa SK, Sinha N, Rudrabhatla SR, Morón JA. Extinction of morphine-dependent conditioned behavior is associated with increased phosphorylation of the GluR1 subunit of AMPA receptors at hippocampal synapses. *Eur J Neurosci*. (2009) **29**:55–64. doi: 10.1111/j.1460-9568.2008.06560.x
130. Morón JA, Gullapalli S, Taylor C, Gupta A, Gomes I, Devi LA. Modulation of opiate-related signaling molecules in morphine-dependent conditioned behavior: conditioned place preference to morphine induces CREB phosphorylation. *Neuropsychopharmacology* (2010) **35**:955–66. doi: 10.1038/npp.2009.199
131. Pahlevani P, Fatahi Z, Moradi M, Haghparast A. Morphine-induced conditioned place preference and the alterations of p-ERK, p-CREB and c-fos levels in hypothalamus and hippocampus: the effects of physical stress. *Cell Mol Biol*. (2014) **60**:48–55.
132. Haghparast A, Fatahi Z, Alamdary SZ, Reisi Z, Khodagholi F. Changes in the levels of p-ERK, p-CREB, and c-fos in rat mesocorticolimbic dopaminergic system after morphine-induced conditioned place preference: the role of acute and subchronic stress. *Cell Mol Neurobiol*. (2014) **34**:277–88. doi: 10.1007/s10571-013-0011-z
133. Lin X, Wang Q, Ji A, Yu LC. Role of MEK-ERK pathway in morphine-induced conditioned place preference in ventral tegmental area of rats. *J Neurosci Res*. (2010) **88**:1595–604. doi: 10.1002/jnr.22326
134. Giuliano C, Robbins TW, Wille DR, Bullmore ET, Everitt BJ. Attenuation of cocaine and heroin seeking by μ-opioid receptor antagonism. *Psychopharmacology* (2013) **227**:137–47. doi: 10.1007/s00213-012-2949-9
135. Burattini C, Burbassi S, Aicardi G, Cervo L. Effects of naltrexone on cocaine- and sucrose-seeking behaviour in response to associated stimuli in rats. *Int J Neuropsychopharmacol*. (2008) **11**:103–9. doi: 10.1017/S1461145707007705
136. Sushchyk S, Xi ZX, Wang JB. (2016). Combination of levo-tetrahydropalmatine and low dose naltrexone: a promising treatment for prevention of cocaine relapse. *J Pharmacol Exp Ther*. (2016) **357**:248–57. doi: 10.1124/jpet.115.229542
137. Ward SJ, Martin TJ, Roberts DCS. Beta-funaltrexamine affects cocaine self-administration in rats responding on a progressive ratio schedule of reinforcement. *Pharmacol Biochem Behav*. (2003) **75**:301–7. doi: 10.1016/S0091-3057(03)00087-X
138. Tang X-C, McFarland K, Cagle S, Kalivas PW. Cocaine-induced reinstatement requires endogenous stimulation of μ-opioid receptors in the ventral pallidum. *J Neurosci*. (2005) **25**:4512–20. doi: 10.1523/JNEUROSCI.0685-05.2005
139. de Vries TJ, Babovic-Vuksanovic D, Elmer G, Shippenberg TS. Lack of involvement of delta-opioid receptors in mediating the rewarding effects of cocaine. *Psychopharmacology* (1995) **120**:442–8. doi: 10.1007/BF02245816
140. Martin TJ, Kim SA, Cannon DG, Sizemore GM, Bian D, Porreca F, et al. Antagonism of delta(2)-Opioid Receptors by Naltrindole-5'- isothiocyanate attenuates heroin self-administration but not antinociception in rats. *J Pharmacol Exp Ther*. (2000) **294**:975–82.
141. Reid LD, Glick SD, Menkens KA, French ED, Bilsky EJand Porreca F. Cocaine self-administration and naltrindole, a delta-selective opioid antagonist. *Neuroreport* (1995) **6**:1409–12. doi: 10.1097/00001756-199507100-00012
142. Simmons D, Self DW. Role of Mu- and delta-opioid receptors in the nucleus accumbens in cocaine-seeking behavior. *Neuropsychopharmacology* (2009) **34**:1946–57. doi: 10.1038/npp.2009.28
143. Mathon DS, Lesscher HM, Gerrits MA, Kamal A, Pintar JE, Schuller AG, et al. Increased gabaergic input to ventral tegmental area dopaminergic neurons associated with decreased cocaine reinforcement in mu-opioid receptor knockout mice. *Neuroscience* (2005) **130**:359–67. doi: 10.1016/j.neuroscience.2004.10.002
144. Gutiérrez-Cuesta J, Burokas A, Mancino S, Kummer S, Martín-García E, Maldonado R. Effects of genetic deletion of endogenous opioid system components on the reinstatement of cocaine-seeking behavior in mice. *Neuropsychopharmacology* (2014) **39149**:2974–88. doi: 10.1038/npp.2014.149
145. Kupchik YM, Scofield MD, Rice KC, Cheng K, Roques BP, Kalivas PW. (2014). Cocaine dysregulates opioid gating of GABA neurotransmission in the ventral pallidum. *J Neurosci*. (2014) **34**:1057–66. doi: 10.1523/JNEUROSCI.4336-13.2014
146. Comer SD, Mogali S, Saccone PA, Askalsky P, Martinez D, Walker EA, et al. Effects of acute oral naltrexone on the subjective and physiological effects of oral D-amphetamine and smoked cocaine in cocaine abusers. *Neuropsychopharmacology* (2013) **38143**:2427–38. doi: 10.1038/npp.2013.143
147. Schmitz JM, Stotts AL, Rhoades HM, Grabowski J. Naltrexone and relapse prevention treatment for cocaine-dependent patients. *Addic Behav*. (2001) **26**:167–80. doi: 10.1016/S0306-4603(00)00098-8
148. Schmitz JM, Lindsay JA, Green CE, Herin DV, Stotts AL, Moeller FG. High-dose naltrexone therapy for cocaine-alcohol dependence. *Am J Addic*. (2009) **18**:356–62. doi: 10.3109/10550490903077929
149. Jayaram-Lindström N, Hammarberg A, Beck O, Franck J. Naltrexone for the treatment of amphetamine dependence: a randomized, placebo-controlled trial. *Am J Psychiatry* (2008a) **165**:1442–8. doi: 10.1176/appi.ajp.2008.08020304
150. Jayaram-Lindström N, Konstenius M, Eksborg S, Beck O, Hammarberg A, Franck J. Naltrexone attenuates the subjective effects of amphetamine in patients with amphetamine dependence. *Neuropsychopharmacology* (2008b) **33**:1856–63. doi: 10.1038/sj.npp.1301572
151. Pettinati HM, Kampman KM, Lynch KG, Xie H, Dackis C, Rabinowitz AR, et al. A double blind, placebo-controlled trial that combines disulfiram and naltrexone for treating co-occurring cocaine and alcohol dependence. *Addict Behav*. (2008a) **33**:651–67. doi: 10.1016/j.addbeh.2007.11.011
152. Pettinati HM, Kampman KM, Lynch KG, Suh JJ, Dackis C A, Oslin DW, et al. Gender differences with high-dose naltrexone in patients with co-occurring cocaine and alcoholdependence. *J Subst Abuse Treat*. (2008b) **34**:378–90. doi: 10.1016/j.jsat.2007.05.011
153. Schmitz JM, Stotts AL, Sayre SL, DeLaune KA, Grabowski J. Treatment of cocaine-alcohol dependence with naltrexone and relapse prevention therapy. *Am J Addict*. (2004) **13**:333–41. doi: 10.1080/10550490490480982
154. Dunn KE, Defulio A, Everly JJ, Donlin WD, Aklin WM, Nuzzo PA, et al. Employment-based reinforcement of adherence to oral naltrexone treatment in unemployed injection drug users. *Exp Clin Psychopharmacol*. (2013) **21**:74–83. doi: 10.1037/a0030743
155. Kreek MJ. Pharmacogenetics and human molecular genetics of opiate and cocaine addictions and their treatments. *Pharmacol Rev*. (2005) **57**:1–26. doi: 10.1124/pr.57.1.1
156. Moeller SJ, Beebe-Wang N, Schneider KE, Konova AB, Parvaz MA, Alia-Klein N, et al. Effects of an opioid (proenkephalin) polymorphism on neural response to errors in health and cocaine use disorder. *Behav Brain Res*. (2015) **293**:18–26. doi: 10.1016/j.bbr.2015.07.004
157. Nikoshkov A, Drakenberg K, Wang X, Horvath MC, Keller E, Hurd YL. Opioid neuropeptide genotypes in relation to heroin abuse: dopamine tone contributes to reversed mesolimbic proenkephalin expression. *Proc Natl Acad Sci USA*. (2008) **105**:786–91. doi: 10.1073/pnas.0710902105
158. Yuferov V, Levran O, Proudnikov D, Nielsen DA, Kreek MJ. Search for genetic markers and functional variants involved in the development of opiate and cocaine addiction and treatment. *Ann N Y Acad Sci*. (2010) **1187**:184–207. doi: 10.1111/j.1749-6632.2009.05275.x

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Overexpression of Thioredoxin-1 Blocks Morphine-Induced Conditioned Place Preference Through Regulating the Interaction of γ-Aminobutyric Acid and Dopamine Systems

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Morphine is one kind of opioid, which is currently the most effective widely utilized pain relieving pharmaceutical. Long-term administration of morphine leads to dependence and addiction. Thioredoxin-1 (Trx-1) is an important redox regulating protein and works as a neurotrophic cofactor. Our previous study showed that geranylgeranylaceton, an inducer of Trx-1 protected mice from rewarding effects induced by morphine. However, whether overexpression of Trx-1 can block morphine-induced conditioned place preference (CPP) in mice is still unknown. In this study, we first examined whether overexpression of Trx-1 affects the CPP after morphine training and further examined the dopamine (DA) and γ-aminobutyric acid (GABA) systems involved in rewarding effects. Our results showed that morphine-induced CPP was blocked in Trx-1 overexpression transgenic (TG) mice. Trx-1 expression was induced by morphine in the ventral tegmental area (VTA) and nucleus accumbens (NAc) in wild-type (WT) mice, which was not induced in Trx-1 TG mice. The DA level and expressions of tyrosine hydroxylase (TH) and D1 were induced by morphine in WT mice, which were not induced in Trx-1 TG mice. The GABA level and expression of $GABA_BR$ were decreased by morphine, which were restored in Trx-1 TG mice. Therefore, Trx-1 may play a role in blocking CPP induced by morphine through regulating the expressions of D1, TH, and $GABA_BR$ in the VTA and NAc.

Keywords: thioredoxin-1, morphine, ventral tegmental area, nucleus accumbens, conditioned place preference

INTRODUCTION

Morphine is the most effective pain relieving pharmaceutical, which repeated use can lead to dependence and addiction. Morphine induces addiction through stimulating dopaminergic neurons in the ventral tegmental area (VTA) (1–3). The activity of the dopaminergic neurons in the VTA is involved in the rewarding effects induced by morphine (4). Morphine first targets γ-aminobutyric acid (GABA) neurons, which results in activation of dopaminergic neurons, then leads to rewarding effects (5). Thus, the rewarding effects are regulated by γ-aminobutyric acid and dopamine (DA) systems in the VTA, which projects to the nucleus accumbens (NAc).

Thioredoxin-1 (Trx-1) has various biological activities, such as regulating redox, activating transcription factors and protecting mice from Parkinson's disease (6–8). Nerve growth factor induces Trx-1 expression *via* activation of the extracellular signal-regulated kinase (ERK) and cAMP-response element binding protein (CREB) (6). Previous studies showed that morphine-induced Trx-1 expression *in vitro* and *in vivo* (9, 10). Geranylgeranylaceton, an inducer of Trx-1, protects mice from rewarding effects induced by morphine (10). Trx-1 overexpressing transgenic (TG) mice resisted the rewarding effects induced by methamphetamine (11). However, whether Trx-1 TG mice resist the rewarding effects induced by morphine is still unknown.

In this study, we examined conditioned place preference (CPP) in both wild-type (WT) mice and Trx-1 TG mice after morphine conditioned training and detected the levels of DA and GABA and the expressions of tyrosine hydroxylase (TH), D1, and GABA receptor B ($GABA_BR$) in the VTA and NAc.

ANIMALS AND METHODS

Reagents

Morphine hydrochloride was obtained from Shenyang First Pharmaceutical Factory, Northeast Pharmaceutical Group Corp. (Shenyang, China). Anti-mouse Trx-1 rabbit polyclonal antibody (14999-1-AP; 1:1,000) was purchased from ProteinTech (Wuhan, China). Antibody β-actin (sc-47778; 1:1,000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody D1 DA receptor (ADR001AN0302; 1:1,000) was purchased from alomone labs (Jerusalem Israel). Antibodies TH (ab137869; 1:1,000) and $GABA_BR$ (ab55051; 1:1,000) were purchased from Abcam (Cambridge, UK).

Animals

Male C57BL/6 mice (22–25 g, 8 weeks) were obtained from Chongqing Medical University, China. Mice were housed in plastic cages under controlled condition: 12 h light/dark cycles, average temperature of 23°C, with free access to food and water. C57BL/6 human Trx-1 overexpression TG mice were constructed by (Cyagen Biosciences Inc., Guangzhou, China). The pronuclei of fertilized eggs from hyperovulated C57BL/6 were microinjected with human Trx-1 cDNA construct. The presence of Trx-1 transgene was confirmed by performing western blot and real-time PCR analysis (Figure S1 in Supplementary Material). Mice were divided into four groups: saline, morphine (20 mg/kg), TG + saline, and TG + morphine ($n = 7$ per group). All procedures and protocols were approved by the animal ethics council of Kunming University of Science and Technology and were in accordance with the National Institutes of Health Guide for the Care and Use of Animals (12). The lab procedures were also approved by the local Committee on Animal Use and Protection of Yunnan province (No. LA2008305).

Western Blot Analysis

The VTA and NAc were dissected out according to the stereotaxic coordinates of Franklin and Paxinos, after the post-conditioning test. The exact coordinates for the two regions based on the center of the punch are: NAc (including core and shell): 1.34 mm anterior to bregma, 4.5 mm ventral to bregma, 0.8 mm lateral to the midline; and VTA: 3.4 mm posterior to bregma, 4.3 mm ventral to bregma, 0.5 mm lateral to the midline. After dissection, tissues were stored at −80°C until assay. Protein lysates was prepared using the solubilizing solution [1 mM EDTA, 20 mM Tris–HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, and 1 mg/ml leupeptin]. Protein concentration was determined using Bio-Rad protein assay reagent (Hercules, CA, USA). Using 12% (for $GABA_BR$, D1, and TH) or 15% (for Trx-1) SDS-PAGE, equal quantity of proteins was separated and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was soaked in 10% skimmed milk (in phosphate-buffered saline, containing 0.1% Tween 20, pH 7.2) for 2 h and incubated at 4°C overnight with the primary antibody. Immunoblots then were processed with the secondary antibodies (peroxidase-conjugated anti-mouse or anti-rabbit IgG) (1: 10,000, KPL, Gaithersburg, MD, USA). The bands were detected using an ECL chemiluminescence reagent kit (Millipore, MA, USA). Finally, densitometry analysis was performed by using ImageJ software.

Conditioned Place Preference

The CPP apparatus (15 cm × 15 cm × 30 cm) consisted of two chambers divided by a Plexiglas sliding door, one chamber is a black wall and a rough floor, and the other one is a white wall and a smooth floor. Mice were given a 15 min pretest to verify that the box configuration did not produce a significant bias for either chamber. However, individual mice tended to spend more time in one chamber or the other during the pretest, thus mice were morphine paired in the chamber in which they spent the least amount of time during the pretest (13). The experimental schedule for the CPP task is shown in **Figure 1**; mice were given 2 days for habituation freely in the apparatus for 15 min/day. On day 3, mice were placed into the chamber and allowed to move freely between the white and the black chamber for 15 min for the pre-conditioning, record the time that the mice spent in each chamber to determine the preference of experimental mice before morphine administrator. On days 4, 6, 8, and 10, mice received a morphine injection (20 mg/kg) and were immediately placed into the appropriate chamber (morphine-paired chamber) of the CPP box for 15 min. Saline group and TG + saline group received a saline injection and were placed in the chamber (morphine paired) of the CPP apparatus for 15 min immediately. On days 5, 7, 9, and 11, mice received a saline injection and were immediately placed in the opposite chamber (saline-paired chamber) for 15 min. On day 12, the post-conditioning test was performed without drug treatment, and the time when the mice spent in each chamber was measured for 15 min and time spent in each chamber was evaluated to determine preference. The standard for us to determine whether mice were addictive (CPP) is that the mice which previously tended to prefer the dark chamber turned to tend to prefer the white chamber after morphine condition. According to the data for the pretest, we found that overall

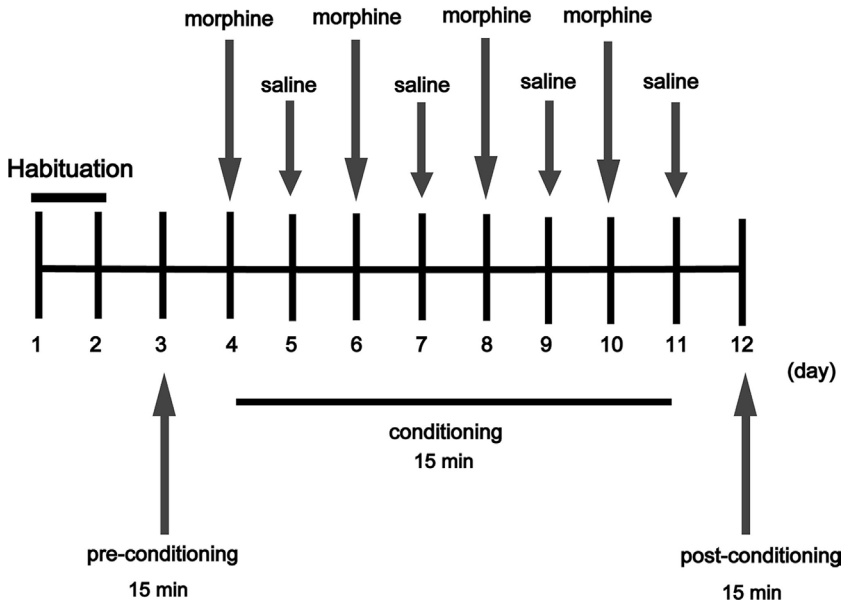

FIGURE 1 | Experimental schedule for measurement of morphine-induced conditioned place preference in mice. Arrows indicate days on which behavioral tests were carried out (morphine 20 mg/kg).

mice tended to prefer the dark chamber over the white chamber, thus most mice received morphine in the white chamber. The formula for calculation is as follows: Post–Pre value (s) = the time the mice spent in the white chamber in Post-CPP test − the time the mice spent in the white chamber in Pre-CPP test.

High Performance Liquid Chromatography (HPLC) Analysis

The VTA and NAc samples ($n = 6$ per group) were ultrasonicated in 0.1 M perchloric acid containing 10 ng/mg of internal standard dihydroxybenzylamine. It was centrifuged at 12,000 rpm for 15 min. The concentrations of DA and GABA were measured by 1100 HPLC system equipped with an ECD-105 electrochemical detection (CoMetro) and XDB-C18 column (150 mm × 4.6 mm, 50 mM, Agilent Technologies). The mobile phase A for separation consisted of the following: Na_2HPO_4, 50 mM; trisodium citrate, 20 mM; triethylamine, 5 mM, pH 4.75; and L-heptanesulfonic acid 0.3 mM. The mobile phase B is methanol (A: B 1/4 95:5). The HPLC systems were controlled, and the data were collected by a computer equipped with ChemStation software from Agilent Technologies (14). The amounts of GABA and DA in each sample were calculated from calibration curves of standards which were run simultaneously with every set of unknown samples.

Statistical Analysis

The data were expressed as mean ± SE values. Statistical analysis was performed using GraphPad Prism5 software. Normality was assessed using the Shapiro–Wilk test. A two-way ANOVA followed by a Bonferroni *post hoc* analysis was used to identify differences between treatment groups. A P value less than 0.05 was considered statistically significant.

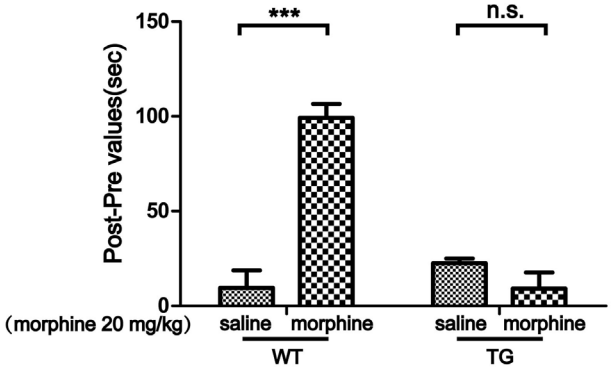

FIGURE 2 | Effects of thioredoxin-1 (Trx-1) overexpression on morphine-induced conditioned place preference (CPP) in mice. The morphine-induced CPP was blocked in Trx-1 overexpressing transgenic (TG) mice. Mice were treated with morphine (20 mg/kg, intraperitoneally). Each bar represents the mean ± SE ($n = 7$). n.s. >0.05, $^{***}P < 0.001$, statistically significant.

RESULTS

Overexpression of Trx-1 Blocked CPP Induced by Morphine

Conditioned place preference is a model to examine the rewarding effects of drugs and other stimuli. The experimental schedule for the CPP is shown in **Figure 1**. The results showed that CPP was induced after morphine treatment in WT mice, while the CPP was not induced by morphine in Trx-1 TG mice (**Figure 2**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,24} = 48.33$, $P < 0.001$) and significant effects of mice ($F_{1,24} = 27.01$, $P < 0.001$) and drug ($F_{1,24} = 26.41$, $P < 0.001$).

Bonferroni *post hoc* test showed that significant difference between the saline and morphine group in WT mice ($P < 0.001$) but not in TG mice ($P > 0.05$).

The Expression of Trx-1 in the VTA and NAc After Morphine-CPP Training

The VTA and NAc are critically involved in CPP for both psychostimulants and opiates (15, 16). Our previous study showed that Trx-1 was induced by morphine *in vitro* and *in vivo* (9, 10). Thus, we first examined the expression of Trx-1 after morphine treatment. The expression of Trx-1 was induced by morphine in the VTA of WT mice, while the expression was not induced by morphine in TG mice (**Figure 3A**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 5.01$, $P < 0.05$) and significant effects of mice ($F_{1,20} = 13.62$, $P < 0.01$) and drug ($F_{1,20} = 9.36$, $P < 0.01$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.01$). The expression of Trx-1 was induced by morphine in the NAc of WT mice; however, the expression of Trx-1 was not induced by morphine in TG mice (**Figure 3B**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 9.59$, $P < 0.01$) and significant effects of mice ($F_{1,20} = 7.82$, $P < 0.05$) and drug ($F_{1,20} = 4.85$, $P < 0.05$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.05$).

The Level of DA and the Expression of TH, D1 in the VTA and NAc After Morphine-CPP Training

Dopaminergic neurons in the VTA and NAc are activated in response to unpredicted rewards or cues that predict reward delivery (17). The DA receptor 1 (D1) is abundantly expressed in the VTA, especially on GABAergic neurons and synaptic afferents (18). Tyrosine hydroxylase (TH) is the maker of dopaminergic neurons (19). In this study, we found that the level of DA was increased by morphine in the VTA of WT mice, while the DA level was not increased by morphine in the VTA in TG mice (**Figure 4A**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 40.5$, $P < 0.001$) and significant effects of mice ($F_{1,20} = 5.61$, $P < 0.05$) and drug ($F_{1,20} = 32.06$, $P < 0.001$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.001$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.001$). The level of DA was increased by morphine in the NAc in WT mice, while the DA level was not increased by morphine in TG mice (**Figure 4B**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 56.95$, $P < 0.001$) and significant effects of mice ($F_{1,20} = 6.33$, $P < 0.05$) and drug ($F_{1,20} = 31.10$, $P < 0.001$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.001$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.001$). We found that the expression TH was increased by morphine in the VTA in WT mice, while the TH expression was not increased by morphine in TG mice (**Figure 4C**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 4.54$, $P < 0.05$) and significant effects of mice ($F_{1,20} = 12.48$, $P < 0.01$) and drug ($F_{1,20} = 6.46$, $P < 0.05$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.01$). The results also showed that TH was increased by morphine in the NAc in WT mice, while the TH expression was not increased

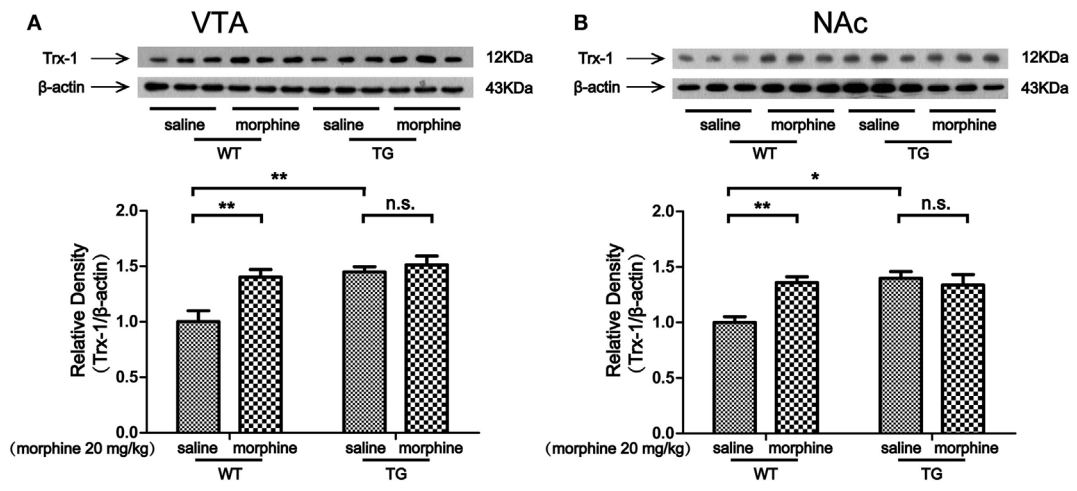

FIGURE 3 | Thioredoxin-1 (Trx-1) expression in the ventral tegmental area (VTA) and nucleus accumbens (NAc). Immediately after the post-conditioning test, the VTA and NAc of mice were dissected out. Trx-1 expression was detected by western blot analysis. Trx-1 overexpression inhibited the further increase of Trx-1 by morphine in the VTA **(A)** and NAc **(B)**. Each bar represents the mean ± SE ($n = 6$). n.s. >0.05, $^{*}P < 0.05$ and $^{**}P < 0.01$, statistically significant.

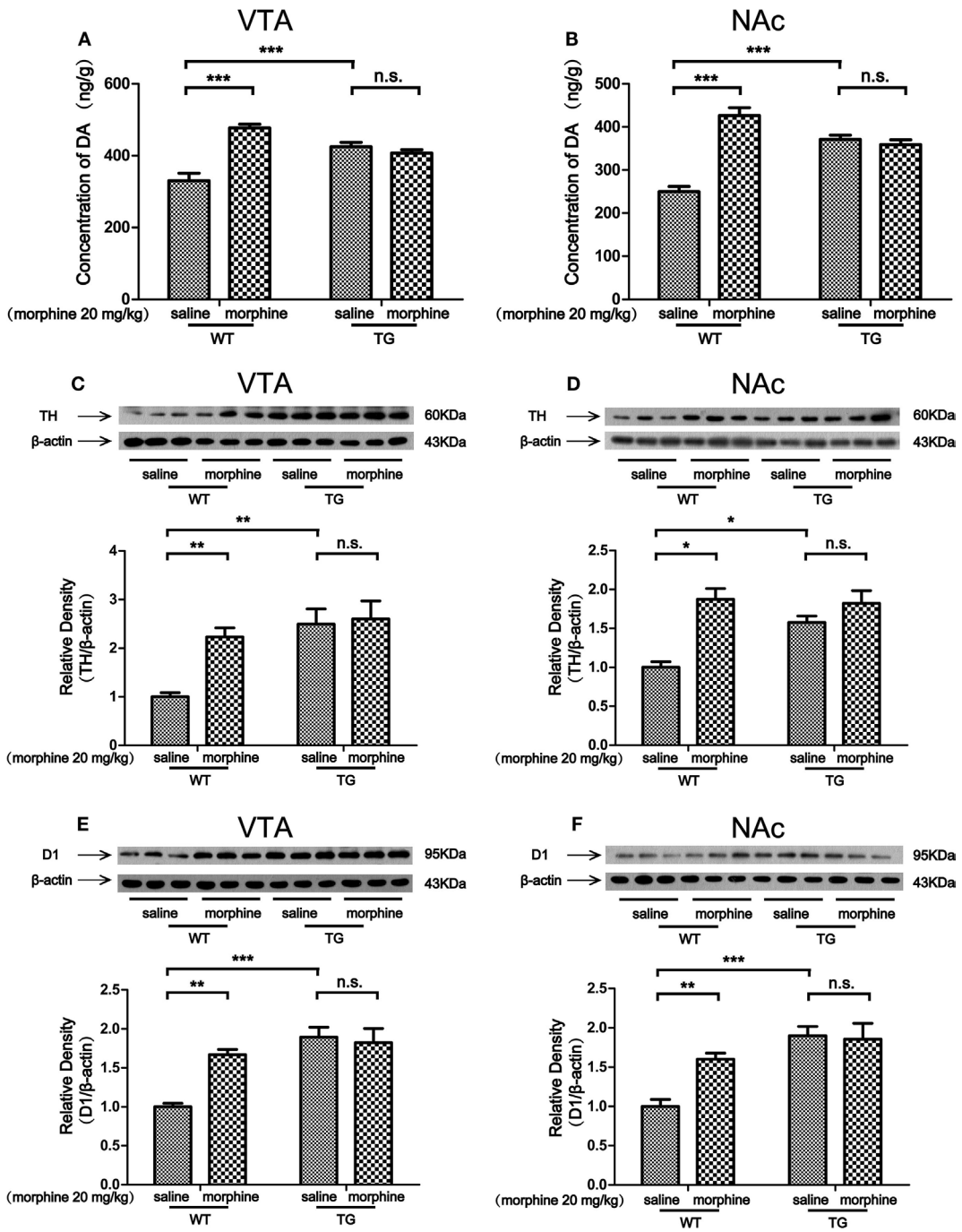

FIGURE 4 | Dopamine (DA) concentration and the expressions of tyrosine hydroxylase (TH) and D1 in the ventral tegmental area (VTA) and nucleus accumbens (NAc). Immediately following the post-conditioning test, the VTA and NAc of mice were dissected out. DA concentration was detected by high performance liquid chromatography in the VTA **(A)** and NAc **(B)**. The expressions of TH and D1 were detected by western blot analysis. Thioredoxin-1 (Trx-1) overexpression inhibited the further increase of TH induced by morphine in the VTA **(C)** and NAc **(D)**. Trx-1 overexpression inhibited the further increase of D1 induced by morphine in the VTA **(E)** and NAc **(F)**. Each bar represents the mean ± SE ($n = 6$). n.s. >0.05, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, statistically significant.

by morphine in TG mice (**Figure 4D**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 6.84$, $P < 0.05$) and significant effects of mice ($F_{1,20} = 4.83$, $P < 0.05$) and drug ($F_{1,20} = 21.81$, $P < 0.001$). Bonferroni *post hoc* test showed significant differences between the saline and morphine group in WT mice ($P < 0.05$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed significant differences between the TG and WT mice that have never been treated with morphine

($P < 0.05$). We found that the expression of D1 was increased by morphine in VTA in WT mice, while the expression of D1 was not increased by morphine in TG mice (**Figure 4E**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 10.08$, $P < 0.01$) and significant effects of mice ($F_{1,20} = 20.17$, $P < 0.001$) and drug ($F_{1,20} = 6.59$, $P < 0.05$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed significant differences between the TG and WT mice that have never been treated with morphine ($P < 0.001$). We found that the expression of D1 was increased by morphine in the NAc of WT mice, while the expression of D1 was not increased by morphine in TG mice (**Figure 4F**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 6.06$, $P < 0.05$) and significant effects of mice ($F_{1,20} = 19.36$, $P < 0.001$) and drug ($F_{1,20} = 4.54$, $P < 0.05$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.001$).

The Level of GABA and the Expression of $GABA_BR$ in the VTA and NAc After Morphine-CPP Training

Morphine targets GABAergic interneurons in the VTA and NAc and decreases their activity, which leads to an indirect increase activity of dopaminergic neurons (2). We found that the level of GABA was decreased by morphine in VTA in WT mice, while the level of GABA was not decreased by morphine in TG mice (**Figure 5A**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 20.28$, $P < 0.001$) and significant effects of mice ($F_{1,20} = 73.71$, $P < 0.001$) and drug ($F_{1,20} = 17.64$, $P < 0.001$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.001$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.001$). The level of GABA was decreased by morphine in the NAc of WT mice, while the level of GABA was not decreased by morphine in TG mice (**Figure 5B**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 16.11$, $P < 0.001$) and significant

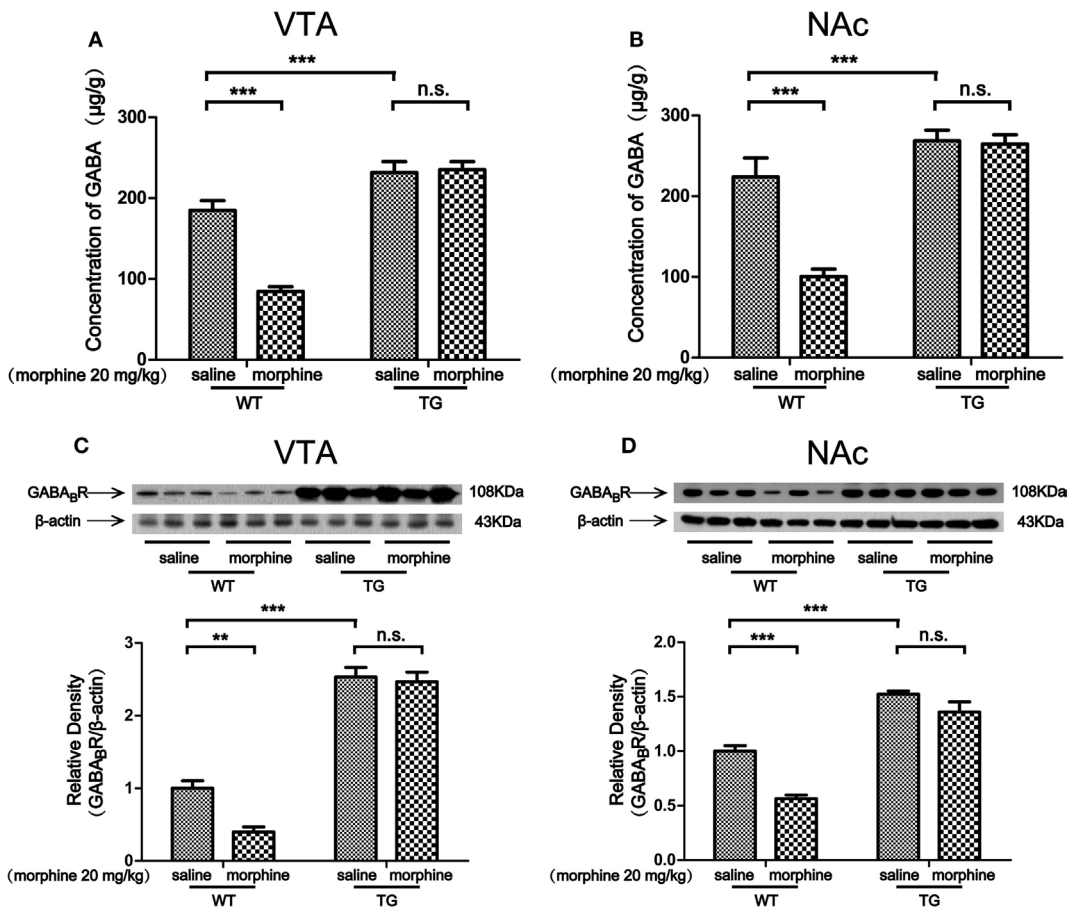

FIGURE 5 | GABA concentration and the expression of $GABA_BR$ in the ventral tegmental area (VTA) and nucleus accumbens (NAc). After the post-conditioning test, the VTA and NAc of mice were dissected out. GABA concentration was detected by high performance liquid chromatography in the VTA **(A)** and NAc **(B)**. The expression of $GABA_BR$ was detected by western blot analysis. Thioredoxin-1 overexpression restored the expression of $GABA_BR$ suppressed by morphine in the VTA **(C)** and NAc **(D)**. Each bar represents the mean ± SE ($n = 6$). n.s. >0.05, $^{**}P < 0.01$ and $^{***}P < 0.001$, statistically significant.

effects of mice ($F_{1,20} = 49.28$, $P < 0.001$) and drug ($F_{1,20} = 18.30$, $P < 0.001$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.001$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.001$). We further detected the expression of $GABA_BR$ in the VTA and NAc. The expression of $GABA_BR$ was decreased by morphine in the VTA of WT mice, while the expression of $GABA_BR$ was not decreased by morphine in TG mice (**Figure 5C**). Two-way ANOVA revealed a significant mice × drug interaction ($F_{1,20} = 5.68$, $P < 0.05$) and significant effects of mice ($F_{1,20} = 256.57$, $P < 0.001$) and drug ($F_{1,20} = 8.78$, $P < 0.001$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.01$). The expression of $GABA_BR$ was decreased by morphine in the NAc in WT mice, while the expression of $GABA_BR$ was not decreased by morphine in TG mice (**Figure 5D**). Two-way ANOVA revealed a significant mouse × drug interaction ($F_{1,20} = 5.51$, $P < 0.05$) and significant effects of mice ($F_{1,20} = 130.30$, $P < 0.001$) and drug ($F_{1,20} = 27.07$, $P < 0.001$). Bonferroni *post hoc* test showed a significant difference between the saline and morphine group in WT mice ($P < 0.01$) but not in TG mice ($P > 0.05$). The *post hoc* test also showed a significant difference between the TG and WT mice that have never been treated with morphine ($P < 0.001$).

DISCUSSION

In this study, we found that overexpression of Trx-1 blocked morphine-induced CPP (**Figure 2**). Trx-1 expression was induced by morphine in the VTA and NAc. This result is consisted with our previous study. Trx-1 was induced by morphine in the VTA, NAc, and prefrontal cortex, as well as the inducer of Trx-1 protected mice from rewarding effects induced by morphine (10).

The VTA and NAc are critically involved in CPP for both psychostimulants and opiates (15, 20). Studies have shown that activation of dopaminergic neurons in the midbrain can induce place preference (21). Dopaminergic neurons in the VTA are predicted to play roles in rewarding effects. The VTA contains substantial heterogeneity in neurotransmitter type, dopaminergic and GABAergic neurons (22). The NAc is the main projection from the VTA (23). Thus, the increased expressions of TH and D1 induced by morphine in the VTA and NAc of WT mice were related to morphine-induced CPP. However, the level of DA as well as the expressions of TH and D1 was not induced by morphine in TG mice in which the CPP was blocked (**Figures 4A–F**). It has been reported that dopaminergic neurons in the VTA are inhibited by endogenous GABA (24). Morphine inhibits GABAergic neurons in the VTA and disinhibits dopaminergic neurons (25). GABAergic inhibition results in tonic and phasic ignition of dopaminergic neurons. Similarly, activation of $GABA_BR$ in the VTA by baclofen blocks both heroin self-administration behavior and DA release in the NAc (26, 27) and morphine-induced CPP (28). Taken together, $GABA_BR$ in the VTA may play an essential role in mediating opiate reinforcement and rewarding effects. The majority of neurons in the NAc are GABAergic. Thus, $GABA_BR$ in the NAc also plays an essential role in mediating opiate reinforcement and rewarding effects. The GABAergic neuronal inhibition in the NAc can be antagonized by elevating endogenous GABA concentration in the VTA. Our results showed that the GABA release and the $GABA_BR$ expression were decreased by morphine in the VTA and NAc of WT mice, while the alterations were inhibited in Trx-1 TG mice (**Figures 5A–D**). Thus, these results suggest that Trx-1 overexpression may block morphine-induced CPP through elevating endogenous GABA concentration and $GABA_BR$ expression.

Interestingly, the expressions of TH, D1, and $GABA_BR$ were upregulated by Trx-1 (**Figures 4C–F** and **5C,D**). These results may be explained by the following studies. Our previous study showed that epinephrine increased TH expression through upregulating Trx-1 expression (14). Trx-1 increases the expressions of TH and D1 through increasing the expressions of c-fos and cAMP-response element binding protein (CREB) (29, 30). Forskolin enhances GABAergic responses (31). Trx-1 may increase $GABA_BR$ expression through upregulating forskolin/CREB. Thus, as our previous study suggested (11), an active defense system may have been built through increasing the levels of TH, D1, and $GABA_BR$ by the overexpression of Trx-1 in TG mice, which may contribute to the resistance of the development of CPP during morphine condition training.

In conclusion, Trx-1 overexpression blocks morphine-induced CPP in mice through regulating dopaminergic and GABAergic systems in the VTA and NAc. Our results indicate that Trx-1 may be a novel therapeutic target for morphine dependence.

ETHICS STATEMENT

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

AUTHOR CONTRIBUTIONS

JB was responsible for the study concept and design. XL, LG, XY, YL, XZ, and NG contributed to the acquisition of animal data. XL, MB, MH, and ZZ performed the western blot analysis. LY measured the concentrations of DA and GABA. XL and JB drafted the manuscript. JB and MH provided a critical revision of the manuscript for important intellectual content. All the authors read and approved the final version.

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REFERENCES

1. Gardner EL. Addiction and brain reward and antireward pathways. *Adv Psychosom Med* (2011) 30:22–60. doi:10.1159/000324065
2. Luscher C, Malenka RC. Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. *Neuron* (2011) 69(4):650–63. doi:10.1016/j.neuron.2011.01.017
3. Yuan K, Sheng H, Song J, Yang L, Cui D, Ma Q, et al. Morphine treatment enhances glutamatergic input onto neurons of the nucleus accumbens via both disinhibitory and stimulating effect. *Addict Biol* (2017) 22(6):1756–67. doi:10.1111/adb.12438
4. Chen J, Zhang R, Chen X, Wang C, Cai X, Liu H, et al. Heterodimerization of human orexin receptor 1 and kappa opioid receptor promotes protein kinase A/cAMP-response element binding protein signaling via a Galphas-mediated mechanism. *Cell Signal* (2015) 27(7):1426–38. doi:10.1016/j.cellsig.2015.03.027
5. Kalivas PW. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. *Brain Res Brain Res Rev* (1993) 18(1):75–113. doi:10.1016/0165-0173(93)90008-N
6. Bai J, Nakamura H, Kwon YW, Hattori I, Yamaguchi Y, Kim YC, et al. Critical roles of thioredoxin in nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells. *J Neurosci* (2003) 23(2):503–9. doi:10.1523/JNEUROSCI.23-02-00503.2003
7. Burke-Gaffney A, Callister ME, Nakamura H. Thioredoxin: friend or foe in human disease? *Trends Pharmacol Sci* (2005) 26(8):398–404. doi:10.1016/j.tips.2005.06.005
8. Zeng XS, Jia JJ, Kwon Y, Wang SD, Bai J. The role of thioredoxin-1 in suppression of endoplasmic reticulum stress in Parkinson disease. *Free Radic Biol Med* (2014) 67(2):10–8. doi:10.1016/j.freeradbiomed.2013.10.013
9. Luo FC, Feng YM, Zhao L, Li K, Wang SD, Song JY, et al. Thioredoxin-1 expression regulated by morphine in SH-SY5Y cells. *Neurosci Lett* (2012) 523(1):50–5. doi:10.1016/j.neulet.2012.06.039
10. Luo FC, Qi L, Lv T, Wang SD, Liu H, Nakamura H, et al. Geranylgeranylacetone protects mice against morphine-induced hyperlocomotion, rewarding effect, and withdrawal syndrome. *Free Radic Biol Med* (2012) 52(7):1218–27. doi:10.1016/j.freeradbiomed.2012.01.004
11. Huang M, Kong L, Yang L, Li X, Zhou X, Li Y, et al. The role of thioredoxin-1 in resisting methamphetamine-induced rewarding effect. *Behav Brain Res* (2018) 337:280–6. doi:10.1016/j.bbr.2017.07.047
12. Yang L, Guo Y, Huang M, Wu X, Li X, Chen G, et al. Thioredoxin-1 protects spinal cord from demyelination induced by methamphetamine through suppressing endoplasmic reticulum stress and inflammation. *Front Neurol* (2018) 9:49. doi:10.3389/fneur.2018.00049
13. Voigt RM, Napier TC. Context-dependent effects of a single administration of mirtazapine on the expression of methamphetamine-induced conditioned place preference. *Front Behav Neurosci* (2011) 5:92. doi:10.3389/fnbeh.2011.00092
14. Jia JJ, Zeng XS, Yang LH, Bai J. The epinephrine increases tyrosine hydroxylase expression through upregulating thioredoxin-1 in PC12 cells. *Biochimie* (2015) 115:52–8. doi:10.1016/j.biochi.2015.04.022
15. Fields HL, Hjelmstad GO, Margolis EB, Nicola SM. Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu Rev Neurosci* (2007) 30:289–316. doi:10.1146/annurev.neuro.30.051606.094341
16. Alizamini MM, Farzinpour Z, Ezzatpanah S, Haghparast A. Role of intra-accumbal orexin receptors in the acquisition of morphine-induced conditioned place preference in the rats. *Neurosci Lett* (2017) 660:1–5. doi:10.1016/j.neulet.2017.09.007
17. Owesson-White C, Belle AM, Herr NR, Peele JL, Gowrishankar P, Carelli RM, et al. Cue-evoked dopamine release rapidly modulates D2 neurons in the nucleus accumbens during motivated behavior. *J Neurosci* (2016) 36(22):6011–21. doi:10.1523/JNEUROSCI.0393-16.2016
18. Cadet JL, Jayanthi S, McCoy MT, Beauvais G, Cai NS. Dopamine D1 receptors, regulation of gene expression in the brain, and neurodegeneration. *CNS Neurol Disord Drug Targets* (2010) 9(5):526–38. doi:10.2174/187152710793361496
19. Perez-Costas E, Melendez-Ferro M, Rice MW, Conley RR, Roberts RC. Dopamine pathology in schizophrenia: analysis of total and phosphorylated tyrosine hydroxylase in the substantia nigra. *Front Psychiatry* (2012) 3:31. doi:10.3389/fpsyt.2012.00031
20. Qi C, Wang X, Ge F, Li Y, Shen F, Wang J, et al. mGluR5 in the nucleus accumbens shell regulates morphine-associated contextual memory through reactive oxygen species signaling. *Addict Biol* (2015) 20(5):927–40. doi:10.1111/adb.12222
21. Pascoli V, Terrier J, Hiver A, Lüscher C. Sufficiency of mesolimbic dopamine neuron stimulation for the progression to addiction. *Neuron* (2015) 88(5):1054–66. doi:10.1016/j.neuron.2015.10.017
22. CB D, AL C. 7(th) ISN special neurochemistry conference 'Synaptic function and dysfunction in brain diseases'. *J Neurochem* (2016) 139(6):918–20. doi:10.1111/jnc.113839
23. Han X, Jing MY, Zhao TY, Wu N, Song R, Li J. Role of dopamine projections from ventral tegmental area to nucleus accumbens and medial prefrontal cortex in reinforcement behaviors assessed using optogenetic manipulation. *Metab Brain Dis* (2017) 32(5):1491–502. doi:10.1007/s11011-017-0023-3
24. Smolders I, De KN, Sarre S, Ebinger G, Michotte Y. Tonic GABA-ergic modulation of striatal dopamine release studied by in vivo microdialysis in the freely moving rat. *Eur J Pharmacol* (1995) 284(284):83–91. doi:10.1016/0014-2999(95)00369-V
25. Jalabert M, Bourdy R, Courtin J, Veinante P, Manzoni OJ, Barrot M, et al. Neuronal circuits underlying acute morphine action on dopamine neurons. *Proc Natl Acad Sci U S A* (2011) 108(39):16446–50. doi:10.1073/pnas.1105418108
26. Xi ZX, Stein EA. Nucleus accumbens dopamine release modulation by mesolimbic GABAA receptors-an in vivo electrochemical study. *Brain Res* (1998) 798(1–2):156–65. doi:10.1016/S0006-8993(98)00406-5
27. Xi ZX, Stein EA. Baclofen inhibits heroin self-administration behavior and mesolimbic dopamine release. *J Pharmacol Exp Ther* (1999) 290(3):1369.
28. Tsuji M, Nakagawa Y, Ishibashi Y, Yoshii T, Takashima T, Shimada M, et al. Activation of ventral tegmental GABAB receptors inhibits morphine-induced place preference in rats. *Eur J Pharmacol* (1996) 313(3):169. doi:10.1016/0014-2999(96)00642-5
29. Lazaroff M, Patankar S, Yoon SO, Chikaraishi DM. The cyclic AMP response element directs tyrosine hydroxylase expression in catecholaminergic central and peripheral nervous system cell lines from transgenic mice. *J Biol Chem* (1995) 270(37):21579–89. doi:10.1074/jbc.270.37.21579
30. Sun B, Tank AW. c-Fos is essential for the response of the tyrosine hydroxylase gene to depolarization or phorbol ester. *J Neurochem* (2003) 85(6):1421–30. doi:10.1046/j.1471-4159.2003.01789.x
31. Nugent FS, Niehaus JL, Kauer JA. PKG and PKA signaling in LTP at GABAergic synapses. *Neuropsychopharmacology* (2009) 34(7):1829–42. doi:10.1038/npp.2009.5

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fneur.2018.00309/full#supplementary-material.

FIGURE S1 | The expression of humanTrx-1 in the VTA and NAc.

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REVIEW
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Pain Therapy Guided by Purpose and Perspective in Light of the Opioid Epidemic

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Prescription opioid misuse is an ongoing and escalating epidemic. Although these pharmacological agents are highly effective analgesics prescribed for different types of pain, opioids also induce euphoria, leading to increasing diversion and misuse. Opioid use and related mortalities have developed in spite of initial claims that OxyContin, one of the first opioids prescribed in the USA, was not addictive in the presence of pain. These claims allayed the fears of clinicians and contributed to an increase in the number of prescriptions, quantity of drugs manufactured, and the unforeseen diversion of these drugs for non-medical uses. Understanding the history of opioid drug development, the widespread marketing campaign for opioids, the immense financial incentive behind the treatment of pain, and vulnerable socioeconomic and physical demographics for opioid misuse give perspective on the current epidemic as an American-born problem that has expanded to global significance. In light of the current worldwide opioid epidemic, it is imperative that novel opioids are developed to treat pain without inducing the euphoria that fosters physical dependence and addiction. We describe insights from preclinical findings on the properties of opioid drugs that offer insights into improving abuse-deterrent formulations. One finding is that the ability of some agonists to activate one pathway over another, or agonist bias, can predict whether several novel opioid compounds bear promise in treating pain without causing reward among other off-target effects. In addition, we outline how the pharmacokinetic profile of each opioid contributes to their potential for misuse and discuss the emergence of mixed agonists as a promising pipeline of opioid-based analgesics. These insights from preclinical findings can be used to more effectively identify opioids that treat pain without causing physical dependence and subsequent opioid abuse.

Keywords: biased agonism, pharmacokinetics, opioid epidemic, chronic pain, opioid use disorder, oxycodone, mixed agonists

OXYCODONE AND OXYCONTIN AT THE CENTER OF THE PRESCRIPTION OPIOID EPIDEMIC

The History of Oxycodone Treatment of Chronic Non-Cancer Pain

Oxycodone, a semisynthetic opioid, was first formulated in 1916 from thebaine, a chemical found in opium poppy plants. The drug was first marketed as a less addictive alternative to "narcotic" drugs, such as morphine and heroin, which were typically prescribed to patients as an analgesic in the early 1900s. Oxycodone was first released in the USA in 1939 by Merck as a combination drug containing scopolamine, oxycodone, and ephedrine, but was discontinued in 1987 (1). Purdue Pharma then developed an extended-release formulation of oxycodone, called OxyContin. The FDA approved OxyContin in 1995 (2), noting that the reduced frequency of dosing was the only advantage of OxyContin over regular oxycodone (3). This drug was aggressively marketed by Purdue Pharma for opioid-based management of moderate-to-severe cancer and non-cancer pain where the use of an opioid analgesic was considered appropriate for more than a few days (2). Purdue used an aggressive marketing strategy to target-specific physicians (4), particularly those with less time to evaluate patients and often with less training in pain-management techniques. This led to more than half of the total OxyContin prescriptions being written by primary care physicians rather than pain specialists (4). In addition, direct-to-consumer pharmaceutical advertising, allowed only in the USA and New Zealand, has contributed to mass consumer awareness of the availability of these drugs with a demonstrated influence on the prescribing practice of physicians (5, 6). This aggressive physician directed marketing, as well as direct-to-consumer marketing, has become a benchmark for the marketing of opioids.

The Financial Incentive for Prescription Opioid Distribution

The Purdue-Frederick company first marketed MsContin (morphine sulfate) as an extended-release opioid-containing formula to treat pain in terminal cancer patients. MsContin generated $475 million in sales over a decade. After the Sackler brothers acquired Purdue-Frederick and rebranded the company as Purdue Pharma, they released OxyContin, which generated $45 million in sales in just the first year after its release in 1996. By 2001, the annual revenue from OxyContin sales reached $1.1 billion and rose to $2.528 billion by 2014 in the USA alone.

Currently, the Purdue Pharma company is 100% owned by members of the Sackler family, who are worth $13 billion and ranked as the 19th wealthiest family in the USA in 2016 (7). In addition to Purdue Pharma and other Sackler holdings, there are several other companies manufacturing oxycodone and related opioid compounds to fill the 259 million annual prescriptions written to patients in the USA, generating an additional $11 billion in opioid sales annually in 2011 (8). These include Abbot Labs, Novartis, Teva, Pfizer, Endo Pharmaceuticals, Impax, Actavis, Sandoz, Janssen Pharmaceuticals, etc. Together, these figures demonstrate the significant financial incentive pharmaceutical companies have to market opioid compounds despite growing concerns of the abuse liability and safety of these drugs.

Recognition of the Abuse Liability of Oxycodone and OxyContin

OxyContin was marketed as a delayed-release formulation allowing 12 h of continuous analgesia with fewer side effects than other opioid-based analgesics if used as directed. This formulation was promising in that the delayed-release would enable patients to sleep through the night, improving the standard of care for chronic pain patients at the time. However, this drug has been widely misused for non-medical purposes. At the time of the release of OxyContin in 1996, it was already known that 68% of an OxyContin tablet could be extracted by crushing the tablet (4). Since the first published reports of OxyContin abuse in 2000 (9), public awareness of its abuse liability has grown. Indeed, Purdue-Frederick, a holding of Purdue Pharma, paid $470 million dollars in fines to federal and state agencies and $130 million of payments in civil suits due to the misbranding of OxyContin as non-addictive in 2007 (10). Three executives of Purdue Pharma also pleaded guilty to OxyContin misbranding charges and paid $34.5 million in fines. By early 2017, there were daily reports of the diversion and misuse of prescription opioids with a number of states and counties across the country filing suit against five pharmaceutical companies, including Purdue. The plaintiffs in these suits claimed that the aggressive marketing campaign of opioid compounds is founded on fraudulent assertions of the safety of these drugs and that this misinformation has contributed to the ongoing opioid crisis. Purdue has responded to these claims by emphasizing that opioids are essential in pain management (2) and that their extended-release abuse-deterrent formulations are evidence of their drive to reduce the diversion of OxyContin (8). In 2018, Purdue stated it will no longer advertise directly to American doctors, a measure that will hopefully reduce overprescription of opioids (11).

The Patterns of Prescription Opioid Misuse and Overdose Mortalities Worldwide

The incidence of lifetime OxyContin abuse in the USA increased from 0.1% in 1999 to 0.4% in 2001 (12). By 2013, over 1,000 Americans were treated daily in emergency departments for prescription opioid misuse and in 2014, 4.3 million people used prescription opioids for non-medical reasons (13, 14). This trend was also seen in the number of deaths attributed to oxycodone, which increased from 14 cases in 1998 to ~14,000 cases in 2006 and 18,000 in 2015 (15). Although not of the same magnitude and somewhat delayed, this increase in opioid abuse and mortality is also occurring in other countries (16, 17). In Australia, oxycodone-related deaths increased sevenfold between 2001 and 2011 (18). In Finland, opioid mortalities increased from 9.5% of all drug overdose deaths in 2000 to 32.4% in 2008 (19), and data from Brazil, China, and the Middle East show similar increases in opioid diversion (17). In the United Kingdom, although tramadol and methadone are misused over oxycodone, the pattern of opioid misuse shows a similar increase to the USA albeit

on a smaller scale (20). While Americans consume 80% of the global opioid supply and 99% of the global hydrocodone supply (21) and the number of overdose mortalities is considerably higher in the USA, the opioid epidemic is growing worldwide.

The Most Vulnerable Populations

The incidence of opioid overdose mortality in the USA shows three hotspots: (1) the Appalachian states of Kentucky, Virginia, West Virginia, Pennsylvania, and Ohio, (2) the Northeast states of Maine, New Hampshire, and Rhode Island, and (3) the Southwest states of Nevada, Utah, New Mexico, and Arizona (15). This could be related to the demographics of these areas and the prescribing habits of the local medical professionals and pharmacies (22–25). Within all of these affected areas, opioid-related deaths are predominately Caucasians of middle age and are a result of drug overdose, alcohol-related disease, suicide, and psychiatric disorders. This has resulted in the first decline in life expectancy in the USA since 1993 (26–29). This has been highlighted in a series of articles that describe this population as subject to the "deaths of despair" (27, 30) and a "toxic stress" response to benign early-life events (31).

The primary factor contributing to these "deaths of despair" is the collapse of the white high-school educated working class from its heyday of the 1970s. This population's struggles in the job market in early adulthood became more difficult over time and are accompanied by health and personal issues that contribute to an increased morbidity from chronic pain, and physical and mental health disorders including opioid use disorder [OUD (32)]. The (USA) National Bureau of Economic Research found that for every 1% increase in unemployment, there is a 3.6% increase in opioid-related deaths, suggesting that macroeconomic conditions have influence over national drug misuse (33). Considering the global economic aftershocks of the USA's recession, we suggest that global economic recession contributed to the developing international opioid epidemic. To this point, a meta-analysis of research published from 1995 to 2015 in South America, the Caribbean, Europe, Asia, the USA, and Australia suggests that economic depression causes mental health issues that exacerbate illicit drug use (34). Case and Deaton additionally report that the use of prescription opioids did not create the vulnerable American profile, but the ease of availability of these compounds and the difficulty in treating opioid misuse in a depressed economy has inflamed the "sea of despair" that extends across the USA (27, 30).

Addressing Chronic Pain in the Midst of the Opioid Epidemic

It is clear that mass production, marketing, and prescription of opioids for pain treatment has contributed to the opioid epidemic in vulnerable demographics, characterized by mental health disorders, socioeconomic challenges, and susceptibility to occupational injury. We discuss the interplay of mental health, pain, and depression, and how these factors contribute to the misuse and addiction of prescription opioids. One of the key marketing claims of pharmaceutical companies was that the presence of pain is protective against opioid misuse. The evidence for this claim is shockingly limited due to evolving diagnostic criterion for opioid misuse and does not account for the influence of mental health on opioid misuse behavior in the pain state. This gives us perspective toward treating pain with the intent to limit the pro-addiction properties and off-target effects of future pharmaceuticals to decrease opioid dependence in the chronic pain state. We look to insights from behavioral research on addiction and reward, and then to mechanistic research on the pharmacokinetic and signaling properties on opioids to address these issues.

ARE CHRONIC PAIN PATIENTS AT RISK FOR OPIOID MISUSE?

The Use of Opioids to Treat Chronic Pain

Opioids are highly effective analgesics for the management of acute pain or pain associated with cancer but it is the opioid treatment of non-cancer pain that is at the root of the opioid epidemic. Before the introduction of OxyContin, patients of all ages suffering from chronic non-cancer pain were commonly under-treated due to a fear of opioid addiction and of other side effects of these drugs (35–37). There were also few viable alternatives, heroin and its metabolite, morphine, had been abused during the Vietnam war and a prevailing public stigma against the use of drugs developed (38). This culminated in an "opioid-phobia" and recurrent under-treatment of pain. Spurred by the promise that the presence of pain protects against opioid addiction in patients with chronic cancer pain (39), the availability of a slow-release opioid (OxyContin) and an aggressive marketing strategy by Purdue, opioid-phobia was replaced by an over-willingness to prescribe opioids. This openness, based on the success of long-term opioid treatment of cancer patients by oncologists and pain specialists (39) was coupled with a lack of adequate physician training in the appropriate use of opioids or evidence for their use in other pain conditions, increasing market pressure and a lack of regulatory control by the government. This timely interplay of multiple factors resulted in the number of opioid prescriptions per 100 persons per year increasing from 61.9 in 2000 to 83.7 in 2009, and to 259 million prescriptions by 2012, almost one per person (40–42). This increase has not been reflected by a change in the percentage of either ambulatory Americans or those reporting to the emergency department in pain, suggesting that the increase in opioid prescriptions is unrelated to the presence or absence of pain (43, 44). However, these large scale epidemiological studies make it difficult to assess whether opioid-based treatment of the ~100 million Americans in pain (45) has influenced the incidence of opioid misuse that affects 4.3 million Americans (14, 46). Assessing the risk of opioid misuse in chronic pain patients provides greater insight into the vulnerability of these patients for addiction.

The Risk of Opioid Misuse in Chronic Pain Patients

At the center of the opioid epidemic lies an unanswered question as to whether pain is protective of opioid misuse, a claim first made by Purdue in the 1990s. This was based on the findings from two studies that suggested the risk of addiction in pain

patients was less than 1% (4). In the first study, Porter and Jick found iatrogenic addiction in 4 of 11,882 patients (47) and in the second, Perry and Heidrich found no addiction among 10,000 burn patients treated with opioids (48). A third study by Portenoy and Foley found no evidence of abuse behaviors in 38 patients treated with different opioids (49). The 5-sentence, 101-word letter to the New England Journal of Medicine in 1980 by Porter and Jick was recently found to have been uncritically cited by 439 authors as proof that addiction was rare in long-term opioid therapy. Despite its limitations, this letter and its citations have made a seminal contribution to the opioid crisis (50).

Before considering the evidence for a protective effect of pain in preventing opioid misuse, the criterion by which to assess opioid misuse must be defined. Initially, opioid dependence and addiction were considered the definitive benchmark. These terms have recently been replaced by the term "opioid misuse" or the use of opioids for any other reason or under any other dosing schedule than originally prescribed. The diagnostic classification system of patients misusing opioids has similarly evolved and the terms "abuse" or "dependence" have been replaced by the diagnosis of OUD. According to the criteria established by the Diagnostic and Standard Manual of Mental Disorders (DSM) V (51), OUD has levels of severity depending on the number of criteria met in several categories. The four categories of criteria that characterize OUD include impaired control, social impairment, risky use, and pharmacological properties (physical tolerance and withdrawal symptoms).

Using these criteria, recent reports clearly show that the incidence of opioid misuse and aberrant drug-related behavior is in fact higher, not lower, in pain patients compared with the general population (52–63). Chronic pain patients have a higher rate of comorbid depression and anxiety, likely contributing to their increased use and misuse of opioids (64). Indeed, 30–80% of chronic pain patients are concurrently diagnosed with both depression and chronic pain, a comorbidity known as the pain-depression dyad (65, 66). Both conditions are closely interwoven in that they respond to similar treatments, aggravate or improve each other, and share common biological mechanisms [for review see Ref. (67)]. Using opioids to relieve pain in the presence of this dyad may in itself drive further psychiatric comorbidities (68). This patient population is unsurprisingly more likely to increasingly misuse opioids (58, 63, 69–71). Patient escalation of opioid doses in response to the progressive interaction between pain and affect or to compensate for tolerance and changes in pain sensitivity over time ("pseudoaddictive" behaviors) (71–73) may explain enhanced aberrant drug-related behaviors in chronic pain patients (61), as well as the positive correlation between baseline pain and the presence of OUD at a 3-year follow-up (74).

WHY ARE OPIOIDS SO ADDICTIVE?

The motivation to continue taking drugs in spite of adverse consequences can be explained by several concurrent theories. The Opponent Process theory (75) results from a balance between two valuationally opposite components, a loss of function within the reward-mediating dopaminergic circuits and an increased function of stress-related circuitry involving the extended amygdala, the kappa/dynorphin opioid and corticotrophin-signaling systems [reviewed in Ref. (76)]. The latter system becomes hyperactive during opioid dependence and manifests as increased anxiety and aggressive behaviors (77, 78). Another, co-occurring theory of the motivation behind continued drug use is the Incentive Sensitization theory that proposes an increase in drug-paired cues with chronic drug taking (79). Together, these systems drive drug-seeking behavior that is a product of (1) a decrease in positive outcome coupled with the promise and pullof drug-associated cues and (2) an increase in dysphoria between drug exposures and during withdrawal (80–82). This is particularly relevant for opioids as these compounds induce a tolerance to repeated exposures of the same dose of the drug. This leads to (1) an escalating intake of opioids over time resulting in compulsive opioid-taking behaviors (83), (2) increasing dependence, and (3) increasing negative affect seen in the absence of the drug (84) that together culminate in further dysregulation of the reward system (85).

The negative affective state of depression and anxiety associated with chronic pain can be relieved temporarily by the analgesic and euphoric properties of acute opioid use, which contributes to their abuse liability in the chronic pain state (86). However, both pain and opioid use create a new homeostasis in the reward and stress-related pathways [reviewed in Ref. (87)], an example of which can be seen in chronic pain patients who misuse opioids and also fail to show a positive affect from natural rewards (84, 88). Preclinical studies in rodent models have been able to examine the interaction between pain and opioids at several levels. Pain does not affect the number of low doses of opioid infusions (of heroin, morphine, and oxycodone) earned in a self-administration model of drug-seeking behavior but does increase heroin self-administration to binge levels at higher doses and during prolonged access to the drug (89–93). By contrast, pain reduces the self-administration of fentanyl (94), a shorter-acting but highly efficacious opioid that rapidly crosses the blood–brain barrier (BBB) (95). Pain also increases drug (morphine)-seeking behavior when the drug is no longer available (96). This result suggests that the abuse liability of opioids in the chronic pain state is not directly motivated by analgesia-seeking and intensifies when the drug is no longer available yet drug-associated cues and environmental stimuli are present. Together, these preclinical findings suggests that chronic pain produces a vulnerability to addiction-like behavior, bearing a similarity to the behavior of opioid addicts in chronic pain who are more likely to relapse once tapering off a maintenance buprenorphine naloxone treatment (97).

THE CURRENT CLINICAL TREATMENT OF CHRONIC PAIN PATIENTS WITH OPIOID USE DISORDER

The current clinical treatment of chronic pain in patients with OUD in the USA relies on 3 FDA-approved medications: buprenorphine naloxone, methadone, and long-acting injectable naltrexone (98). These strategies seek to antagonize or minimize the agonist properties of opioids to reduce the likability of

opioids. The use of methadone in the USA for OUD is limited to highly restricted methadone programs, but buprenorphine can be prescribed for office-based treatment by certified physicians. Buprenorphine, an opioid partial agonist, has analgesic effects and can be used to treat co-occurring chronic pain and OUD. While outcomes for OUD treatment with buprenorphine are similar for patients with and without chronic pain (99), poorly controlled pain during buprenorphine treatment is a risk for opioid relapse (97, 100, 101). Buprenorphine combined with naloxone, an opioid antagonist added to reduce diversion of buprenorphine for intravenous abuse, is FDA approved for OUD (e.g., Suboxone®), while a transdermal formulation (Butrans®) and a buccal film (Belbuca®), both without added naloxone, are approved for chronic pain. There are several novel compounds and approaches under development to treat pain, non-opioid compounds such as those that target cannabinoid receptors (102) and non-pharmaceutical, behavioral-based options to treat pain patients (103). However, for patients with chronic pain who continue to prefer a "quick fix from pain pills," the development of analgesic compounds that are not rewarding and have minimal off-target effects remains a challenge in the current context of the opioid epidemic.

NOT ALL OPIOID ANALGESICS ARE THE SAME: EXPLORING NOVEL PHARMACEUTICAL APPROACHES TO GUIDE THERAPEUTIC INTERVENTIONS FOR CHRONIC PAIN

Opioids have been used for centuries as the treatment of choice for pain but "abuse-deterrent" formulations may decrease opioid misuse and deaths if strategically developed. Abuse-deterrent formulations of existing opioids are one strategy to reduce misuse, but they have been demonstrated to be modifiable, necessitating the consideration of additional properties to minimize abuse liability and fatalities. We suggest that therapeutics that do not produce reward are most likely to reduce diversion for misuse. Focusing on this approach, we discuss novel interventions that maximize analgesic properties while minimizing reward-promoting effects on the affective state. To provide background information for this section, we have included a table (**Table 1**) of the clinical use and pharmacological properties of opioids commonly used in the clinic and those that are often abused. This table shows that most opioids used clinically to relieve pain are either full or partial agonists of the mu opioid receptor (MOR) with some activity at other members of the family of opioid receptors.

Biased Agonism of the Mu Opioid Receptor

Over the years, many opioid compounds have been classified by their efficacy to activate a downstream pathway (such as cAMP), their selectivity for a specific opioid receptor, and ability to desensitize, internalize, and re-sensitize the ligand-bound receptor. More recently, many opioids have been further classified by their ability to induce a specific ligand-receptor conformation to recruit and activate different downstream signaling cascades [reviewed in Ref. (153)]. This bias toward either activation of G-protein-dependent or G-protein-independent, arrestin signaling pathways is known as "biased agonism" (154). This is an exciting discovery with obvious translational significance if specific pathways can indeed be activated to reduce non-analgesic opioid signaling. For MORs and other G-protein coupled receptors, such as the Cannabinoid 1 receptor, agonists biased toward arrestin-mediated signaling rather than G-protein-dependent signaling pathways seem to produce greater adverse side effects (155, 156). This has led to an emphasis on developing compounds that do not recruit either of the non-visual arrestin isoforms very well. Herkinorin was the first example of a MOR agonist showing that reduction of arrestin-signaling bias is associated with reduced adverse side effects. Using this logic, novel MOR agonists such as TRV130, TRV0109101, and PZM21, have been developed that do not recruit arrestin very well and also induce fewer adverse side effects (156–159). In particular, TRV130 has been shown to be G-protein biased, has a greater or equal analgesic potency to morphine, and induces less tolerance (160, 161). However, it is controversial as to whether TRV130 causes less rewarding behaviors, inhibition of gastrointestinal transit, or induction of respiratory depression than morphine (158, 160, 161). This compound is now in a Phase III clinical trial for parenteral treatment of acute pain (NCT02656875). TRV0109101 is also biased toward G-protein signaling and does not induce hyperalgesia, a common side effect of chronic opioid use (159). PZM21 similarly does not recruit β-arrestin 2 but is less potent than morphine and appears to induce less constipation, less respiratory depression, and reduced reward-seeking behaviors (158).

Using agonist bias profiles to predict the abuse of the commonly abused semisynthetic and synthetic opioids yields mixed results. For example, morphine shows the same or greater arrestin bias than oxycodone (162, 163), yet oxycodone has a greater abuse liability than morphine (136). Fentanyl and its analogs are highly abused yet this class shows no overt bias for either signaling pathway. This suggests that biased agonism alone cannot be used to separate the analgesic from rewarding properties of opioids.

Pharmacokinetics

The action of opioids in the central nervous system facilitates analgesia mediated at supraspinal sites, such as those in the rostral ventral medulla, but also induces euphoria due to signaling at different central opioid receptor populations mediating reward (164). These central effects of opioids are also the major cause of overdose lethality due to respiratory depression (165), which is mediated by opioid receptors in breath-pattern generating neurons such as those in the pre-Bötzinger's complex of the medulla (166). Limiting the access of opioids to the central nervous system is a beneficial pharmacokinetic manipulation that may bypass these off-target effects while preserving the potential for analgesia mediated by signaling at opioid receptors in the spinal cord or primary nociceptive afferent neurons.

This relationship between the pharmacokinetic profile of opioids and their abuse liability was first described in the 1970s (167, 168) and resulted in the use of buprenorphine and methadone as a non-rewarding analgesic or to treat OUD (104, 167, 169). It is now well-known that the intrinsic abuse liability of an opioid

TABLE 1 | Descriptive and clinically relevant information of common opioids including clinical formulations, class of opioid, clinical uses, and cellular targets.

Drug [brand or alternative name(s)]	Common clinical formulation(s) (USA unless stated otherwise)	Classification; origin	Clinical use	Cellular target
Buprenorphine (e.g., Suboxone, Subutex, Buprenex)	Buprenorphine hydrochloride, buprenorphine naloxone (4:1)	Semisynthetic opiate; thebaine of the opium poppy (104)	Analgesia and maintenance therapy or opiate addiction treatment (Step 3 pain medication) (104)	Partial MOR agonist, KOR antagonist, nociceptin receptor agonist and antagonist (105, 106)
Fentanyl (e.g., Actiq, Duragesic, Fentora)	Fentanyl citrate	Synthetic opioid; *N*-phenethyl-piperidone (95)	Chronic and acute pain; administered orally, IV, transdermal patches (Step 3 pain medication) (107, 108)	Full MOR agonist, weak KOR agonist (109)
Heroin (i.e., diamorphine)	Diamorphine (UK) (110), diacetylmorphine (Canada/Switzerland) (111)	Opiate; morphine, and opium poppy (112)	Strong analgesic (Step 3 pain medication) (113, 114), opiate addiction treatment (Switzerland, Netherlands, Germany, England, Denmark) (115)	Partial MOR agonist (116) acts as prodrug (see active metabolites).
Hydrocodone (i.e., dihydrocodeinone) (e.g., Zohydro ER, Vicodin)	Hydrocodone bitartrate, hydrocodone bitartrate, and acetaminophen (117)	Semisynthetic opioid (118, 119); codeine (from opium poppy)	Chronic pain and opioid maintenance therapy (117)	Full MOR agonist (118)
Hydromorphone (e.g., Dilaudid)	Hydromorphone hydrochloride (120)	A semisynthetic opioid; the hydrogenated ketone of morphine (121)	Acute and chronic analgesia (Step 3 pain medication) (122), 5–8× more potent than morphine (123)	Full MOR agonist, partial DOR agonist, and weak KOR agonist (124, 125)
Methadone (e.g., Dolophine)	Methadone hydrochloride [(R) or racemic mixture] (126, 127)	Synthetic opioid (128); diphenylacetonitrile (129); and 1-dimethylamino-2-chloropropane (130)	Opioid dependence treatment (detoxification), chronic pain (131, 132)	Levo: full MOR agonist (109); dextro (D) NMDA antagonist (127).
Morphine (e.g., morphine sulfate ER, Roxanol, MsContin)	Morphine sulfate	Opiate; opium poppy (133)	Acute and chronic pain (Step 3 pain medication) (134)	Partial MOR agonist, weak DOR agonist (109, 135)
Oxycodone (e.g., Oxycontin, Norco, etc.)	Oxycodone hydrochloride, oxycodone acetaminophen, and oxycodone aspirin	Semisynthetic opiate; thebaine of the (136) opium poppy (137, 138)	Acute and chronic pain; may be superior than morphine for some types of pain (Step 3 pain medication) (139, 140)	Medium MOR agonist, partial KOR agonist (141), and partial DOR agonist (137, 142)
Remifentanil (e.g., Ultiva)	Remifentanil hydrochloride (always administered IV)	Synthetic opioid (143); derivative of fentanyl (144)	Acute pain or sedation (50–100× more potent than morphine) often used for surgical procedures (145–148)	Full MOR agonist (143)
Tramadol (e.g., Ultram)	Tramadol hydrochloride [racemic (+/–)], Tramadol hydrochloride, and acetaminophen	Synthetic opioid; salicylic acid with addition of 3-methoxyphenyl magnesium halide (149)	Moderate pain (Step 2 pain medication) (149, 150). Analgesic potency is 10% that of morphine (149)	(+/–) MOR agonist (151), (–) monoamine reuptake inhibitor (152)

Alternative names refer to either the chemical name (referred to as i.e.) or brand name (referred as e.g.). Pain medication steps of analgesia are derived from World Health Organization classifications.
MOR; mu opioid receptor, DOR; delta opioid receptor, KOR; kappa opioid receptor.

is a product of different pharmacokinetic parameters such as the time to peak plasma concentration, lipid solubility, BBB transport (a combination of passive diffusion and active transport in and out of the brain), and the presence of bioactive metabolites. Abuse liability may also be influenced by availability, with some compounds such as remifentanil being less available than others, such as methadone and heroin. We have outlined these pharmacokinetic properties and the abuse potential of commonly abused opioids and those used clinically (**Table 2**). This shows that fentanyl is one of the most rapidly bio-available opioids but has the same elimination half-life as morphine. However, it is highly lipid soluble (580× that of morphine) and so more easily crosses the BBB in both directions, shortening its effective duration of action. Heroin is a prodrug that is quickly transported across the BBB and converted to 6-acetyl-morphine, morphine, and demethylated to hydromorphone (170). Both of these opioids have a high abuse liability, but fentanyl and its derivatives are both more potent and have a longer elimination half-life making the fentanyl family of opioids fatal if taken in unknown or high quantities, as has often been the case (171). In comparison, morphine is hydrophilic, has poor protein binding capacity and its transport across the BBB is regulated, making it less likely to be abused. Compared with morphine, oxycodone is actively transported across the BBB, has a more rapid onset of effect and several active metabolites that all contribute to its greater abuse profile. At the other end of the spectrum are methadone and buprenorphine with medium-to-low abuse liabilities explained by low BBB permeability and a longer elimination half-life, in addition to differences in receptor selectivity and pharmacological profiles (**Tables 1** and **2**).

The positive correlation between BBB permeability/transport with abuse liability is the cornerstone of the strategically designed novel mu-opioid agonist, NKTR-181, which is analgesic but has limited abuse liability in humans (208, 209). This compound has a poly-ethylene glycol side chain and shows delayed transfer across

TABLE 2 | The abuse liability, aspects of the pharmacokinetic profile, and bioavailability of select clinical and abused opioid compounds.

Drug	Abuse liability	Onset of effect and time to peak plasma concentration (min to h)	Elimination half-life (generally oral/human)	Metabolite(s)	Metabolite half-life	Bioavailability and blood–brain barrier (BBB) permeability/transport
Buprenorphine	Low in relation to other opioids (172, 173)	Sublingual onset of 0.25–0.75 h, peak plasma concentration at 2 h (174)	3–48 h (175), variable	Buprenorphine-3-glucuronide, norbuprenorphine-3-glucuronide (106)	Unknown (106)	28–51% bioavailability (176), low BBB permeability (177)
Fentanyl	Very high (178–180)	2–5 min onset of action, and peak plasma concentrations of 20 min after oral and 12 min after intranasal administration (95)	1.5–7 h (181)	Norfentanyl; minimal activity (182)	N/A	50–90%, highly lipophilic and high BBB permeability through passive and active transport (178, 183). Transfer half-life of 4.7–6.6 min (95)
Heroin	Very high (184)	45 s to onset of effect, heroin undetectable in blood and CSF by 20 min in rats (185)	3 min (IV) (170)	6-Monoacetylmorphine (6-MAM), morphine, and morphine's metabolites (182)	6-MAM < 10 min after BBB crossing (116). Plasma conversion to morphine: 1.5–4.5 h, hydromorphone: 5 h, M6G: 2 h, M3G: 1.5 h	High (lipophilic) 60% or greater BBB permeability (116, 186)
Hydrocodone	High (136, 179, 187)	10 min to onset of effect and peak effects within 30–60 min (188)	3–9 h (189)	Hydromorphone and norhydrocodone (190).	Hydromorphone: 5 norhydrocodone: 8 (191)	25% bioavailability; 50% BBB permeability (187)
Hydromorphone	High (179)	5–30 min to onset of action, 30 min to peak effect (125, 189)	2–3 h (192)	Hydromorphone-3-glucuronide (182)	1.5–3 h (193)	55% bioavailability (194), higher BBB permeability than morphine; transfer half-life; 18–38 min (191)
Methadone	Medium (172)	30 min for onset of action, 1–5 h (132, 185)	4–6 h (132) or longer (195)	None (196)	N/A	41–99% bioavailability (195), 40% permeability (186)
Morphine	High (136, 179)	15–60 min (125, 139)	1.5–4.5 h (IV and IM) (121, 137)	Active: morphine-6-glucuronide (M6G) and hydromorphone. Inactive: morphine-3-glucuronide (M3G) (182).	M6G: 2 h (197); hydromorphone: 5 h (191); M3G: 1.5 h (198)	30% bioavailability, low BBB permeability; transfer half-life; 1.6–4.8 h (191)
Oxycodone	Very high (greater than morphine and hydrocodone) (136, 179)	10–30 min for onset of action (199), peak plasma levels occur ~1 h (137)	2–3 h (199), 3–5 h plasma after oral (137)	Noroxycodone (low activity) and oxymorphone (potency > morphine) (141), both metabolize into noroxymorphone (8–30× morphine's activity, BBB impermeable) (141)	Noroxycodone is converted slowly into noroxymorphone (200), oxymorphone (7–8 h) (141), noroxymorphone significantly longer than oxycodone (3–5 h but limited BBB permeability) (201)	60–90% bioavailability (142), active transport across the BBB and can reach 3× higher levels in the brain than blood (140, 202)
Remifentanil	Medium, possibly due to low availability (few cases) (203, 204)	1–2 min (143)	3–4 min (IV) (143)	Remifentanil acid, relatively inactive (205)	Negligible (205)	50% bioavailability and BBB equilibration half-life is 2–5 min (205)
Tramadol (e.g., Ultram)	Medium (179, 206);	2–3 h (149, 187)	5.1 h (149)	*O*-desmethyltramadol (M1), an MOR agonist (149)	9 h (149)	Actively transported (207)

the BBB (208). It is currently in Phase III clinical trials to treat chronic lower back pain or non-cancer pain (NCT02367820).

Mixed Opioid Agonists

Another interesting development is the use of ligands that simultaneously bind to and activate multiple receptors to relieve pain. Careful design of these bivalent ligands and their linkers has been shown to increase signaling efficacy of the target receptors, allowing a lower dose of the ligand to be used to achieve the same analgesic effect. Such bitopicity, or action at two sites, was first described for biphalin, a dual enkephalin analog that showed greater analgesic efficacy than enkephalin alone (210). Furthermore, incorporating the pharmacological properties of an opioid that has a reduced abuse liability, i.e., a slow onset of action, a long half-life and low BBB permeability, would result in an effective analgesic that is not rewarding. Several such mixed

ligands have now been generated that are based on the structure of buprenorphine (211–213), a partial MOR agonist, kappa opioid receptor (KOR)antagonist and nociceptin receptor ligand (214, 215) with reduced reward liability (172, 173). There are also other bivalent compounds that activate MOR and delta opioid receptors (DOR) (216), MOR and mGluR5 (217), and MOR, DOR and KORs (218).

In summary, we propose that the preclinical examination of novel opioid agonists that are pharmacologically designed to be (1) biased and so able to influence one signaling pathway over another, or (2) show a pharmacological profile that reduces a central duration of action, or (3) are able to signal selectively through mixed receptors, may provide better insight into and predictability of their abuse and lethality profiles. Such novel agonists may also incorporate aspects of each of these designs to obtain the desired clinical outcome. An example of this multi-faceted approach is the family of mixed ligands that are based on the structure of buprenorphine, which may target multiple receptors to enhance analgesia but have a buprenorphine-like pharmacological profile of reduced reward and overdose liability. The specificity and effects of these novel pharmaceutical compounds may be further influenced by the use of a positive allosteric modulator for which a conserved site has been found on MORs, DORs, and KORs (219).

TREATING PAIN WITH PERSPECTIVE AND WITH THE PURPOSE OF REDUCING HARM DURING THE OPIOID EPIDEMIC

Many pain patients have now found themselves physically and psychologically dependent on their opioid prescriptions, as both fail to relieve their pain in the chronic setting but are also now known to be addictive and harmful with long-term use. We have described the etiology of the opioid epidemic from the financial motivation for the over-prescription of these drugs, to the socioeconomic and physical issues that contribute to pain and addiction-prone populations worldwide. Navigating through the devastation caused by the opioid epidemic requires some perspective. While acknowledging that many opioids are harmful and addictive, they are still the most efficacious class of drugs for analgesia. Here, we aim to guide the refinement of prescription opioid compounds by improving upon the currently available abuse-deterrent formulations. These treatments should maximize analgesic properties by directing ligand bias toward signaling through G-proteins rather than β-arrestins, delaying or minimizing the BBB entry of drugs, minimizing metabolites with pro-addictive or off-target properties and using mixed agonists to provide more specific clinical effects. These strategies have led to the development of some promising compounds that may provide pain relief while minimizing the likelihood of addiction and misuse. Of course, these pharmaceutical agents should only be used following a comprehensive screening strategy to both exclude patients likely to misuse their medications and to identify those who may respond to alternate, non-opioid-based pain-management strategies.

AUTHOR CONTRIBUTIONS

This review is the result of a collaboration between basic research scientists and medical professionals. ALS and WW contributed to the conceptualization of the manuscript; edited and wrote the sections of the manuscript concerning the context of the opioid epidemic, pain, biased agonism, and pharmacokinetics. ALS and KH wrote the sections of the manuscript concerning medical interventions, psychiatric comorbidities to pain and addiction, and the epidemiology. ALS, WW, and JH produced the tables of drug properties. OC and GS contributed to writing and editing of the manuscript. All the authors contributed to the final editing and revision of the paper.

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REFERENCES

1. Defalque RJ, Wright AJ. Scophedal (SEE) was it a fad or a miracle drug? *Bull Anesth Hist* (2003) 21:12–4. doi:10.1016/S1522-8649(03)50051-8
2. Rappaport B. *Overview of the November 13, 2008 ALSDAC Meeting to Discuss NDA 22-324 for Remoxy (Oxycodone Hydrochloride Controlled-Release) Capsules*. A. Division of Anesthesia, and Rheumatology Products, Office of Drug Evaluation II, editor (2008).
3. Aquina CT, Marques-Baptista A, Bridgeman P, Merlin MA. OxyContin abuse and overdose. *Postgrad Med* (2009) 121(2):163–7. doi:10.3810/pgm.2009.03.1988
4. Van Zee A. The promotion and marketing of oxycontin: commercial triumph, public health tragedy. *Am J Public Health* (2009) 99(2):221–7. doi:10.2105/AJPH.2007.131714
5. Ventola CL. Direct-to-consumer pharmaceutical advertising: therapeutic or toxic? *P T* (2011) 36:669–84.
6. Fain KM, Alexander GC. Mind the gap: understanding the effects of pharmaceutical direct-to-consumer advertising. *Med Care* (2014) 52:291–3.
7. *Sackler Family [Online]*. Forbes (2016). Available from: https://www.forbes.com/profile/sackler/ (Accessed: October 12, 2017)
8. Eban K. OxyContin: Purdue Pharma's painful medicine. *Fortune* (2011). Available from: https://fortune.com/2011/11/09/oxycontin-purdue-pharmas-painful-medicine/
9. *Drug Overdose Deaths in the United States Continue to Increase in 2015 [Online]*. (2016). Available from: https://www.cdc.gov/drugoverdose/epidemic/ (Accessed: October 30, 2017).
10. Jones JP. *United States of America v. The Purdue Frederick Company, INC., et al*. In the United States District Court for the Western District of Virginia Abingdon Division (2007). 07CR00029.
11. Whalen J. Purdue Pharma to Stop Promoting OxyContin to U.S. Doctors. In: *The Wall Street Journal* (2018). New York: The Wall Street Journal. Available from:

https://www.wsj.com/articles/purdue-pharma-to-stop-promoting-oxycontin-to-u-s-doctors-1518307565
12. Sees KL, Di Marino ME, Ruediger NK, Sweeney CT, Shiffman S. Non-medical use of oxycontin tablets in the United States. *J Pain Palliat Care Pharmacother* (2005) 19:13–23. doi:10.1080/J354v19n02_04
13. *The DAWN Report: Highlights of the 2011 Drug Abuse Warning Network (DAWN) Findings on Drug-Related Emergency Department Visits*. Rockville, MD: Substance Abuse and Mental Health Services Administration (2013).
14. Zielinska M, Ben Haddou T, Cami-Kobeci G, Salaga M, Jarmuz A, Padysz M, et al. Anti-inflammatory effect of dual nociceptin and opioid receptor agonist, BU08070, in experimental colitis in mice. *Eur J Pharmacol* (2015) 765:582–90. doi:10.1016/j.ejphar.2015.09.021
15. Rudd RA, Seth P, David F, Scholl L. Increases in drug and opioid-involved overdose deaths – United States, 2010-2015. *MMWR Morb Mortal Wkly Rep* (2016) 65:1445–52. doi:10.15585/mmwr.mm655051e1
16. Helmerhorst GT, Teunis T, Janssen SJ, Ring D. An epidemic of the use, misuse and overdose of opioids and deaths due to overdose, in the United States and Canada: is Europe next? *Bone Joint J* (2017) 99-B:856–64. doi:10.1302/0301-620X.99B7.BJJ-2016-1350.R1
17. Martins SS, Ghandour LA. Nonmedical use of prescription drugs in adolescents and young adults: not just a western phenomenon. *World Psychiatry* (2017) 16:102–4. doi:10.1002/wps.20350
18. Pilgrim JL, Yafistham SP, Gaya S, Saar E, Drummer OH. An update on oxycodone: lessons for death investigators in Australia. *Forensic Sci Med Pathol* (2015) 11:3–12. doi:10.1007/s12024-014-9624-x
19. Hakkinen M, Launiainen T, Vuori E, Ojanpera I. Comparison of fatal poisonings by prescription opioids. *Forensic Sci Int* (2012) 222:327–31. doi:10.1016/j.forsciint.2012.07.011
20. Giraudon I, Lowitz K, Dargan PI, Wood DM, Dart RC. Prescription opioid abuse in the UK. *Br J Clin Pharmacol* (2013) 76:823–4. doi:10.1111/bcp.12133
21. Manchikanti L, Singh A. Therapeutic opioids: a ten-year perspective on the complexities and complications of the escalating use, abuse, and nonmedical use of opioids. *Pain Physician* (2008) 11:S63–88.
22. Hall AJ, Logan JE, Toblin RL, Kaplan JA, Kraner JC, Bixler D, et al. Patterns of abuse among unintentional pharmaceutical overdose fatalities. *JAMA* (2008) 300:2613–20. doi:10.1001/jama.2008.802
23. Paulozzi LJ, Mack KA, Hockenberry JM. Variation among states in prescribing of opioid pain relievers and benzodiazepines – United States, 2012. *J Safety Res* (2014) 51:125–9. doi:10.1016/j.jsr.2014.09.001
24. Paulozzi LJ, Strickler GK, Kreiner PW, Koris CM; Centers for Disease Conrol and Prevention. Controlled substance prescribing patterns – prescription behavior surveillance system, eight states, 2013. *MMWR Surveill Summ* (2015) 64:1–14. doi:10.15585/mmwr.ss6409a1
25. Woolley SM, Meacham SL, Balmert LC, Talbott EO, Buchanich JM. Comparison of mortality disparities in central appalachian coal- and non-coal-mining counties. *J Occup Environ Med* (2015) 57:687–94. doi:10.1097/JOM.0000000000000435
26. Becker WC, Sullivan LE, Tetrault JM, Desai RA, Fiellin DA. Non-medical use, abuse and dependence on prescription opioids among U.S. adults: psychiatric, medical and substance use correlates. *Drug Alcohol Depend* (2008) 94:38–47. doi:10.1016/j.drugalcdep.2007.09.018
27. Case A, Deaton A. Rising morbidity and mortality in midlife among white non-Hispanic Americans in the 21st century. *Proc Natl Acad Sci U S A* (2015) 112:15078–83. doi:10.1073/pnas.1518393112
28. Wilkerson RG, Kim HK, Windsor TA, Mareiniss DP. The opioid epidemic in the United States. *Emerg Med Clin North Am* (2016) 34:e1–23. doi:10.1016/j.emc.2015.11.002
29. Hedegaard HW, Warner M, Minio AM. *Drug Overdose Deaths in the United States, 1999-2015*. Hyattsville, MD: N.C.F.H. Statistics (2017).
30. Case A, Deaton A. Mortality and morbidity in the 21st century. *Brookings Papers on Economic Activity*. (2017).
31. Shern DL, Blanch AK, Steverman SM. Toxic stress, behavioral health, and the next major era in public health. *Am J Orthopsychiatry* (2016) 86:109–23. doi:10.1037/ort0000120
32. Modrek S, Hamad R, Cullen MR. Psychological well-being during the great recession: changes in mental health care utilization in an occupational cohort. *Am J Public Health* (2015) 105:304–10. doi:10.2105/AJPH.2014.302219
33. Hollingsworth A, Ruhm CJ, Simon K. Macroeconomic conditions and opioid abuse. *J Health Econ* (2017) 56:222–33. doi:10.1016/j.jhealeco.2017.07.009
34. Nagelhout GE, Hummel K, De Goeij MCM, De Vries H, Kaner E, Lemmens P. How economic recessions and unemployment affect illegal drug use: a systematic realist literature review. *Int J Drug Policy* (2017) 44:69–83. doi:10.1016/j.drugpo.2017.03.013
35. Friedman DP. Perspectives on the medical use of drugs of abuse. *J Pain Symptom Manage* (1990) 5:S2–5.
36. Tucker C. Acute pain and substance abuse in surgical patients. *J Neurosci Nurs* (1990) 22:339–49. doi:10.1097/01376517-199012000-00003
37. Tunca M, Yelken J. Undertreatment of cancer pain. *Lancet* (1991) 337:1294. doi:10.1016/0140-6736(91)92968-8
38. Zinberg NE. Heroin use in Vietnam and the United States. A contrast and a critique. *Arch Gen Psychiatry* (1972) 26:486–8. doi:10.1001/archpsyc.1972.01750230096019
39. Manchikanti L, Helm S II, Fellows B, Janata JW, Pampati V, Grider JS, et al. Opioid epidemic in the United States. *Pain Physician* (2012) 15:ES9–38.
40. Ping A, Xi J, Prasad BM, Wang MH, Kruzich PJ. Contributions of nucleus accumbens core and shell GluR1 containing AMPA receptors in AMPA- and cocaine-primed reinstatement of cocaine-seeking behavior. *Brain Res* (2008) 1215:173–82. doi:10.1016/j.brainres.2008.03.088
41. Kenan K, Mack K, Paulozzi L. Trends in prescriptions for oxycodone and other commonly used opioids in the United States, 2000-2010. *Open Med* (2012) 6:e41–7.
42. Cummins S, Leischow S, Bailey L, Bush T, Wassum K, Copeland L, et al. Knowledge and beliefs about electronic cigarettes among quitline cessation staff. *Addict Behav* (2016) 60:78–83. doi:10.1016/j.addbeh.2016.03.031
43. Daubresse M, Chang HY, Yu Y, Viswanathan S, Shah ND, Stafford RS, et al. Ambulatory diagnosis and treatment of nonmalignant pain in the United States, 2000-2010. *Med Care* (2013) 51:870–8. doi:10.1097/MLR.0b013e3182a95d86
44. Chang HY, Daubresse M, Kruszewski SP, Alexander GC. Prevalence and treatment of pain in EDs in the United States, 2000 to 2010. *Am J Emerg Med* (2014) 32:421–31. doi:10.1016/j.ajem.2014.01.015
45. Committee on Advancing Pain Research, Care, And Education. Summary. *Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research (2011)*. Washington, DC: National Academies Press (2011).
46. Volkow ND. *America's Addiction to Opioids: Heroin and Prescription Drug Abuse*. N.I.o.D. Abuse, editors. Washington DC: NIDA (2014).
47. Porter J, Jick H. Addiction rare in patients treated with narcotics. *N Engl J Med* (1980) 302:123. doi:10.1056/NEJM198001103020221
48. Perry S, Heidrich G. Management of pain during debridement: a survey of U.S. burn units. *Pain* (1982) 13:267–80. doi:10.1016/0304-3959(82)90016-1
49. Portenoy RK, Foley KM. Chronic use of opioid analgesics in non-malignant pain: report of 38 cases. *Pain* (1986) 25:171–86. doi:10.1016/0304-3959(86)90091-6
50. Leung PTM, Macdonald EM, Stanbrook MB, Dhalla IA, Juurlink DN. A 1980 letter on the risk of opioid addiction. *N Engl J Med* (2017) 376:2194–5. doi:10.1056/NEJMc1700150
51. Brezing C, Bisaga A. *Opioid Use Disorder: Update on Diagnosis and Treatment [Online]*. Psychiatric Times Editorial (2015). Available from: http://www.psychiatrictimes.com/special-reports/opioid-use-disorder-update-diagnosis-and-treatment (Accessed: August 11, 2017).
52. Fishbain DA, Rosomoff HL, Rosomoff RS. Drug abuse, dependence, and addiction in chronic pain patients. *Clin J Pain* (1992) 8:77–85. doi:10.1097/00002508-199206000-00003
53. Hoffmann NG, Olofsson O, Salen B, Wickstrom L. Prevalence of abuse and dependency in chronic pain patients. *Int J Addict* (1995) 30:919–27. doi:10.3109/10826089509055820
54. Chabal C, Erjavec MK, Jacobson L, Mariano A, Chaney E. Prescription opiate abuse in chronic pain patients: clinical criteria, incidence, and predictors. *Clin J Pain* (1997) 13:150–5. doi:10.1097/00002508-199706000-00009
55. Kouyanou K, Pither CE, Wessely S. Medication misuse, abuse and dependence in chronic pain patients. *J Psychosom Res* (1997) 43:497–504. doi:10.1016/S0022-3999(97)00171-2
56. Reid MC, Engles-Horton LL, Weber MB, Kerns RD, Rogers EL, O'Connor PG. Use of opioid medications for chronic noncancer pain syndromes in primary care. *J Gen Intern Med* (2002) 17:173–9. doi:10.1046/j.1525-1497.2002.10435.x
57. Katz NP, Sherburne S, Beach M, Rose RJ, Vielguth J, Bradley J, et al. Behavioral monitoring and urine toxicology testing in patients receiving long-term

opioid therapy. *Anesth Analg* (2003) 97:1097–102. doi:10.1213/01.ANE.0000080159.83342.B5
58. Edlund MJ, Sullivan M, Steffick D, Harris KM, Wells KB. Do users of regularly prescribed opioids have higher rates of substance use problems than nonusers? *Pain Med* (2007) 8:647–56. doi:10.1111/j.1526-4637.2006.00200.x
59. Hojsted J, Sjogren P. Addiction to opioids in chronic pain patients: a literature review. *Eur J Pain* (2007) 11:490–518. doi:10.1016/j.ejpain.2006.08.004
60. Michna E, Jamison RN, Pham LD, Ross EL, Janfaza D, Nedeljkovic SS, et al. Urine toxicology screening among chronic pain patients on opioid therapy: frequency and predictability of abnormal findings. *Clin J Pain* (2007) 23:173–9. doi:10.1097/AJP.0b013e31802b4f95
61. Fishbain DA, Cole B, Lewis J, Rosomoff HL, Rosomoff RS. What percentage of chronic nonmalignant pain patients exposed to chronic opioid analgesic therapy develop abuse/addiction and/or aberrant drug-related behaviors? A structured evidence-based review. *Pain Med* (2008) 9:444–59. doi:10.1111/j.1526-4637.2007.00370.x
62. Vowles KE, Mcentee ML, Julnes PS, Frohe T, Ney JP, Van Der Goes DN. Rates of opioid misuse, abuse, and addiction in chronic pain: a systematic review and data synthesis. *Pain* (2015) 156:569–76. doi:10.1097/01.j.pain.0000460357.01998.f1
63. Feingold D, Goor-Aryeh I, Bril S, Delayahu Y, Lev-Ran S. Problematic use of prescription opioids and medicinal cannabis among patients suffering from chronic pain. *Pain Med* (2017) 18(2):294–306. doi:10.1093/pm/pnw134
64. Berrocoso E, Sanchez-Blazquez P, Garzon J, Mico JA. Opiates as antidepressants. *Curr Pharm Des* (2009) 15:1612–22. doi:10.2174/138161209788168100
65. Yalcin I, Barrot M. The anxiodepressive comorbidity in chronic pain. *Curr Opin Anaesthesiol* (2014) 27:520–7. doi:10.1097/ACO.0000000000000116
66. Cicero TJ, Ellis MS. Abuse-deterrent formulations and the prescription opioid abuse epidemic in the United States: lessons learned from oxycontin. *JAMA Psychiatry* (2015) 72:424–30. doi:10.1001/jamapsychiatry.2014.3043
67. Li JX. Pain and depression comorbidity: a preclinical perspective. *Behav Brain Res* (2015) 276:92–8. doi:10.1016/j.bbr.2014.04.042
68. Scherrer JF, Salas J, Copeland LA, Stock EM, Schneider FD, Sullivan M, et al. Increased risk of depression recurrence after initiation of prescription opioids in noncancer pain patients. *J Pain* (2016) 17:473–82. doi:10.1016/j.jpain.2015.12.012
69. Wasan AD, Michna E, Edwards RR, Katz JN, Nedeljkovic SS, Dolman AJ, et al. Psychiatric comorbidity is associated prospectively with diminished opioid analgesia and increased opioid misuse in patients with chronic low back pain. *Anesthesiology* (2015) 123:861–72. doi:10.1097/ALN.0000000000000768
70. Arteta J, Cobos B, Hu Y, Jordan K, Howard K. Evaluation of how depression and anxiety mediate the relationship between pain catastrophizing and prescription opioid misuse in a chronic pain population. *Pain Med* (2016) 17:295–303.
71. Edwards RR, Dolman AJ, Michna E, Katz JN, Nedeljkovic SS, Janfaza D, et al. Changes in pain sensitivity and pain modulation during oral opioid treatment: the impact of negative affect. *Pain Med* (2016) 17(10):1882–91. doi:10.1093/pm/pnw010
72. Chow MJ, Ambre JJ, Ruo TI, Atkinson AJ Jr, Bowsher DJ, Fischman MW. Kinetics of cocaine distribution, elimination, and chronotropic effects. *Clin Pharmacol Ther* (1985) 38:318–24. doi:10.1038/clpt.1985.179
73. De Pinto M, Cahana A. Medical management of acute pain in patients with chronic pain. *Expert Rev Neurother* (2012) 12:1325–38. doi:10.1586/ern.12.123
74. Blanco C, Wall MM, Okuda M, Wang S, Iza M, Olfson M. Pain as a predictor of opioid use disorder in a nationally representative sample. *Am J Psychiatry* (2016) 173(12):1189–95. doi:10.1176/appi.ajp.2016.15091179
75. Solomon RL, Corbit JD. An opponent-process theory of motivation. I. Temporal dynamics of affect. *Psychol Rev* (1974) 81:119–45.
76. Zhou Y, Leri F. Neuroscience of opiates for addiction medicine: from stress-responsive systems to behavior. *Prog Brain Res* (2016) 223:237–51. doi:10.1016/bs.pbr.2015.09.001
77. Gerra G, Zaimovic A, Moi G, Bussandri M, Bubici C, Mossini M, et al. Aggressive responding in abstinent heroin addicts: neuroendocrine and personality correlates. *Prog Neuropsychopharmacol Biol Psychiatry* (2004) 28:129–39. doi:10.1016/j.pnpbp.2003.09.029
78. Kakko J, Von Wachenfeldt J, Svanborg KD, Lidstrom J, Barr CS, Heilig M. Mood and neuroendocrine response to a chemical stressor, metyrapone, in buprenorphine-maintained heroin dependence. *Biol Psychiatry* (2008) 63:172–7. doi:10.1016/j.biopsych.2007.05.001
79. Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev* (1993) 18:247–91. doi:10.1016/0165-0173(93)90013-P
80. Kalivas PW, Volkow ND. The neural basis of addiction: a pathology of motivation and choice. *Am J Psychiatry* (2005) 162:1403–13. doi:10.1176/appi.ajp.162.8.1403
81. Koob GF, Volkow ND. Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* (2016) 3:760–73. doi:10.1016/S2215-0366(16)00104-8
82. Volkow ND, Koob GF, Mclellan AT. Neurobiologic advances from the brain disease model of addiction. *N Engl J Med* (2016) 374:363–71. doi:10.1056/NEJMra1511480
83. Koob GF, Le Moal M. Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* (2001) 24:97–129. doi:10.1016/S0893-133X(00)00195-0
84. Garland EL, Bryan CJ, Nakamura Y, Froeliger B, Howard MO. Deficits in autonomic indices of emotion regulation and reward processing associated with prescription opioid use and misuse. *Psychopharmacology (Berl)* (2017) 234:621–9. doi:10.1007/s00213-016-4494-4
85. Huhn AS, Meyer RE, Harris JD, Ayaz H, Deneke E, Stankoski DM, et al. Evidence of anhedonia and differential reward processing in prefrontal cortex among post-withdrawal patients with prescription opiate dependence. *Brain Res Bull* (2016) 123:102–9. doi:10.1016/j.brainresbull.2015.12.004
86. Leitl MD, Negus SS. Pharmacological modulation of neuropathic pain-related depression of behavior: effects of morphine, ketoprofen, bupropion and [INCREMENT]9-tetrahydrocannabinol on formalin-induced depression of intracranial self-stimulation in rats. *Behav Pharmacol* (2016) 27:364–76. doi:10.1097/FBP.0000000000000207
87. Finan PH, Remeniuk B, Dunn KE. The risk for problematic opioid use in chronic pain: what can we learn from studies of pain and reward? *Prog Neuropsychopharmacol Biol Psychiatry* (2017). doi:10.1016/j.pnpbp.2017.07.029
88. Elvemo NA, Landro NI, Borchgrevink PC, Haberg AK. Reward responsiveness in patients with chronic pain. *Eur J Pain* (2015) 19:1537–43. doi:10.1002/ejp.687
89. Woller SA, Moreno GL, Hart N, Wellman PJ, Grau JW, Hook MA. Analgesia or addiction? Implications for morphine use after spinal cord injury. *J Neurotrauma* (2012) 29:1650–62. doi:10.1089/neu.2011.2100
90. Hipolito L, Wilson-Poe A, Campos-Jurado Y, Zhong E, Gonzalez-Romero J, Virag L, et al. Inflammatory pain promotes increased opioid self-administration: role of dysregulated ventral tegmental area mu opioid receptors. *J Neurosci* (2015) 35:12217–31. doi:10.1523/JNEUROSCI.1053-15.2015
91. Kaufling J, Aston-Jones G. Persistent adaptations in afferents to ventral tegmental dopamine neurons after opiate withdrawal. *J Neurosci* (2015) 35:10290–303. doi:10.1523/JNEUROSCI.0715-15.2015
92. Park PE, Schlosburg JE, Vendruscolo LF, Schulteis G, Edwards S, Koob GF. Chronic CRF1 receptor blockade reduces heroin intake escalation and dependence-induced hyperalgesia. *Addict Biol* (2015) 20:275–84. doi:10.1111/adb.12120
93. Wade CL, Vendruscolo LF, Schlosburg JE, Hernandez DO, Koob GF. Compulsive-like responding for opioid analgesics in rats with extended access. *Neuropsychopharmacology* (2015) 40:421–8. doi:10.1038/npp.2014.188
94. Wade CL, Krumenacher P, Kitto KF, Peterson CD, Wilcox GL, Fairbanks CA. Effect of chronic pain on fentanyl self-administration in mice. *PLoS One* (2013) 8:e79239. doi:10.1371/journal.pone.0079239
95. Lotsch J, Walter C, Parnham MJ, Oertel BG, Geisslinger G. Pharmacokinetics of non-intravenous formulations of fentanyl. *Clin Pharmacokinet* (2013) 52:23–36. doi:10.1007/s40262-012-0016-7
96. Hou YY, Cai YQ, Pan ZZ. Persistent pain maintains morphine-seeking behavior after morphine withdrawal through reduced MeCP2 repression of GluA1 in rat central amygdala. *J Neurosci* (2015) 35:3689–700. doi:10.1523/JNEUROSCI.3453-14.2015
97. Worley MJ, Heinzerling KG, Shoptaw S, Ling W. Volatility and change in chronic pain severity predict outcomes of treatment for prescription opioid addiction. *Addiction* (2017) 112:1202–9. doi:10.1111/add.13782
98. Sharma A, Kelly SM, Mitchell SG, Gryczynski J, O'Grady KE, Schwartz RP. Update on barriers to pharmacotherapy for opioid use disorders. *Curr Psychiatry Rep* (2017) 19:35. doi:10.1007/s11920-017-0783-9
99. Weiss RD, Potter JS, Fiellin DA, Byrne M, Connery HS, Dickinson W, et al. Adjunctive counseling during brief and extended buprenorphine-naloxone

treatment for prescription opioid dependence: a 2-phase randomized controlled trial. *Arch Gen Psychiatry* (2011) 68:1238–46. doi:10.1001/archgenpsychiatry.2011.121

100. Worley MJ, Heinzerling KG, Shoptaw S, Ling W. Pain volatility and prescription opioid addiction treatment outcomes in patients with chronic pain. *Exp Clin Psychopharmacol* (2015) 23:428–35. doi:10.1037/pha0000039
101. Griffin ML, Mcdermott KA, Mchugh RK, Fitzmaurice GM, Jamison RN, Weiss RD. Longitudinal association between pain severity and subsequent opioid use in prescription opioid dependent patients with chronic pain. *Drug Alcohol Depend* (2016) 163:216–21. doi:10.1016/j.drugalcdep.2016.04.023
102. Seltzman HH, Shiner C, Hirt EE, Gilliam AF, Thomas BF, Maitra R, et al. Peripherally selective cannabinoid 1 receptor (cb1r) agonists for the treatment of neuropathic pain. *J Med Chem* (2016) 59:7525–43. doi:10.1021/acs.jmedchem.6b00516
103. Kaiser RS, Mooreville M, Kannan K. Psychological interventions for the management of chronic pain: a review of current evidence. *Curr Pain Headache Rep* (2015) 19:43. doi:10.1007/s11916-015-0517-9
104. Heel RC, Brogden RN, Speight TM, Avery GS. Buprenorphine: a review of its pharmacological properties and therapeutic efficacy. *Drugs* (1979) 17:81–110. doi:10.2165/00003495-197917020-00001
105. Lutfy K, Cowan A. Buprenorphine: a unique drug with complex pharmacology. *Curr Neuropharmacol* (2004) 2:395–402. doi:10.2174/1570159043359477
106. Brown SM, Holtzman M, Kim T, Kharasch ED. Buprenorphine metabolites, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide, are biologically active. *Anesthesiology* (2011) 115:1251–60. doi:10.1097/ALN.0b013e318238fea0
107. Lane ME. The transdermal delivery of fentanyl. *Eur J Pharm Biopharm* (2013) 84:449–55. doi:10.1016/j.ejpb.2013.01.018
108. Smith HS, Colson J, Sehgal N. An update of evaluation of intravenous sedation on diagnostic spinal injection procedures. *Pain Physician* (2013) 16:SE217–28.
109. Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, et al. Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol* (1994) 45:330–4.
110. Bell J. Pharmacological maintenance treatments of opiate addiction. *Br J Clin Pharmacol* (2014) 77:253–63. doi:10.1111/bcp.12051
111. Oviedo-Joekes E, Guh D, Brissette S, Marchand K, Macdonald S, Lock K, et al. Hydromorphone compared with diacetylmorphine for long-term opioid dependence: a randomized clinical trial. *JAMA Psychiatry* (2016) 73:447–55. doi:10.1001/jamapsychiatry.2016.0109
112. Oye KA, Lawson JC, Bubela T. Drugs: regulate 'home-brew' opiates. *Nature* (2015) 521:281–3. doi:10.1038/521281a
113. *Opioids in Palliative Care: Safe and Effective Prescribing of Strong Opioids for Pain in Palliative Care of Adults*. Cardiff UK: National Collaborating Centre for Cancer (2012).
114. Friedrichsdorf SJ, Postier A. Management of breakthrough pain in children with cancer. *J Pain Res* (2014) 7:117–23. doi:10.2147/JPR.S58862
115. Uchtenhagen AA. Heroin maintenance treatment: from idea to research to practice. *Drug Alcohol Rev* (2011) 30:130–7. doi:10.1111/j.1465-3362.2010.00266.x
116. Gottas A, Oiestad EL, Boix F, Vindenes V, Ripel A, Thaulow CH, et al. Levels of heroin and its metabolites in blood and brain extracellular fluid after i.v. heroin administration to freely moving rats. *Br J Pharmacol* (2013) 170:546–56. doi:10.1111/bph.12305
117. Vadivelu N, Schermer E, Kodumudi G, Berger JM. The clinical applications of extended-release abuse-deterrent opioids. *CNS Drugs* (2016) 30:637–46. doi:10.1007/s40263-016-0357-0
118. Vallejo R, Barkin RL, Wang VC. Pharmacology of opioids in the treatment of chronic pain syndromes. *Pain Physician* (2011) 14:E343–60.
119. Harris SC, Cipriano A, Colucci SV, Kapil RP, Geoffroy P, Hopyan T, et al. Intranasal abuse potential, pharmacokinetics, and safety of once-daily, single-entity, extended-release hydrocodone (HYD) in recreational opioid users. *Pain Med* (2016) 17:820–31. doi:10.1093/pm/pnv004
120. *Dilaudid® Oral Liquid and Dilaudid® Tablets [Online]*. (2007). Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/019892s015lbl.pdf (Accessed: August 15, 2017).
121. MORPHINE. *Toxinet: Toxicology Data Network [Online]*. NIH Division of Specialized Information Services: US National Library of Medicine (2016). Available from: https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+2134 (Accessed: August 15, 2017).
122. Bao YJ, Hou W, Kong XY, Yang L, Xia J, Hua BJ, et al. Hydromorphone for cancer pain. *Cochrane Database Syst Rev* (2016) 10:CD011108.
123. Sarhill N, Walsh D, Nelson KA. Hydromorphone: pharmacology and clinical applications in cancer patients. *Support Care Cancer* (2001) 9:84–96. doi:10.1007/s005200000183
124. Ananthan S, Khare NK, Saini SK, Seitz LE, Bartlett JL, Davis P, et al. Identification of opioid ligands possessing mixed micro agonist/delta antagonist activity among pyridomorphinans derived from naloxone, oxymorphone, and hydromorphone [correction of hydropmorphone]. *J Med Chem* (2004) 47:1400–12. doi:10.1021/jm0400597
125. Trescot AM, Datta S, Lee M, Hansen H. Opioid pharmacology. *Pain Physician* (2008) 11:S133–53.
126. Schwertschlag U, Schrier RW, Wilson P. Beneficial effects of calcium channel blockers and calmodulin binding drugs on in vitro renal cell anoxia. *J Pharmacol Exp Ther* (1986) 238:119–24.
127. Inturrisi CE. Pharmacology of methadone and its isomers. *Minerva Anestesiol* (2005) 71:435–7.
128. Pathan H, Williams J. Basic opioid pharmacology: an update. *Br J Pain* (2012) 6:11–6. doi:10.1177/2049463712438493
129. Cardos SF, Florman AL, Simberkoff MS, Lanier L. *Serratia marcescens*: use of detailed characterization of strains to evaluate an increase of isolates in an intensive care unit. *Am J Med Sci* (1973) 266:447–52. doi:10.1097/00000441-197312000-00006
130. Casy AF, Parfitt RT. *Opioid Analgesics [Online]*. 1 ed. US: Springer (1986).
131. Ternes JW, O'Brien CP. The opioids: abuse liability and treatments for dependence. *Adv Alcohol Subst Abuse* (1990) 9:27–45. doi:10.1300/J251v09n01_03
132. Brown R, Kraus C, Fleming M, Reddy S. Methadone: applied pharmacology and use as adjunctive treatment in chronic pain. *Postgrad Med J* (2004) 80:654–9. doi:10.1136/pgmj.2004.022988
133. Onoyovwe A, Hagel JM, Chen X, Khan MF, Schriemer DC, Facchini PJ. Morphine biosynthesis in opium poppy involves two cell types: sieve elements and laticifers. *Plant Cell* (2013) 25:4110–22. doi:10.1105/tpc.113.115113
134. Gupta M, Msambichaka L, Ballas SK, Gupta K. Morphine for the treatment of pain in sickle cell disease. *ScientificWorldJournal* (2015) 2015:540154. doi:10.1155/2015/540154
135. Yekkirala AS, Kalyuzhny AE, Portoghese PS. Standard opioid agonists activate heteromeric opioid receptors: evidence for morphine and [d-Ala(2)-MePhe(4)-Glyol(5)]enkephalin as selective mu-delta agonists. *ACS Chem Neurosci* (2010) 1:146–54. doi:10.1021/cn9000236
136. Wightman R, Perrone J, Portelli I, Nelson L. Likeability and abuse liability of commonly prescribed opioids. *J Med Toxicol* (2012) 8:335–40. doi:10.1007/s13181-012-0263-x
137. Ordonez Gallego A, Gonzalez Baron M, Espinosa Arranz E. Oxycodone: a pharmacological and clinical review. *Clin Transl Oncol* (2007) 9:298–307. doi:10.1007/s12094-007-0057-9
138. Kimishima A, Umihara H, Mizoguchi A, Yokoshima S, Fukuyama T. Synthesis of (-)-oxycodone. *Org Lett* (2014) 16:6244–7. doi:10.1021/ol503175n
139. Lugo RA, Kern SE. Clinical pharmacokinetics of morphine. *J Pain Palliat Care Pharmacother* (2002) 16:5–18. doi:10.1080/J354v16n04_02
140. Okura T, Hattori A, Takano Y, Sato T, Hammarlund-Udenaes M, Terasaki T, et al. Involvement of the pyrilamine transporter, a putative organic cation transporter, in blood-brain barrier transport of oxycodone. *Drug Metab Dispos* (2008) 36:2005–13. doi:10.1124/dmd.108.022087
141. Prommer E. Oxymorphone: a review. *Support Care Cancer* (2006) 14:109–15. doi:10.1007/s00520-005-0917-1
142. Olkkola KT, Kontinen VK, Saari TI, Kalso EA. Does the pharmacology of oxycodone justify its increasing use as an analgesic? *Trends Pharmacol Sci* (2013) 34:206–14. doi:10.1016/j.tips.2013.02.001
143. Michelsen LG, Hug CC Jr. The pharmacokinetics of remifentanil. *J Clin Anesth* (1996) 8:679–82. doi:10.1016/S0952-8180(96)00179-1
144. Feldman PL, Chorghade MS. Discovery and development of the ultrashort-acting analgesic remifentanil. In: Chorghade MS, editor. *Drug Discovery and Development*. Wiley online library (2006). p. 339–42.
145. Gesztesi Z, Mootz BL, White PF. The use of a remifentanil infusion for hemodynamic control during intracranial surgery. *Anesth Analg* (1999) 89:1282–7. doi:10.1097/00000539-199911000-00038

146. Grottke O, Dietrich PJ, Wiegels S, Wappler F. Intraoperative wake-up test and postoperative emergence in patients undergoing spinal surgery: a comparison of intravenous and inhaled anesthetic techniques using short-acting anesthetics. *Anesth Analg* (2004) 99:1521–7. doi:10.1213/01.ANE.0000134684.25322.26
147. Knapik M, Knapik P, Nadziakiewicz P, Misiolek H, Saucha W, Walaszczyk M, et al. Comparison of remifentanil or fentanyl administration during isoflurane anesthesia for coronary artery bypass surgery. *Med Sci Monit* (2006) 12:I33–8.
148. De Baerdemaeker LE, Jacobs S, Pattyn P, Mortier EP, Struys MM. Influence of intraoperative opioid on postoperative pain and pulmonary function after laparoscopic gastric banding: remifentanil TCI vs sufentanil TCI in morbid obesity. *Br J Anaesth* (2007) 99:404–11. doi:10.1093/bja/aem164
149. Dayer P, Desmeules J, Collart L. [Pharmacology of tramadol]. *Drugs* (1997) 53(Suppl 2):18–24. doi:10.2165/00003495-199700532-00006
150. Grond S, Sablotzki A. Clinical pharmacology of tramadol. *Clin Pharmacokinet* (2004) 43:879–923. doi:10.2165/00003088-200443130-00004
151. Hennies HH, Friderichs E, Schneider J. Receptor binding, analgesic and antitussive potency of tramadol and other selected opioids. *Arzneimittelforschung* (1988) 38:877–80.
152. Leppert W. Tramadol as an analgesic for mild to moderate cancer pain. *Pharmacol Rep* (2009) 61:978–92. doi:10.1016/S1734-1140(09)70159-8
153. Williams JT, Ingram SL, Henderson G, Chavkin C, Von Zastrow M, Schulz S, et al. Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* (2013) 65:223–54. doi:10.1124/pr.112.005942
154. Shukla AK, Violin JD, Whalen EJ, Gesty-Palmer D, Shenoy SK, Lefkowitz RJ. Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors. *Proc Natl Acad Sci U S A* (2008) 105:9988–93. doi:10.1073/pnas.0804246105
155. Priestley R, Glass M, Kendall D. Functional selectivity at cannabinoid receptors. *Adv Pharmacol* (2017) 80:207–21. doi:10.1016/bs.apha.2017.03.005
156. Siuda ER, Carr R III, Rominger DH, Violin JD. Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. *Curr Opin Pharmacol* (2017) 32:77–84. doi:10.1016/j.coph.2016.11.007
157. Soergel DG, Subach RA, Burnham N, Lark MW, James IE, Sadler BM, et al. Biased agonism of the mu-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: a randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain* (2014) 155:1829–35. doi:10.1016/j.pain.2014.06.011
158. Manglik A, Lin H, Aryal DK, Mccorvy JD, Dengler D, Corder G, et al. Structure-based discovery of opioid analgesics with reduced side effects. *Nature* (2016) 537:185–90. doi:10.1038/nature19112
159. Koblish M, Carr R III, Siuda ER, Rominger DH, Gowen-Macdonald W, Cowan CL, et al. TRV0109101, a G protein-biased agonist of the micro-opioid receptor, does not promote opioid-induced mechanical allodynia following chronic administration. *J Pharmacol Exp Ther* (2017) 362:254–62. doi:10.1124/jpet.117.241117
160. Dewire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, et al. A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* (2013) 344:708–17. doi:10.1124/jpet.112.201616
161. Altarifi AA, David B, Muchhala KH, Blough BE, Akbarali H, Negus SS. Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. *J Psychopharmacol* (2017) 31:730–9. doi:10.1177/0269881116689257
162. Raehal KM, Bohn LM. The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* (2011) 60:58–65. doi:10.1016/j.neuropharm.2010.08.003
163. Thompson GL, Kelly E, Christopoulos A, Canals M. Novel GPCR paradigms at the mu-opioid receptor. *Br J Pharmacol* (2015) 172:287–96. doi:10.1111/bph.12600
164. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev* (2009) 89:1379–412. doi:10.1152/physrev.00005.2009
165. Lalley PM. Mu-opioid receptor agonist effects on medullary respiratory neurons in the cat: evidence for involvement in certain types of ventilatory disturbances. *Am J Physiol Regul Integr Comp Physiol* (2003) 285:R1287–304. doi:10.1152/ajpregu.00199.2003
166. Schwarzacher SW, Rub U, Deller T. Neuroanatomical characteristics of the human pre-Botzinger complex and its involvement in neurodegenerative brainstem diseases. *Brain* (2011) 134:24–35. doi:10.1093/brain/awq327
167. Farre M, Cami J. Pharmacokinetic considerations in abuse liability evaluation. *Br J Addict* (1991) 86:1601–6. doi:10.1111/j.1360-0443.1991.tb01754.x
168. Comer SD, Ashworth JB, Sullivan MA, Vosburg SK, Saccone PA, Foltin RW. Relationship between rate of infusion and reinforcing strength of oxycodone in humans. *J Opioid Manag* (2009) 5:203–12. doi:10.5055/jom.2009.0022
169. Berkowitz BA. The relationship of pharmacokinetics to pharmacological activity: morphine, methadone and naloxone. *Clin Pharmacokinet* (1976) 1:219–30. doi:10.2165/00003088-197601030-00004
170. Sawynok J. The therapeutic use of heroin: a review of the pharmacological literature. *Can J Physiol Pharmacol* (1986) 64:1–6. doi:10.1139/y86-001
171. Ciccarone D, Ondocsin J, Mars SG. Heroin uncertainties: exploring users' perceptions of fentanyl-adulterated and -substituted 'heroin'. *Int J Drug Policy* (2017) 46:146–55. doi:10.1016/j.drugpo.2017.06.004
172. Bonhomme J, Shim RS, Gooden R, Tyus D, Rust G. Opioid addiction and abuse in primary care practice: a comparison of methadone and buprenorphine as treatment options. *J Natl Med Assoc* (2012) 104:342–50. doi:10.1016/S0027-9684(15)30175-9
173. Jones JD, Sullivan MA, Vosburg SK, Manubay JM, Mogali S, Metz V, et al. Abuse potential of intranasal buprenorphine versus buprenorphine/naloxone in buprenorphine-maintained heroin users. *Addict Biol* (2015) 20:784–98. doi:10.1111/adb.12163
174. Gilbert IA, Regnard J, Lenner KA, Nelson JA, Mcfadden ER Jr. Intrathoracic airstream temperatures during acute expansions of thoracic blood volume. *Clin Sci (Lond)* (1991) 81:655–61. doi:10.1042/cs0810655
175. Elkader A, Sproule B. Buprenorphine: clinical pharmacokinetics in the treatment of opioid dependence. *Clin Pharmacokinet* (2005) 44:661–80. doi:10.2165/00003088-200544070-00001
176. Kuhlman JJ Jr, Lalani S, Magluilo J Jr, Levine B, Darwin WD. Human pharmacokinetics of intravenous, sublingual, and buccal buprenorphine. *J Anal Toxicol* (1996) 20:369–78. doi:10.1093/jat/20.6.369
177. Brown SM, Campbell SD, Crafford A, Regina KJ, Holtzman MJ, Kharasch ED. P-glycoprotein is a major determinant of norbuprenorphine brain exposure and antinociception. *J Pharmacol Exp Ther* (2012) 343:53–61. doi:10.1124/jpet.112.193433
178. Cobaugh DJ, Gainor C, Gaston CL, Kwong TC, Magnani B, Mcpherson ML, et al. The opioid abuse and misuse epidemic: implications for pharmacists in hospitals and health systems. *Am J Health Syst Pharm* (2014) 71:1539–54. doi:10.2146/ajhp140157
179. Dart RC, Surratt HL, Cicero TJ, Parrino MW, Severtson SG, Bucher-Bartelson B, et al. Trends in opioid analgesic abuse and mortality in the United States. *N Engl J Med* (2015) 372:241–8. doi:10.1056/NEJMsa1406143
180. Suzuki J, El-Haddad S. A review: fentanyl and non-pharmaceutical fentanyls. *Drug Alcohol Depend* (2017) 171:107–16. doi:10.1016/j.drugalcdep.2016.11.033
181. Mather LE. Clinical pharmacokinetics of fentanyl and its newer derivatives. *Clin Pharmacokinet* (1983) 8:422–46. doi:10.2165/00003088-198308050-00004
182. Smith HS. Opioid metabolism. *Mayo Clin Proc* (2009) 84:613–24. doi:10.1016/S0025-6196(11)60750-7
183. Schug SA, Ting S. Fentanyl formulations in the management of pain: an update. *Drugs* (2017) 77:747–63. doi:10.1007/s40265-017-0727-z
184. Jones CM. Heroin use and heroin use risk behaviors among nonmedical users of prescription opioid pain relievers – United States, 2002-2004 and 2008-2010. *Drug Alcohol Depend* (2013) 132:95–100. doi:10.1016/j.drugalcdep.2013.01.007
185. Osborne R, Joel S, Grebenik K, Trew D, Slevin M. The pharmacokinetics of morphine and morphine glucuronides in kidney failure. *Clin Pharmacol Ther* (1993) 54:158–67. doi:10.1038/clpt.1993.127
186. Oldendorf WH, Hyman S, Braun L, Oldendorf SZ. Blood-brain barrier: penetration of morphine, codeine, heroin, and methadone after carotid injection. *Science* (1972) 178:984–6. doi:10.1126/science.178.4064.984

187. Firestein GS, Budd R, Gabriel SE, Mcinnes IB, O'Dell JR. *Kelley and Firestein's Textbook of Rheumatology E-Book*. 10th ed. Elsevier (2017).
188. Brucker MC, King TL. *Pharmacology for Women's Health*. 2nd ed. J.a.B. Learning, editor. Burlington MA: Jones and Bartlett Learning (2017).
189. Vallner JJ, Stewart JT, Kotzan JA, Kirsten EB, Honigberg IL. Pharmacokinetics and bioavailability of hydromorphone following intravenous and oral administration to human subjects. *J Clin Pharmacol* (1981) 21:152–6. doi:10.1002/j.1552-4604.1981.tb05693.x
190. Barakat NH, Atayee RS, Best BM, Ma JD, Jones JD, Sullivan MA, et al. Urinary hydrocodone and metabolite distributions in pain patients abuse potential of intranasal buprenorphine versus buprenorphine/naloxone in buprenorphine-maintained heroin users. *J Anal Toxicol* (2014) 38:404–9. doi:10.1093/jat/bku051
191. Toyama K, Uchida N, Ishizuka H, Sambe T, Kobayashi S. Single-dose evaluation of safety, tolerability and pharmacokinetics of newly formulated hydromorphone immediate-release and hydrophilic matrix extended-release tablets in healthy Japanese subjects without co-administration of an opioid antagonist. *J Clin Pharmacol* (2015) 55:975–84. doi:10.1002/jcph.501
192. Inturrisi CE. Clinical pharmacology of opioids for pain. *Clin J Pain* (2002) 18:S3–13. doi:10.1097/00002508-200207001-00002
193. Wright AW, Mather LE, Smith MT. Hydromorphone-3-glucuronide: a more potent neuro-excitant than its structural analogue, morphine-3-glucuronide. *Life Sci* (2001) 69:409–20. doi:10.1016/S0024-3205(01)01133-X
194. Coda BA, Rudy AC, Archer SM, Wermeling DP. Pharmacokinetics and bioavailability of single-dose intranasal hydromorphone hydrochloride in healthy volunteers. *Anesth Analg* (2003) 97:117–23. doi:10.1213/01.ANE.0000066311.40978.4F
195. Ferrari A, Coccia CP, Bertolini A, Sternieri E. Methadone – metabolism, pharmacokinetics and interactions. *Pharmacol Res* (2004) 50:551–9. doi:10.1016/j.phrs.2004.05.002
196. Preston KL, Epstein DH, Davoudzadeh D, Huestis MA. Methadone and metabolite urine concentrations in patients maintained on methadone. *J Anal Toxicol* (2003) 27:332–41. doi:10.1093/jat/27.6.332
197. Kilpatrick GJ, Smith TW. Morphine-6-glucuronide: actions and mechanisms. *Med Res Rev* (2005) 25:521–44. doi:10.1002/med.20035
198. Hasselstrom J, Sawe J. Morphine pharmacokinetics and metabolism in humans. Enterohepatic cycling and relative contribution of metabolites to active opioid concentrations. *Clin Pharmacokinet* (1993) 24:344–54.
199. Takala A, Kaasalainen V, Seppala T, Kalso E, Olkkola KT. Pharmacokinetic comparison of intravenous and intranasal administration of oxycodone. *Acta Anaesthesiol Scand* (1997) 41(2):309–12.
200. Lalovic B, Kharasch E, Hoffer C, Risler L, Liu-Chen LY, Shen DD. Pharmacokinetics and pharmacodynamics of oral oxycodone in healthy human subjects: role of circulating active metabolites. *Clin Pharmacol Ther* (2006) 79:461–79. doi:10.1016/j.clpt.2006.01.009
201. Lemberg KK, Siiskonen AO, Kontinen VK, Yli-Kauhaluoma JT, Kalso EA. Pharmacological characterization of noroxymorphone as a new opioid for spinal analgesia. *Anesth Analg* (2008) 106:463–70. doi:10.1213/ane.0b013e3181605a15
202. Bostrom E, Simonsson US, Hammarlund-Udenaes M. In vivo blood-brain barrier transport of oxycodone in the rat: indications for active influx and implications for pharmacokinetics/pharmacodynamics. *Drug Metab Dispos* (2006) 34:1624–31. doi:10.1124/dmd.106.009746
203. Baylon GJ, Kaplan HL, Somer G, Busto UE, Sellers EM. Comparative abuse liability of intravenously administered remifentanil and fentanyl. *J Clin Psychopharmacol*(2000)20:597–606.doi:10.1097/00004714-200012000-00002
204. Levine AI, Bryson EO. Intranasal self-administration of remifentanil as the foray into opioid abuse by an anesthesia resident. *Anesth Analg* (2010) 110:524–5. doi:10.1213/ANE.0b013e3181c5f069
205. Hoke JF, Cunningham F, James MK, Muir KT, Hoffman WE. Comparative pharmacokinetics and pharmacodynamics of remifentanil, its principle metabolite (GR90291) and alfentanil in dogs. *J Pharmacol Exp Ther* (1997) 281:226–32.
206. Christrup LL. Morphine metabolites. *Acta Anaesthesiol Scand* (1997) 41:116–22. doi:10.1111/j.1399-6576.1997.tb04625.x
207. Kitamura A, Higuchi K, Okura T, Deguchi Y. Transport characteristics of tramadol in the blood-brain barrier. *J Pharm Sci* (2014) 103:3335–41. doi:10.1002/jps.24129
208. Miyazaki T, Choi IY, Rubas W, Anand NK, Ali C, Evans J, et al. NKTR-181: a novel mu-opioid analgesic with inherently low abuse potential. *J Pharmacol Exp Ther* (2017) 363:104–13. doi:10.1124/jpet.117.243030
209. Webster L, Henningfield J, Buchhalter AR, Siddhanti S, Lu L, Odinecs A, et al. Human abuse potential of the new opioid analgesic molecule NKTR-181 compared with oxycodone. *Pain Med* (2018) 19(2):307–18. doi:10.1093/pm/pnw344
210. Lipkowski AW, Konecka AM, Sroczynska I, Przewlocki R, Stala L, Tam SW. Bivalent opioid peptide analogues with reduced distances between pharmacophores. *Life Sci* (1987) 40:2283–8. doi:10.1016/0024-3205(87)90065-8
211. Toll L, Khroyan TV, Polgar WE, Jiang F, Olsen C, Zaveri NT. Comparison of the antinociceptive and antirewarding profiles of novel bifunctional nociceptin receptor/mu-opioid receptor ligands: implications for therapeutic applications. *J Pharmacol Exp Ther* (2009) 331:954–64. doi:10.1124/jpet.109.157446
212. Zaveri NT, Jiang F, Olsen C, Polgar WE, Toll L. Designing bifunctional NOP receptor-mu opioid receptor ligands from NOP receptor-selective scaffolds. Part I. *Bioorg Med Chem Lett* (2013) 23:3308–13. doi:10.1016/j.bmcl.2013.03.101
213. Bird MF, Cerlesi MC, Brown M, Malfacini D, Vezzi V, Molinari P, et al. Characterisation of the novel mixed Mu-NOP peptide ligand dermorphin-N/OFQ (DeNo). *PLoS One* (2016) 11:e0156897. doi:10.1371/journal.pone.0156897
214. Bloms-Funke P, Gillen C, Schuettler AJ, Wnendt S. Agonistic effects of the opioid buprenorphine on the nociceptin/OFQ receptor. *Peptides* (2000) 21:1141–6. doi:10.1016/S0196-9781(00)00252-7
215. Lutfy K, Eitan S, Bryant CD, Yang YC, Saliminejad N, Walwyn W, et al. Buprenorphine-induced antinociception is mediated by mu-opioid receptors and compromised by concomitant activation of opioid receptor-like receptors. *J Neurosci* (2003) 23:10331–7.
216. Vardanyan RS, Cain JP, Haghighi SM, Kumirov VK, Mcintosh MI, Sandweiss AJ, et al. Synthesis and investigation of mixed mu-opioid and delta-opioid agonists as possible bivalent ligands for treatment of pain. *J Heterocycl Chem* (2017) 54:1228–35. doi:10.1002/jhet.2696
217. Peterson CD, Kitto KF, Akgun E, Lunzer MM, Riedl MS, Vulchanova L, et al. Bivalent ligand that activates mu opioid receptor and antagonizes mGluR5 receptor reduces neuropathic pain in mice. *Pain* (2017) 158:2431–41. doi:10.1097/j.pain.0000000000001050
218. Khroyan TV, Cippitelli A, Toll N, Lawson JA, Crossman W, Polgar WE, et al. In vitro and in vivo profile of PPL-101 and PPL-103: mixed opioid partial agonist analgesics with low abuse potential. *Front Psychiatry* (2017) 8:52. doi:10.3389/fpsyt.2017.00052
219. Livingston KE, Stanczyk MA, Burford NT, Alt A, Canals M, Traynor JR. Pharmacologic evidence for a putative conserved allosteric site on opioid receptors. *Mol Pharmacol* (2018) 93:157–67. doi:10.1124/mol.117.109561

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Critical Role for $G_{i/o}$-Protein Activity in the Dorsal Striatum in the Reduction of Voluntary Alcohol Intake in C57Bl/6 Mice

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The transition from non-dependent alcohol use to alcohol dependence involves increased activity of the dorsal striatum. Interestingly, the dorsal striatum expresses a large number of inhibitory G-protein-coupled receptors (GPCRs), which when activated may inhibit alcohol-induced increased activity and can decrease alcohol consumption. Here, we explore the hypothesis that dorsal striatal $G_{i/o}$-protein activation is sufficient to reduce voluntary alcohol intake. Using a voluntary, limited-access, two-bottle choice, drink-in-the-dark model of alcohol (10%) consumption, we validated the importance of $G_{i/o}$ signaling in this region by locally expressing neuron-specific, adeno-associated-virus encoded $G_{i/o}$-coupled muscarinic M_4 designer receptors exclusively activated by designer drugs (DREADD) in the dorsal striatum and observed a decrease in alcohol intake upon DREADD activation. We validated our findings by activating $G_{i/o}$-coupled delta-opioid receptors (DORs), which are natively expressed in the dorsal striatum, using either a G-protein biased agonist or a β-arrestin-biased agonist. Local infusion of TAN-67, an *in vitro*-determined $G_{i/o}$-protein biased DOR agonist, decreased voluntary alcohol intake in wild-type and β-arrestin-2 knockout (KO) mice. SNC80, a β-arrestin-2 biased DOR agonist, increased alcohol intake in wild-type mice; however, SNC80 decreased alcohol intake in β-arrestin-2 KO mice, thus resulting in a behavioral outcome generally observed for $G_{i/o}$-biased agonists and suggesting that β-arrestin recruitment is required for SNC80-increased alcohol intake. Overall, these results suggest that activation $G_{i/o}$-coupled GPCRs expressed in the dorsal striatum, such as the DOR, by G-protein biased agonists may be a potential strategy to decrease voluntary alcohol consumption and β-arrestin recruitment is to be avoided.

Keywords: dorsal striatum, alcohol intake, biased signaling, delta-opioid receptor, β-arrestin, designer receptors exclusively activated by designer drugs, C57BL/6 mice

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INTRODUCTION

Alcoholism and alcohol abuse is a widespread health issue, placing a large burden at both the individual and societal level. Yet, pharmacological treatment options are still limited. Currently, only three drugs have been approved by the Food and Drug Administration for the treatment of alcohol use disorders (AUD), and each come with their own limitations in therapeutic efficacy (1); therefore,

it is imperative to identify novel targets for more effective drug development, with hopes of increasing the number of treatment options and compliance for AUD management.

One potential AUD treatment approach is to increase inhibition of the dorsal striatum, a brain region with observed increasing activation upon alcohol tasting in heavy alcohol drinking human subjects (2). In contrast to the ventral striatum, which is implicated in reward-associated learning and behavior, the dorsal striatum is heavily involved in the transition to compulsive drug or alcohol seeking and taking (2–5). In rats, habitual alcohol self-administration increases habit-like responding with decreased sensitivity to alcohol devaluation (6). This shift toward habit-like responding, as well as reports of increased hyperexcitability and altered glutamatergic and GABAergic transmission in the dorsomedial striatum upon alcohol exposure (7–9), suggests molecular alterations in this brain region lead to behavioral reinforcement of alcohol intake resulting in habitual, excessive alcohol intake (3, 7, 9). We hypothesized that one conceivable strategy to inhibit this alcohol-induced neuronal excitability is by activation of metabotropic, inhibitory $G_{i/o}$-protein signaling pathways *via* G-protein-coupled receptors (GPCRs) expressed on neurons in this region.

Interestingly, a large number of GPCRs expressed in the dorsal striatum couple to inhibitory G proteins ($G_{i/o}$) (10, 11), thereby providing an ideal target for inhibiting this hyperexcitability observed in the dorsal striatum following persistent alcohol use. To investigate our hypothesis, $G_{i/o}$-coupled designer receptors exclusively activated by designer drugs (DREADDs) can provide powerful tools (12, 13) to increase $G_{i/o}$ signaling in a specific brain region, such as the dorsal striatum, on an experimenter's predetermined time point to determine the role of the dorsal striatum in modulating alcohol consumption. In addition to artificially increasing $G_{i/o}$ signaling using viral DREADD strategies, agonists have been developed to preferentially activate $G_{i/o}$-protein signal pathways over the competing β-arrestin recruitment and signaling pathways for receptors endogenously expressed in the dorsal striatum, with recent advances in opioid receptor pharmacology being a prime example (14–16). For this study, the delta-opioid receptor (DOR), a $G_{i/o}$-coupled GPCR with strong expression in the dorsal striatum (17), provided us with a powerful tool to investigate our hypothesis that $G_{i/o}$ signaling in the dorsal striatum can reduce alcohol use. DORs are thought to play a protective role in AUD, as DOR knockout (KO) mice display increased alcohol consumption and preference compared with wild-type, kappa-, or mu-opioid receptor KO mice, suggesting that DOR expression prevents escalated alcohol intake compared with other opioid receptor subtypes (18). Moreover, DORs are heavily expressed in the dorsal striatum presynaptically on corticostriatal glutamatergic inputs (19), both pre- and postsynaptically on interneurons within this brain region, and enriched on D_2 receptor-expressing MSNs (as compared with D_1 receptor-expressing MSNs) (20–22). Furthermore, direct activation (23) or indirect activation of DORs *via* alcohol-induced release of endogenous enkephalins (24) in the dorsal striatum induces long-term depression (LTD).

The importance of the activation of dorsal striatal DORs in the modulation of alcohol intake was first evident in a report by Nielsen et al., where infusion of the DOR-selective agonist SNC80 into the dorsal striatum increased alcohol intake in rats while the DOR antagonist naltrindole reduced intake (25). This finding that DOR agonist SNC80 increased alcohol was somewhat surprising as DOR expression was previously mentioned to be protective against increased alcohol intake (18). Yet, our recent work investigating a panel of DOR agonists suggests that SNC80 prefers to recruit β-arrestin protein through a mechanism called biased signaling (also termed functional selectivity) (26, 27), where we have additionally correlated *in vitro* β-arrestin recruitment with *in vivo* increased alcohol intake (28). In that same study investigating the behavioral effects of DOR biased signaling, we also observed that DOR agonists that weakly recruit β-arrestin, particularly TAN-67 (and thus are G-protein-biased), decreased alcohol intake in mice in a limited-access, drinking-in-the-dark (DID) protocol to 10% alcohol (28).

Therefore, here we hypothesized that activation of $G_{i/o}$ signaling in the dorsal striatum would be beneficial in reducing alcohol intake, whereas β-arrestin signaling will lead to enhanced alcohol use. To begin to investigate this hypothesis, we first utilized hM_4Di DREADD technology (12) to identify the broad role of $G_{i/o}$-coupled receptor activation in the dorsal striatal on voluntary alcohol intake in C57Bl/6 male mice using a two-bottle choice, limited-access DID protocol. In addition, we selectively infused our previously identified differentially biased DOR agonists in wild-type and β-arrestin-2 KO mice to more specifically investigate the effect of increased dorsal striatal DOR $G_{i/o}$-protein signaling (versus β-arrestin) on voluntary alcohol intake.

MATERIALS AND METHODS

Drugs and Chemicals

SNC80 and SB205607 (TAN-67) were purchased from Tocris, R&D systems (Minneapolis, MN, USA); naltrindole hydrochloride, forskolin, 200 proof ethyl alcohol, leu-enkephalin, sodium chloride, DMSO, cocaine hydrochloride, and clozapine-*N*-oxide (CNO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For dorsal striatal infusion studies, TAN-67 and SNC80 were diluted in 0.9% saline to a concentration of 10 μM; for cellular assays, drugs were dissolved in water. Cocaine was dissolved in 0.9% saline for an administered dose of 15 mg/kg, and CNO was dissolved in 100% DMSO and diluted to a concentration of 0.2 mg/ml in saline (final DMSO concentration of 0.5% and administered dose of 2 mg/kg). Both cocaine and CNO were injected intraperitoneally (i.p.) during experimentation. Non-Cre-dependent AAV8-hSyn-hM_4Di-mCherry (7.4×10^{12} vg/ml) virus and AAV8-hSyn-EGFP (3.9×10^{12} vg/ml) virus were obtained from the University of North Carolina Vector Core. Both viruses were chosen as they specifically express in neurons through use of the synapsin promoter. A 100 mg/kg ketamine (Henry Schein, Dublin, OH, USA):10 mg/kg xylazine (Sigma-Aldrich) cocktail was administered to induce anesthesia for cannulation surgeries and before transcardial perfusion. All systemic drugs were injected at a volume of 10 ml/kg.

Cell Culture and Biased Signaling Assays

Competition binding assays were performed using the Tag-lite assay according to the manufacturer's protocol (Cis-Bio, Bedford, MA, USA). In short, Tb-labeled HEK293-SNAP-hDOR cells/well (4,000 cells/well) were plated in 10 µl Tag-lite medium into low-volume 384-well plates in the presence of 5 µl 8 nM fluorescent naltrexone (final concentration) and 5 µl of an increasing concentration of TAN-67, leu-enkephalin, or SNC80 and incubated at room temperature for 3 h. cAMP inhibition and β-arrestin-2 recruitment assays were performed as previously described (28). In brief, for cAMP inhibition assays HEK293 (Life Technologies, Grand Island, NY, USA) cells (15,000 cells/well, 7.5 µl) transiently expressing FLAG-mDOR (29, 30), SNAP-rDOR, or SNAP-hDOR (Cis-Bio), and pGloSensor22F-cAMP plasmids (Promega, Madison, WI, USA) were incubated with Glosensor reagent (Promega, 7.5 µl, 2% final concentration) for 90 min at 37°C/5% CO_2. Cells were stimulated with 5 µl DOR agonist 20 min before 30 µM forskolin (5 µl) stimulation for an additional 15 min. For β-arrestin-2 recruitment assays, CHO-hDOR PathHunter β-arrestin-2 cells (DiscoverX, Fremont, CA, USA) were plated (2,500 cells/well, 10 µl) before stimulation with 2.5 µl DOR agonists for 90 min at 37°C/5% CO_2, after which cells were incubated with 6 µl cell assay buffer for 60 min at room temperature as per the manufacturer's protocol. Luminescence and fluorescence for each of the assays were measured using a FlexStation3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

SNAP-rDOR Construction

Rat DOR cDNA was amplified from the pUC17-rDOR plasmid (Versaclone cDNA NP_036749, R&D systems) using the following forward (5′-CTTCGATATCTTGGAGCCGGTGCCTTCTG-3′) and a standard M13 reverse primer using the Pfu Ultra II Hotstart PCR Mastermix (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. The amplified rDOR PCR product and the pSNAP-hDOR plasmid (Cis-Bio) were restricted using EcoRV and XhoI restriction enzymes (New England BioLabs, Ipswich, MA, USA), and the rDOR construct was exchanged with the hDOR gene followed by ligation with T4 DNA Ligase (New England BioLabs) and transformation into NEB5α competent cells (New England BioLabs). The SNAP-rDOR was fully sequenced to ensure correct orientation and absence of point mutations introduced during amplification.

Animals

37 male C57BL/6 mice (age 6 weeks) were purchased from Harlan and habituated for to the facility 1 week before surgery. For β-arrestin-2 KO animals, animals were bred in house, and 16 animals were selected for surgery [for complete details on strain origin see Ref. (28)]. Throughout the experiment, animals were kept in at ambient temperature of 21°C in a room maintained on a reversed 12L:12D cycle (lights off at 10:00, lights on at 22:00) in Purdue University's animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol (#1305000864) was approved by the Purdue University Institutional Animal Care and Use Committee.

Surgical Cannulation

Directly before surgery, mice were anesthetized with ketamine/xylazine (i.p.). A Kopf model 1900 stereotaxic alignment system (David Kopf Instruments, Tujunga, CA, USA) was used to drill two holes using Kyocera #69 drill bits at the following coordinates from bregma: AP = +1 mm, ML = ±1.5 mm, DV: −3.25 mm (31, 32). For experiments involving drug infusion, an additional two holes were drilled using Kyocera #60 drill bits at the following coordinates from bregma: AP = −2.4 mm, ML = ±1.6 mm, and 1 mm screws were positioned to ensure head-cap stability. A bilateral 22-gage guide cannula (cut 1.5 mm below pedestal, PlasticsOne, Roanoke, VA, USA) was attached to the skull using Geristore dental cement (DenMat, Lompoc, CA, USA). In total, two animals did not wake up from surgery, and eight animals were euthanized after their cannulas came off postoperation or throughout alcohol training and/or experimentation.

Viral Injection

After cannulation surgery, animals were single housed in double grommet cages to allow recovery and individual measurement of fluid intake. One-week postsurgery, mice were anesthetized as previously described and injected bilaterally with 450 nl of virus using a Harvard Apparatus infusion pump at a speed of 50 nl/min *via* internal cannula with 0.5 mm projection (PlasticsOne). The internal cannula was left in place for an additional 5 min to allow viral dispersion and prevent backflow of the viral solution into the injection syringe. All biohazard work was approved by the Institutional Biohazard Committee at Purdue University (#13-013-16).

Voluntary Alcohol Intake

One-week postsurgery and/or 1-week post-viral injection, mice were exposed to a limited-access (4 h/day), two-bottle choice (water versus 10% ethanol), DID protocol 3 h into their active cycle (dark phase) until the alcohol intake was stable as previously described (29). This model has previously shown that TAN-67 administration before the 4-h session decreases alcohol intake with a correlated decrease in blood ethanol concentration (with no TAN-67 effects on alcohol metabolism) (29). Mice were trained for 3 weeks during which the mice initially increased their alcohol intake before reaching steady state consumption. Bottle weights were measured directly before and after the 4-h access period to the second decimal point to determine fluid intake during this access period. Weights of bottles were corrected for any spillage with fluid bottles placed on empty cages.

Drug Infusion or Injection

After 3 weeks of exposure to the drinking model described earlier, alcohol and water intake on the day of infusion (Friday) was compared with the average alcohol intake over the preceding 3 days (Tuesday–Thursday) to determine if either drug injection or infusion altered voluntary alcohol intake in the following manipulations. For experiments involving viral expression, the

AAV injected mice were injected with i.p. saline (with 0.5% DMSO) for vehicle measurements in week 4 and 2 mg/kg CNO (i.p.) the following week (week 5). The dose of CNO of 2 mg/kg was utilized as it has previously been shown to be effective in mice in activating expressed DREADDs (33, 34). Also, this relatively low dose limits high concentrations of clozapine caused by metabolism of CNO (35). For experiments involving direct drug infusion into the dorsal striatum, animals received a 150 nl bilateral infusion of saline into the dorsal striatum on Friday of the fourth week of alcohol exposure. In weeks 5 and 6, animals received either a 150 nl infusion of 10 μM TAN-67 or SNC80, respectively, thereby allowing for a within subjects' analysis. The order of the drug infusions was chosen to mitigate potential DOR internalization and/or degradation as SNC80 is a high internalizing agonist *in vitro* and *in vivo* (36, 37). Doses of TAN-67 and SNC80 were determined based on previous studies of SNC80 infusions in rats (25) and *in vivo* specificity of TAN-67 and SNC80 for the DOR over MOR or KOR had been previously established using KO animals (29, 38). Importantly, no seizure behavior was observed up SNC80 infusion (39) following any dorsal striatal infusions.

Locomotor Activity

Square locomotor boxes from Med Associates (*L* 27.3 cm × *W* 27.3 cm × *H* 20.3 cm, St. Albans, VT, USA) were used to monitor locomotor activity during the active/dark phase as previously described (28). For AAV experiments, animals were placed in the locomotor box 15 min before CNO (2 mg/kg, i.p.) injection to allow baseline locomotor activity scoring. After 15 min, all animals were injected with CNO and then placed back into the box for an additional 60 min of testing to measure the total distance traveled in 60 min following drug injection. For intra-dorsal striatal infusion of SNC80, animals received either 10 μM SNC80 or vehicle (saline 0.9%) infusion and were placed immediately in the boxes for 90 min; locomotor data were analyzed 30 min after drug infusion as this is when drinking experiments began in the previously described alcohol intake studies.

Cannula Location and Immunohistochemical Analysis

For animals undergoing drug infusions, animals were sacrificed *via* transcardial perfusion within 1 week following their final drinking session. During analysis, it was determined that the cannula of one mouse from these experiments was not positioned properly and this animal was removed from analysis (placement was too ventral). Cannulation location and viral expression was verified with confocal microscopy (Nikon A1, Nikon, Melville, NY, USA) with an area of capture of 1.69 mm^2. The experimenter performing analysis was blind to the experimental conditions; all images were evaluated in greyscale to prevent unintentional bias.

Cocaine-Induced c-Fos Activation in DREADD-Expressing Animals

For viral expression studies, both groups of mice were injected with 2 mg/kg CNO (i.p.) during the dark/active phase for each animal. Twenty minutes later, animals were injected with 15 mg/kg cocaine (i.p.) to induce expression of immediate-early gene c-Fos. Brains were collected 90 min following cocaine exposure *via* transcardial perfusion. Extracted brain samples embedded and frozen in Tissue-Tek® O.C.T. compound (VWR, Radnor, PA, USA) in tissue molds (VWR) and sliced into 50 μm coronal sections *via* cryostat (Leica Microsystems Inc., Buffalo Grove, IL, USA). Immunohistochemical staining was conducted using primary rabbit anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology, Dallas, TX, USA), diluted 1:1,000. Control-GFP animal brains were applied Alexa-Fluor 594 goat anti-rabbit antibody (A-11012, Life Technologies, Grand Island, NY, USA) diluted 1:1,000. hM_4Di-mCherry animal brains were applied Alexa-Fluor 488 goat anti-rabbit (A-11008, Life Technologies, Grand Island, NY, USA) diluted 1:1,000. Brain slices were mounted onto microscope slides (Fisher Scientific, Hampton, NH, USA) for confocal microscopy with an area of the capture of 0.40 mm^2. Images were processed using ImageJ software (NIH, Bethesda, MD, USA) for the number of c-Fos positive cells in the dorsal striatum surrounding the viral injection site in infected cell populations. The experimenter performing analysis was blind to the experimental conditions; all images were evaluated in greyscale to prevent unintentional bias.

Statistical Analysis

All data are presented as means ± SEM and was performed using GraphPad Prism7 software (GraphPad Software, La Jolla, CA, USA). Differences between control-GFP and hM_4Di-mCherry animals were analyzed by student two-tailed *t*-test for differences in baseline water intake, alcohol intake, alcohol preference, locomotion after CNO injection, and c-Fos expression in the dorsal striatum. Differences in alcohol intake after saline injection and CNO injection were evaluated by repeated measures, multiple comparisons (Bonferroni) two-way ANOVA. For *in vitro* assays, non-linear regression using a dose–response to either inhibition (binding, cAMP) or stimulation (β-arrestin-2 recruitment) was conducted to determine pIC50 or pEC50, respectively. In direct dorsal striatal drug infusion experiments, differences in voluntary alcohol intake, water intake, and alcohol preference were analyzed by repeated measures, multiple comparisons (Tukey) two-way ANOVA. The Grubb's outlier test (alpha = 0.05) was used to identify potential outliers throughout the study. Statistical analysis was conducted in guidance with and approved by Purdue University's Department of Statistics.

RESULTS

Activation of a $G_{i/o}$-Coupled DREADD in the Dorsal Striatum Decreases Alcohol Intake

Cannula placement was verified postmortem (n = 10–11) through immunohistochemical analysis of viral expression (**Figure 1A**). Viral infusions of control-GFP (green fluorescent protein) or hM_4Di-mCherry in the dorsal striatum did not alter baseline alcohol intake, water intake, or alcohol preference when comparing the two groups [**Figure 1B**, $t(20) = 0.81$, $p = 0.32$; **Figure 1C**, $t(20) = 0.60$, $p = 0.42$; Figure S1A in Supplementary Material, $t(20) = 1.01$, $p = 0.55$]. Vehicle injection (0.5% DMSO,

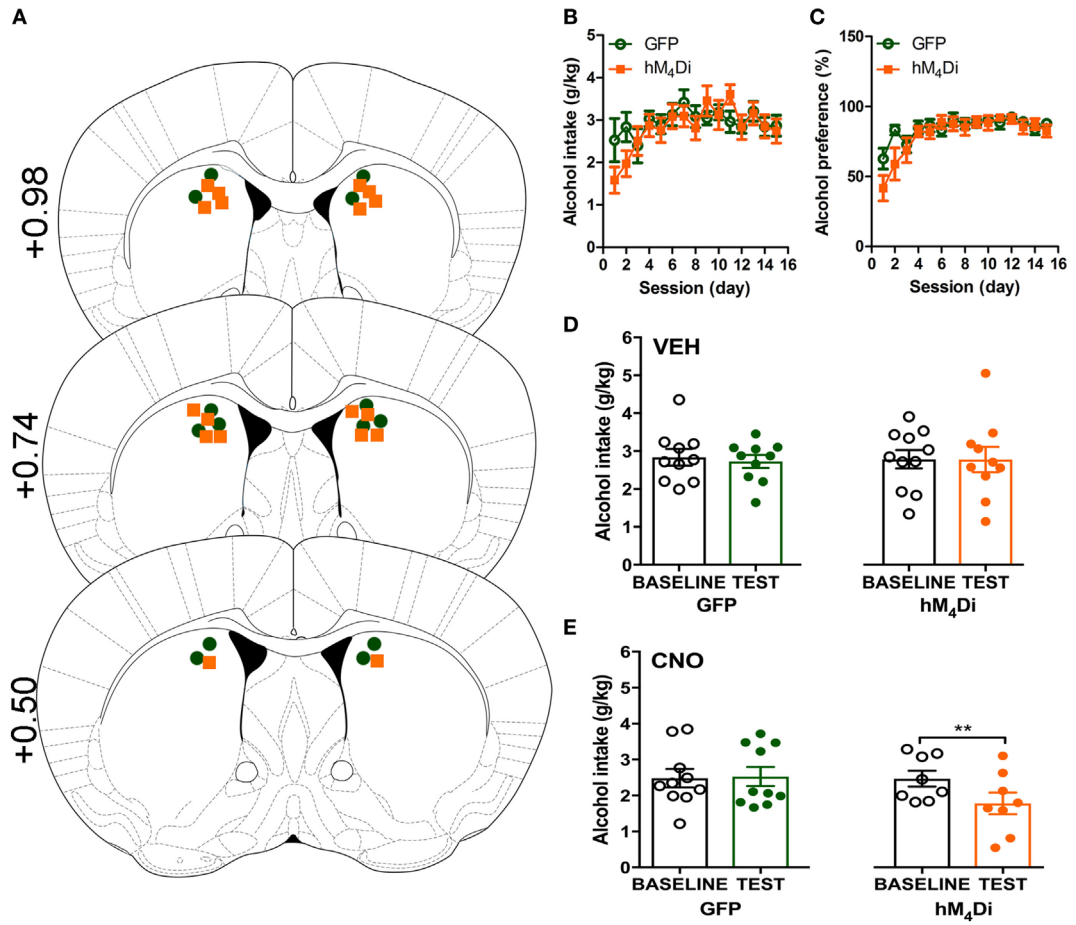

FIGURE 1 | Activation of virally expressed hM_4Di in the dorsal striatum decreases alcohol intake in mice. Cannula placement was verified for all animals included in behavioral analysis **(A)**. C57BL/6 mice (n = 10–11) injected in the dorsal striatum with either AAV8-hSyn-EGFP (GFP) or AAV8-hSyn-hM_4Di-mCherry (hM_4Di) were trained to consume alcohol in a two-bottle, drinking-in-the-dark protocol. Both groups of animals displayed a similar increase in alcohol intake **(B)** and preference **(C)**. Vehicle injection (saline 0.9%, i.p.) did not change alcohol intake **(D)**. Systemic clozapine-*N*-oxide (CNO) injection (2 mg/kg i.p.) significantly decreased alcohol intake in mice expressing hM_4Di, but not GFP, in the dorsal striatum **(E)**. Significance by unpaired, Student's t-test for AUC or two-way ANOVA with Bonferroni posttest for matching, $^{**}p < 0.01$.

i.p.) did not affect alcohol intake for control or hM_4Di-expressing animals in alcohol intake (**Figure 1D**; see **Table 1** for full statistical analysis for experimental group), water intake (Figure S1B in Supplementary Material), or alcohol preference in control-GFP or hM_4Di-mCherry mice (Figure S1E in Supplementary Material). Unlike saline injection, CNO injection (2 mg/kg, i.p.) significantly reduced alcohol intake in hM_4Di-expressing mice compared with GFP-control, as evaluated by two-way ANOVA (**Figure 1E**; Figure S1D in Supplementary Material, effect of drug × virus: $p = 0.03$), where Bonferroni posttest analysis revealed that CNO significantly reduced alcohol intake only in hM_4Di-expressing animals and not control-GFP-expressing mice ($p < 0.002$). No significant change in water intake was observed after CNO injection in the testing period for in either group of animals (Figure S1C in Supplementary Material). CNO injection did not alter alcohol preference in control-GFP or hM_4Di-mCherry mice (Figure S1F in Supplementary Material).

Both viruses properly expressed in the dorsal striatum (**Figure 2A**). Differences in visualization of the control-GFP and hM_4Di-mCherry expression may potentially result from differences in viral load and protein expression or inherent differences in quantum yield and extinction coefficients between GFP and mCherry (40). Considering that the striatum is part of the basal ganglia that controls movement (6, 41), we determined whether CNO activation of dorsal striatal hM_4Di altered locomotor activity where we observed that CNO did not alter locomotor activity between control-GFP and hM_4Di-mCherry expressing mice in a 60-min locomotor period after injection [**Figure 2B**, $t(19) = 0.78$, $p = 0.45$]. To confirm the inhibitory functionality of hM_4Di expression, we determined if CNO activation of hM_4Di could prevent cocaine-induced c-Fos expression (42, 43), an acceptable approach previously used in other studies to validate functionality of inhibitory DREADDs (42–45). We observed that activation of striatal hM_4Di with CNO (2 mg/kg, i.p.) before a cocaine challenge (15 mg/kg, i.p.) significantly inhibited c-Fos activation in animals expressing hM_4Di versus GFP controls (control were also administered CNO before cocaine challenge) [**Figures 2C,D**; $t(13) = 2.78$, $p < 0.02$], suggesting that activation of hM_4Di *via*

TABLE 1 | Analysis of alcohol-related behaviors in control-GFP versus hM_4Di-mCherry designer receptors exclusively activated by designer drugs (DREADD)-expressing mice.

	df	Alcohol intake	Water intake	Alcohol preference
Baseline[a] (Student's *t*-test)	20	$t = 0.812$ $p = 0.32$	$t = 0.603$ $p = 0.42$	$t = 1.01$ $p = 0.55$
Vehicle[b]				
Drug	1, 19	$F = 0.18$ $p = 0.86$	$F = 0.52$ $p = 0.48$	$F = 3.82$ $p = 0.07$
Virus		$F = 0.04$ $p = 0.85$	$F = 0.00$ $p = 0.96$	$F = 0.26$ $p = 0.42$
Drug × virus		$F = 0.00$ $p = 0.99$	$F = 0.26$ $p = 0.61$	$F = 2.85$ $p = 0.11$
Clozapine-N-oxide[b]				
Drug	1, 19	$F = 4.00$ $p = 0.06$	$F = 1.20$ $p = 0.29$	$F = 0.42$ $p = 0.52$
Virus		$F = 1.26$ $p = 0.28$	$F = 2.37$ $p = 0.14$	$F = 0.90$ $p = 0.35$
Drug × virus		$F = 5.17$ $p = 0.03$ Control versus DREADD $p < 0.002$	$F = 0.28$ $p = 0.60$	$F = 1.01$ $p = 0.33$

[a]*Student's t-test.*
[b]*Two-way, repeated measures (Bonferroni) ANOVA.*

CNO before cocaine challenge inhibited cAMP pathway activity by $G_{i/o}$-coupled inhibition. The variability in c-Fos expression in control-GFP may be a result of intrinsic differences in response to psychostimulants between animals, which has been commonly observed in C57Bl/6 mice (46).

In Vitro Characterization of the β-Arrestin-2 Biased DOR Agonist, SNC80

We have previously established that systemic activation of the $G_{i/o}$-coupled DOR with TAN-67, an agonist that only weakly recruits β-arrestin-2 to hDOR (**Figure 3A**), reduces voluntary intake in mice, but that SNC80, an hDOR agonist that strongly recruits β-arrestin-2 (**Figure 3A**) increases alcohol intake (28). However, we previously had not determined if a difference in receptor binding was observed between TAN-67 and SNC80 at hDOR to potentially explain differences in ligand bias. Using a SNAP-tag HTRF® (Cis-Bio) approach we found that hDOR, TAN-67 exhibited a pKi = 7.7 ± 0.1 and SNC80 a pKi = 7.2 ± 0.2, with pKi = 5.8 ± 0.1 for leu-enkephalin (**Figure 3B**), suggesting that the only clear difference between TAN-67 and SNC80 is β-arrestin-2 recruitment efficacy. The surprisingly low affinity observed for leu-enkephalin may be an artifact of the fluorescent binding assay that relies on a large N-terminal SNAP-tag, which may potential interfere with the binding of relatively large peptide ligand, such as leu-enkephalin, but not small molecules.

Expanding from our previous study, we determined the equiactive bias factors for TAN-67 and SNC80 at hDOR using leu-enkephalin as a reference ligand (47) for β-arrestin-2 recruitment compared with $G_{i/o}$-stimulated cAMP inhibition (a more positive bias factor = indicative of bias toward β-arrestin-2, more negative bias factor = indicative of bias toward cAMP activity). TAN-67 displayed a bias factor of −1.4 (cAMP biased) versus a +0.85 bias factor for SNC80 (β-arrestin-2-biased) (**Figure 3C**). To estimate what concentration of SNC80 to infuse *in vivo*, we relied on the Nielsen et al. reported findings in rat (25). Our *in vitro* assays suggest minimal differences in cAMP inhibition between human hDOR (**Figure 3D**), rat rDOR (**Figure 3E**), and mDOR (**Figure 3F**) for SNC80 ($pIC_{50} = 7.8 \pm 0.3$, $n = 3$, $pIC_{50} = 8.4 \pm 0.1$, $n = 5$, $pIC_{50} = 8.4 \pm 0.4$, $n = 3$, respectively) and leu-enkephalin ($pIC_{50} = 8.7 \pm 0.2$, $n = 5$, $pIC_{50} = 8.9 \pm 0.2$, $n = 5$, $pIC_{50} = 8.3 \pm 0.1$, $n = 6$, respectively). Because the affinity and efficacy of TAN-67 is very comparably to SNC80 (**Figures 3A,B**), we decided to infuse 10 nM TAN-67 and SNC80 into the mouse dorsal striatum to investigate the role of $G_{i/o}$ signaling versus β-arrestin-2 recruitment in the modulation of alcohol use.

Differential Modulation of Alcohol Intake Following Dorsal Striatal DOR Activation by $G_{i/o}$-Biased Versus β-Arrestin-2-Biased DOR Agonists

Cannula terminus location and patency were validated *via* trypan blue dye infusion into the dorsal striatum upon experimental completion (**Figure 4A**). Wild-type male animals ($n = 9$–10) were successfully trained to consume alcohol using a limited-access, two-bottle-choice (water versus 10% alcohol), DID protocol as shown by increased daily alcohol intake and preference (**Figures 4B,C**) compared with water intake (Figure S2A in Supplementary Material). For intra-striatal infusions, a significant drug ($p = 0.03$, see **Table 2** for full statistical analysis for experimental group and Table S1 in Supplementary Material for Tukey comparisons between infusion weeks) and drug × test session effect ($p < 0.0001$) was observed, with no effect of test session alone, where Tukey multiple comparisons test revealed that 10 μM of TAN-67 significantly decreased voluntary alcohol intake ($p = 0.04$) while 10 μM SNC80 significantly increased alcohol intake ($p = 0.0005$). Importantly, vehicle (saline 0.9%) infusion did not affect alcohol intake (**Figure 4D**). No changes in water intake or alcohol preference were noted during these drug infusion testing sessions (Figures S2B,C in Supplementary Material).

Genetic KO of β-Arrestin-2 Provides Additional Support for the Critical Role of DOR-Mediated $G_{i/o}$-Coupling in the Dorsal Striatum in Decreasing Alcohol Intake

β-Arrestin-2 KO male C57Bl/6 mice ($n = 12$) were surgically implanted with a bilateral cannula into the dorsal striatum before alcohol training, and cannula terminus location and patency were validated *via* trypan blue dye infusion upon experimental completion (**Figure 5A**). KO animals were successfully trained to consume alcohol using a limited-access, two-bottle-choice (water versus 10% alcohol), DID protocol (**Figures 5B,C**) compared with water intake (Figure S3A in Supplementary Material). A significant effect of drug ($p = 0.003$, see **Table 3** for full statistical analysis for experimental group and Table S2 in

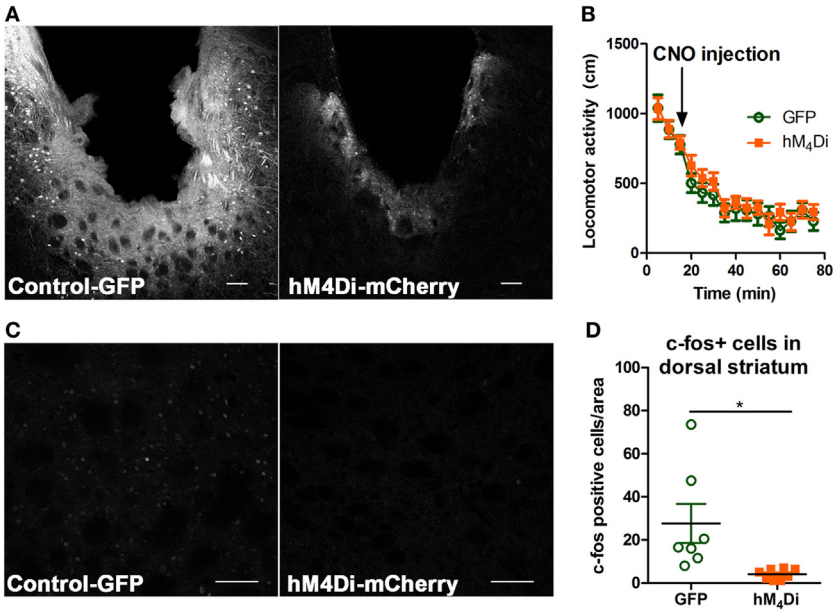

FIGURE 2 | Verification of viral expression and functionality of control-GFP or hM_4Di-mCherry in the dorsal striatum. Viral expression verification *via* confocal microscopy of control-GFP (left) and hM_4Di-mCherry (right) in the dorsal striatum (scale bar = 100 μm) **(A)**. C57BL/6 mice (n = 10–11 per group) expressing GFP or hM_4Di in the dorsal striatum did not display significant clozapine-*N*-oxide (CNO) (2 mg/kg, i.p.) induced locomotor activity in the 60-min session after CNO injection **(B)**. Immunohistochemical representation of c-Fos activation in the dorsal striatum in animals expressing control-GFP (left) and hM_4Di-mCherry (right) in the dorsal striatum (scale bar = 100 μm) **(C)**. Decreased c-Fos expression in dorsal striatum after cocaine challenge (15 mg/kg, i.p.) in C57BL/6 mice (n = 7–8) expressing hM_4Di-mCherry versus control-GFP observed confocal microscopy **(D)**. Significance by unpaired two-tailed t-test, $^*p < 0.05$.

Supplementary Material for Tukey comparisons between infusion weeks), test session ($p = 0.002$), and drug × test session ($p = 0.0021$) was identified for intra-dorsal striatal infusions, where multiple comparisons test found no effect of vehicle (saline 0.9%, $p = 0.968$) on alcohol intake. 10 μM of TAN-67 significantly decreased voluntary alcohol intake ($p = 0.0113$), and 10 μM of SNC80 also significantly decreased alcohol intake ($p = 0.0021$) (**Figure 5D**). This decrease was in contrast with that observed in wild-type animals and is the first report of SNC80's ability to decrease voluntary alcohol intake, further suggesting that β-arrestin-2 functionality is key for SNC80-increased voluntary alcohol intake. No changes in water intake were noted during testing periods (Figure S3B in Supplementary Material), but a decrease in alcohol preference was noted for SNC80 infusion ($p = 0.0018$, Figure S3C in Supplementary Material). We have previously observed hyperlocomotion upon systemic SNC80 administration in both wild-type and β-arrestin-2 KO mice with increased alcohol intake or no change in alcohol intake, respectively (28, 48). Therefore, we questioned whether the decrease in alcohol intake upon dorsal striatal SNC80 infusion in the β-arrestin-2 KO was the result of changes in locomotion. However, SNC80 (10 μM) infusion into the dorsal striatum of β-arrestin-2 KO animals did not cause hyperlocomotion compared with vehicle infusion [Figures S4A,B in Supplementary Material, paired two-tailed Student's t-test: $t(6) = 1.68$, $p = 0.14$], although the trend (albeit not significant) toward a decrease in locomotor activity suggests that there may be a potential influence of SNC80 on locomotor activity with respect to the decrease in alcohol intake observed upon SNC80 infusion in β-arrestin-2 KO animals.

DISCUSSION

Through both chemogenetic and pharmacologic activation of $G_{i/o}$-protein signaling, we observed that activation of $G_{i/o}$-protein-coupled receptors in the dorsal striatum significantly decreases alcohol intake in male C57BL/6 mice by either inhibitory DREADD activation or activation of endogenously expressed DORs using a G-protein biased agonist. We specifically targeted the dorsal striatum as it plays an important role in modulating habitual alcohol use (2, 3, 7, 9), has strong DOR expression (20), and, crucially, is a region where DOR agonist SNC80 has been shown to increase alcohol intake in rats (25). Here, activation of virally expressed $G_{i/o}$-coupled DREADDs in the dorsal striatum was capable of decreasing alcohol intake while no effect was observed in control-GFP animals upon CNO administration (**Figure 1**). For activation of endogenous dorsal striatal DORs, our findings that local dorsal striatal infusion of TAN-67 decreased alcohol intake and SNC80 increased alcohol intake (**Figure 4**; Figure S3 in Supplementary Material) agreed with our systemic findings (28) and also confirmed the previously observed alcohol intake increase following local dorsal striatal infusion of SNC80 in rats (25). Furthermore, through the use of β-arrestin-2 KO mice, we were able to shift the direction of alcohol intake modulation by SNC80 from significantly increasing intake to significantly decreasing consumption when β-arrestin-2 signaling pathways is

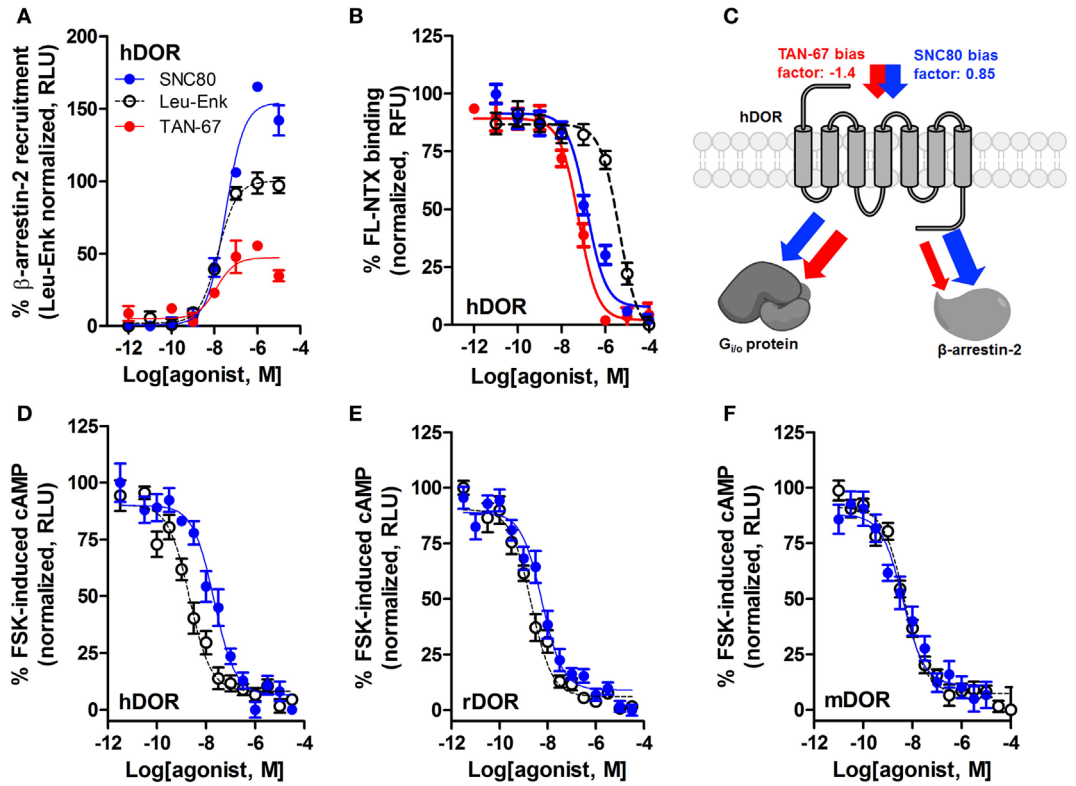

FIGURE 3 | SNC80 is a β-arrestin-biased agonist with comparable potency across species in heterologous cell systems. At the hDOR, SNC80 acts as a β-arrestin-2 super-agonist compared with the endogenous agonist leu-enkephalin and the weak β-arrestin-2 recruiter TAN-67 **(A)**. SNC80 and TAN-67 bind to hDOR with similar affinity **(B)**. Schematic representation of the observed ligand bias of TAN-67 and SNC80 at hDOR, with calculated bias factor **(C)**. SNC80 and Leu-enkephalin have similar potency to inhibit forskolin-induced cAMP production at hDOR **(D)**, rDOR **(E)**, and mDOR **(F)**. A representative summation is shown ($n \geq 3$).

not present (**Figure 5**; Figure S3 in Supplementary Material). This was expected as TAN-67 and SNC80 displayed similar binding and G-protein pathway efficacy at DOR *in vitro,* suggesting that the removal of potential β-arrestin-2 recruitment would allow the agonists to behave similarly (**Figure 3**). This shift is in agreement with our hypothesis that DOR-mediated $G_{i/o}$ signaling is a potential strategy to reduce alcohol use, whereas DOR-mediated β-arrestin signaling is to be avoided.

While the dorsal striatum as a region in general is implicated in procedural learning (49–51), the dorsolateral striatum subregion is heavily associated with habitual behavior (behavioral actions that persist despite reward devaluation) (52) and the dorsomedial striatum with goal-directed learning (53). Chronic alcohol exposure may preferentially activate the dorsolateral striatum versus the dorsomedial striatum, as observed by increased glutamatergic transmission (54) and decreased GABAergic transmission (54, 55) in this subregion in animals exposed to chronic intermittent alcohol. Moreover, in rats, alcohol self-administration upregulates brain-derived neurotrophic factor (BDNF) in both the DLS and DMS, but with more robust increases in BDNF in the DLS (56, 57). Furthermore, infusion of BDNF in the DLS decreases alcohol self-administration (57). In rats, initial alcohol seeking was attenuated upon inactivation of the DMS (with no effect upon inactivation of the DLS). However, upon longer exposure to operant alcohol training, animals became insensitive to alcohol devaluation, and inactivation of the DLS re-sensitizes the animals to devaluation (6). Our results presented here did not differentiate between the DMS and the DLS, although future studies warrant investigation of $G_{i/o}$-protein activity in these dorsal striatal subregions for potential subregion-specific differences in alcohol intake upon $G_{i/o}$-protein activation.

To broadly validate the role of the dorsal striatum in alcohol consumption, we first virally expressed a $G_{i/o}$-coupled DREADD (hM_4Di) to artificially activate $G_{i/o}$-protein signaling pathways in this region to determine how increased $G_{i/o}$-protein activity altered alcohol intake. In the present study, activation by the hM_4Di DREADD ligand CNO decreased alcohol intake of animals expressing hM_4Di in the dorsal striatum and had no effect on control-GFP animals (**Figure 1**). Despite recent concerns on the use of DREADD technology and CNO's conversion to clozapine *in vivo*, the low dose of 2 mg/kg was specific in its behavioral effects on the hM_4Di-expressing mice compared with control GFP-expressing animals in drinking behavior (35), thus ruling out the potential issue that decreased consumption resulted from CNO (or clozapine following CNO conversion) activating endogenous muscarinic M_4 receptors, which are also highly expressed in the striatum (58). In addition, as previously mentioned, no differences in locomotor activity were observed upon

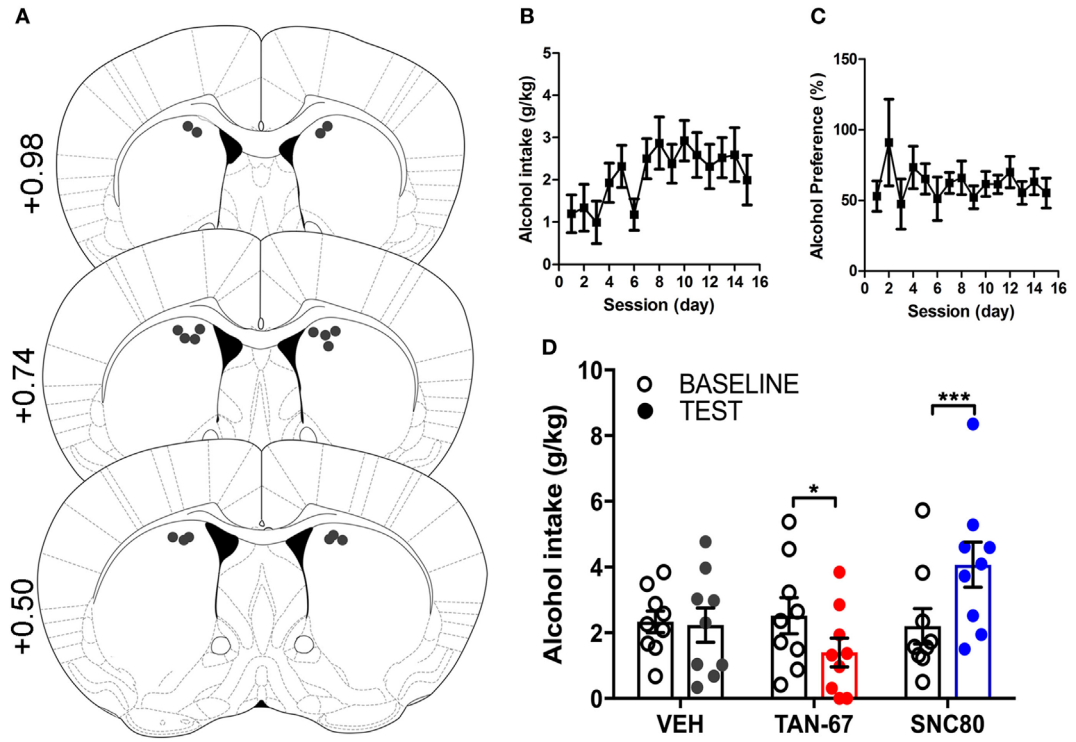

FIGURE 4 | Dorsal striatal infusion of $G_{i/o}$-protein biased delta-opioid receptor (DOR) agonist TAN-67 decreases voluntary alcohol intake, while β-arrestin-2 biased DOR agonist SNC80 increases alcohol intake in wild-type mice. Cannula placement was verified for all animals included in behavioral analysis **(A)**. C57BL/6 male, wild-type mice (n = 9–10) were trained to consume 10% alcohol over the course of 3 weeks, during which they increased their alcohol intake **(B)** and alcohol preference **(C)**. Vehicle saline (0.9%) infusion did not change alcohol intake while TAN-67 (10 μM) significantly decreased alcohol intake and SNC80 (10 μM) significantly increased alcohol intake **(D)**. Significance by repeated measures, multiple comparisons (Tukey) two-way ANOVA, $^{*}p < 0.05$ and $^{***}p < 0.001$.

TABLE 2 | Two-way, repeated measures ANOVA of alcohol-related behaviors in wild-type mice upon biased delta-opioid receptor agonist infusion in the dorsal striatum.

Two-way ANOVA	df	Drug injection, alcohol intake	Drug injection, water intake	Drug injection, alcohol preference
Drug	2, 16	$F = 4.38$ $p = 0.03$	$F = 0.63$ $p = 0.56$	$F = 1.40$ $p = 0.28$
Test session	1, 8	$F = 0.39$ $p = 0.55$	$F = 2.11$ $p = 0.18$	$F = 0.33$ $p = 0.58$
Drug × test session	2, 16	$F = 20.22$ $p < 0.0001$	$F = 0.49$ $p = 0.62$	$F = 1.39$ $p = 0.28$
Multiple comparisons (Tukey)		VEH $p > 0.99$ TAN-67 $p = 0.042$ SNC80 $p = 0.0005$	VEH $p > 0.96$ TAN-67 $p > 0.99$ SNC80 $p = 0.54$	VEH $p > 0.97$ TAN-67 $p = 0.85$ SNC80 $p = 0.88$

CNO administration in either control or DREADD-expressing mice, suggesting that the observed decrease in consumption did not result from hypolocomotion (**Figure 2**). Our viral AAV8-DREADD construct was expressed under a human synapsin promoter which specifically targets neurons (59), and given that the majority of the dorsal striatum consists of MSNs and AAV8 has been shown to infect GABAergic neurons in the mouse striatum (60), activation of virally expressed striatal hM_4Di receptors in our experimental design likely inhibited both the D_1R-MSNs and D_2R-MSNs of the direct and indirect pathways, respectively. This net inhibition may be responsible for the observed no net change in locomotor activity and a modest—albeit significant—decrease in alcohol intake (3, 6, 8, 35, 41). We did not verify the potential of preferential tropism of the AAV8-DREADD construct [although AAV8 transduction in the striatum suggests that serotype 8 successfully transduces GABAergic neurons in the mouse striatum (60, 61)], thus limiting our conclusions on the specificity of increased $G_{i/o}$-protein activity by DREADD activation on striatal GABAergic and/or cholinergic neurons. Furthermore, while our DREADD strategy was successful in confirming that inhibition of dorsal striatum by increased $G_{i/o}$-protein signaling can decrease alcohol consumption, CNO is known to be an unbiased ligand for DREADD receptors (62, 63). Therefore, we next continued with an approach where we could more selectively activate endogenous $G_{i/o}$-protein signaling over β-arrestin pathways.

Because of the limitations of potential tropism and possible β-arrestin-2 recruitment in our DREADD strategy, we next investigated changes in alcohol intake upon activation of $G_{i/o}$-protein activity by infusing DOR agonists into the dorsal striatum, where DORs are endogenously expressed presynaptically on

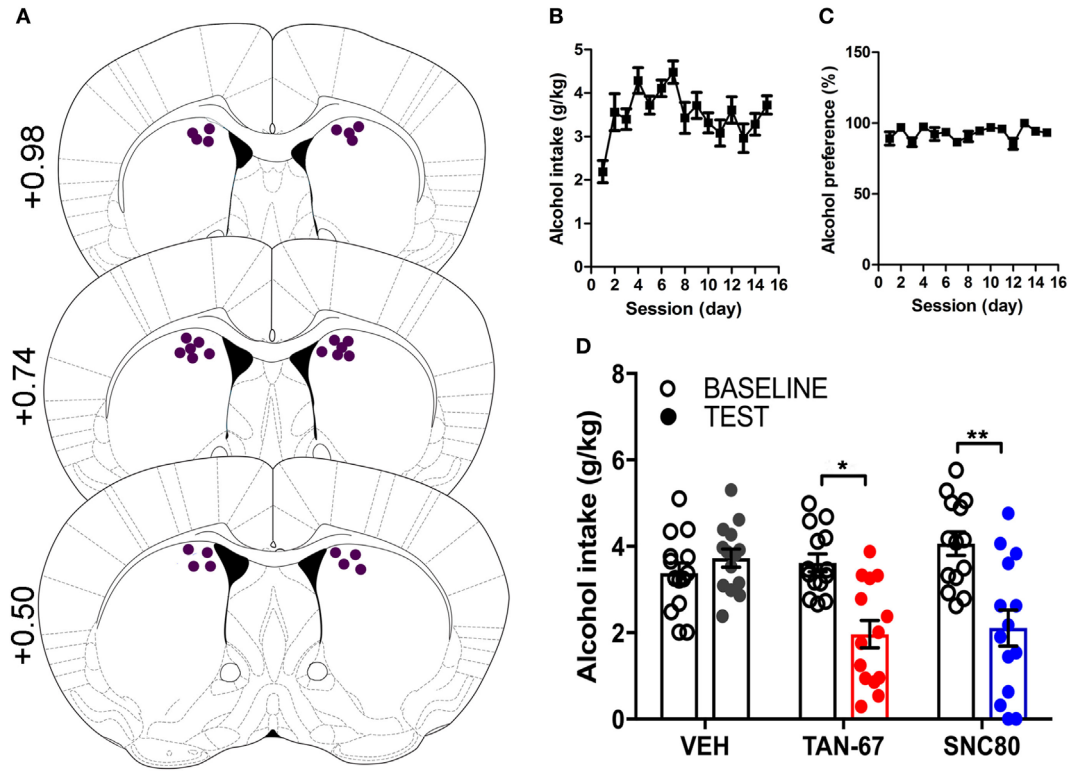

FIGURE 5 | Genetic knockout (KO) of β-arrestin-2 reveals critical role of $G_{i/o}$ signaling in reducing alcohol intake *via* dorsal striatal delta-opioid receptor activation. Cannula placement was verified for all animals included in behavioral analysis **(A)**. C57BL/6 male, β-arrestin-2 KO mice (n = 12) were trained to consume 10% alcohol over the course of 3 weeks, during which they increased their alcohol intake **(B)** and alcohol preference **(C)**. Vehicle saline (0.9%) infusion did not change alcohol intake, but both TAN-67 and SNC80 (10 μM) significantly decreased alcohol intake **(D)**. Significance by repeated measures, multiple comparisons (Tukey) by two-way ANOVA, $^{*}p < 0.05$ and $^{**}p < 0.01$.

TABLE 3 | Two-way, repeated measures ANOVA of alcohol-related behaviors in β-arrestin-2 knockout mice upon biased delta-opioid receptor agonist infusion in the dorsal striatum.

Two-way ANOVA	df	Drug injection, alcohol intake	Drug injection, water intake	Drug injection, alcohol preference
Drug	2, 26	$F = 7.31$ $p = 0.003$	$F = 1.07$ $p = 0.36$	$F = 3.11$ $p = 0.062$
Test session	1, 13	$F = 25.28$ $p = 0.0002$	$F = 0.93$ $p = 0.35$	$F = 7.19$ $p = 0.019$
Drug × test session	2, 26	$F = 7.92$ $p = 0.0021$	$F = 2.46$ $p = 0.11$	$F = 4.03$ $p = 0.03$
Multiple comparisons (Tukey)		VEH $p > 0.97$ TAN-67 $p = 0.011$ SNC80 $p = 0.0021$	VEH $p > 0.99$ TAN-67 $p = 0.95$ SNC80 $p = 0.28$	VEH $p > 0.99$ TAN-67 $p > 0.99$ SNC80 $p = 0.018$

corticostriatal glutamatergic inputs (19), pre- and postsynaptically on cholinergic interneurons, and on D_2-MSNs (20–22). In designing our DOR drug infusion experiments, we infused known DOR agonists into the dorsal striatum of either wild-type or β-arrestin-2 KO mice once a week (following 3 weeks of alcohol drinking) to assess changes in voluntary alcohol intake in response to drug infusion. In the first infusion test week, we infused vehicle (saline 0.9%) to ensure that handling and infusion alone did not change voluntary alcohol intake (**Figures 4** and **5**). In the second infusion test week, TAN-67 was infused, followed by SNC80 infusion in the third infusion test week. This specific order of drug infusion was determined based upon the *in vitro* β-arrestin-2 recruitment profiles of TAN-67 and SNC80 (**Figure 3**) and previously published work on SNC80's ability to cause rapid DOR internalization [and potential degradation (64)] *in vitro* and *in vivo* (in the striatum) (20, 37). Thus, we infused TAN-67 first to prevent potential SNC80-induced desensitization of the DOR system and we did not counterbalance our drug infusions, thus limiting our conclusions on how observed SNC80 responses may be confounded by potential inflammation upon repeated drug infusion into this brain region. Because we specifically observe different behavioral effects with SNC80, which was injected last in both wild-type and β-arrestin-2 KO mice, we would argue that the observed responses represent a true pharmacological effect and are not a negative or positive consequence of repeated infusions.

Our findings that activation of $G_{i/o}$ signaling in the dorsal striatum reduces alcohol intake would suggest a role for adenylyl cyclase and cAMP in this behavior. Recently, reductions in cAMP levels in the dorsal striatum by adenylyl cyclase type 1 (AC1)

inhibition and AC1-KO have been associated with decreased ethanol-induced locomotor sensitization (65). Furthermore, blockade of dorsal striatal G_s-coupled dopamine D_1 receptors (but not blockade of $G_{i/o}$-coupled dopamine D_2) attenuates alcohol consumption (8), suggesting indeed that inhibition of cAMP production in the dorsal striatum may contribute to reduced alcohol use. In the dorsal striatum, alcohol can induce LTD of fast spiking interneuron-medium spiny neuron synapses *via* a mechanism involving DORs, as this LTD was blocked by a DOR antagonist and the effect was mimicked when using the DOR agonist DPDPE (24). Moreover, the effects of DPDPE can also be blocked by activating adenylyl cyclases with forskolin (24). In our hands, we find that DPDPE is relatively unbiased and thus also efficiently recruits β-arrestin (28). This may be relevant as it has been shown that LTD may also rely on functional β-arrestin-2 expression: activation of hippocampal metabotropic glutamatergic receptors attenuated LTD in β-arrestin-2 KO animals (66, 67) and, upon metabotropic glutamate receptor activation, β-arrestin-2 scaffolding proteins increase the synaptic strength of hippocampal neurons (68). Currently, no studies have investigated the role of β-arrestin-2 in alcohol and DOR-mediated LTD in the dorsal striatum, nor have studies investigated if contributions of cAMP and β-arrestin to LTD change in alcohol-exposed or alcohol-dependent animals.

The observation that β-arrestin-2 activation in the dorsal striatum increases alcohol intake in mice is in agreement with reported elevated expression levels of β-arrestin-2 gene (*Arrb2*) and β-arrestin-2 protein levels in the striatum of ethanol-preferring alko alcohol rats in comparison with alko non-alcohol rat counterparts, as well as decreased voluntary alcohol intake in β-arrestin-2 KO (69). Despite these connections of β-arrestin expression and voluntary alcohol intake, conflicting results exist on how alcohol intake is altered in β-arrestin-2 KO animals. Li et al. (70) observed that their β-arrestin-2 KO mice displayed increased voluntary alcohol consumption compared with wild-type mice, in line with behavior by our β-arrestin-2 KO mice which also showed slightly higher alcohol intake than wild-type mice (**Figure 5**) (28). One potential explanation is that the Björk et al. study used alcohol solutions that contained saccharin (28, 69, 70). Importantly, as a number of these aforementioned studies (including ours presented here) utilize global β-arrestin-2 KO animal models, we are limited in our interpretation on how global β-arrestin-2 expression affects general alcohol behavior because of potential compensatory expression of the β-arrestin-1 isoform, particularly because isoform-selective differences in behavior have been observed (71, 72). The effect of β-arrestin expression on alcohol intake is noteworthy as altered levels of β-arrestin-2 have been observed as a result of acute and/or chronic morphine exposure in rats (73), elevated glucocorticoid activity *in vitro* (74), during inflammation *in vivo* in synoviocytes, and after cerebral hypoxia/ischemia (75). It is possible that alcohol intake and preference by subjects in these situations is enhanced, and that effectiveness of therapeutic drugs may be altered in these subjects, i.e., an unbiased drug may become β-arrestin-biased and increase alcohol use.

The dorsal striatum contains a large variety of $G_{i/o}$-coupled GPCRs besides DORs, including the muscarinic M_4 and serotonin 5-HT_{1B} receptors (10, 11). In line with our current findings, all three $G_{i/o}$-coupled receptors the respective KO animals (DOR KO, M_4R KO, and 5-HT_{1B} KO mice) consume more alcohol compared with wild-type littermates (18, 76, 77). Here, our findings indicate that activation of dorsal striatal $G_{i/o}$-coupled receptors, either *via* endogenous DORs or by virally expressed DREADDs, is sufficient to decrease voluntary alcohol intake in C57Bl/6 male mice. As β-arrestin-2 recruitment is associated with rapid internalization of DORs *in vitro* and *in vivo* [where DORs are degraded upon internalization (36, 37, 64)], we hypothesize that β-arrestin-2 recruitment to DORs by SNC80 can lead to rapid desensitization of endogenously expressed DORs, resulting in increased alcohol similar to that observed in DOR KO mice (18). In addition, SNC80-induced β-arrestin-2 recruitment may lead to β-arrestin-dependent signaling events (78), such as increased phosphorylation of ERK (79, 80). Previously, we discovered that agonists of the $G_{i/o}$-coupled DOR can either decrease or increase alcohol intake in mice (29, 81, 82), and closer examination of the pharmacology of the DOR agonists revealed that agonists that strongly recruit β-arrestin-2 increased alcohol intake, whereas agonists that were $G_{i/o}$-protein biased decreased alcohol intake in mice (28), suggesting that $G_{i/o}$-protein biased ligands may be a therapeutic option in treating AUD. Combined with our current results, these studies suggest a potentially broad role for striatal $G_{i/o}$-coupled signaling to decrease alcohol intake, which could be accomplished *via* G-protein biased ligands that activate $G_{i/o}$-coupled receptors robustly expressed in the dorsal striatum, such as the DOR. Therefore, the development of $G_{i/o}$-protein biased DOR agonists or agonists for other striatal $G_{i/o}$-coupled receptors, such as the M_4, 5-HT_{1b}, dopamine D_2 (83), kappa-opioid (84), and/or GPR88 receptor (85), could present a novel strategy to treat AUD by decreasing excessive alcohol consumption.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol (#1305000864) was approved by the Purdue University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MR performed cannulation surgeries, alcohol intake studies, locomotor studies, perfusions, cocaine challenge, and immunohistochemistry, and wrote the main draft of the manuscript. TC performed alcohol intake studies and bred and genotyped β-arrestin-2 KO mice. KM cloned the rDOR construct and performed the *in vitro* assays. DA performed locomotor studies and also bred and genotyped β-arrestin-2 KO mice. RR designed the experiments, assisted with the *in vitro* assays, cannulation surgeries, and alcohol intake studies, and wrote the manuscript.

All the authors analyzed and interpreted data and proofread the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fpsyt.2018.00112/full#supplementary-material.

REFERENCES

1. Zindel LR, Kranzler HR. Pharmacotherapy of alcohol use disorders: seventy-five years of progress. *J Stud Alcohol Drugs Suppl* (2014) 75(Suppl 17):79–88. doi:10.15288/jsads.2014.75.79
2. Filbey FM, Claus E, Audette AR, Niculescu M, Banich MT, Tanabe J, et al. Exposure to the taste of alcohol elicits activation of the mesocorticolimbic neurocircuitry. *Neuropsychopharmacology* (2008) 33(6):1391–401. doi:10.1038/sj.npp.1301513
3. Everitt BJ, Robbins TW. From the ventral to the dorsal striatum: devolving views of their roles in drug addiction. *Neurosci Biobehav Rev* (2013) 37(9 Pt A):1946–54. doi:10.1016/j.neubiorev.2013.02.010
4. DePoy L, Daut R, Brigman JL, MacPherson K, Crowley N, Gunduz-Cinar O, et al. Chronic alcohol produces neuroadaptations to prime dorsal striatal learning. *Proc Natl Acad Sci U S A* (2013) 110(36):14783–8. doi:10.1073/pnas.1308198110
5. Vollstädt-Klein S, Wichert S, Rabinstein J, Bühler M, Klein O, Ende G, et al. Initial, habitual and compulsive alcohol use is characterized by a shift of cue processing from ventral to dorsal striatum. *Addiction* (2010) 105(10):1741–9. doi:10.1111/j.1360-0443.2010.03022.x
6. Corbit LH, Nie H, Janak PH. Habitual alcohol seeking: time course and the contribution of subregions of the dorsal striatum. *Biol Psychiatry* (2012) 72(5):389–95. doi:10.1016/j.biopsych.2012.02.024
7. Wang J, Carnicella S, Phamluong K, Jeanblanc J, Ronesi JA, Chaudhri N, et al. Ethanol induces long-term facilitation of NR2B-NMDA receptor activity in the dorsal striatum: implications for alcohol drinking behavior. *J Neurosci* (2007) 27(13):3593–602. doi:10.1523/JNEUROSCI.4749-06.2007
8. Wang J, Cheng Y, Wang X, Roltsch Hellard E, Ma T, Gil H, et al. Alcohol elicits functional and structural plasticity selectively in dopamine D1 receptor-expressing neurons of the dorsomedial striatum. *J Neurosci* (2015) 35(33):11634–43. doi:10.1523/JNEUROSCI.0003-15.2015
9. Cheng Y, Huang CCY, Ma T, Wei X, Wang X, Lu J, et al. Distinct synaptic strengthening of the striatal direct and indirect pathways drives alcohol consumption. *Biol Psychiatry* (2017) 81(11):918–29. doi:10.1016/j.biopsych.2016.05.016
10. Xie K, Martemyanov KA. Control of striatal signaling by g protein regulators. *Front Neuroanat* (2011) 5:49. doi:10.3389/fnana.2011.00049
11. Komatsu H, Maruyama M, Yao S, Shinohara T, Sakuma K, Imaichi S, et al. Anatomical transcriptome of G protein-coupled receptors leads to the identification of a novel therapeutic candidate GPR52 for psychiatric disorders. *PLoS One* (2014) 9(2):e90134. doi:10.1371/journal.pone.0090134
12. Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A* (2007) 104(12):5163–8. doi:10.1073/pnas.0700293104
13. Vardy E, Robinson JE, Li C, Olsen RHJ, DiBerto JF, Giguere PM, et al. A new DREADD facilitates the multiplexed chemogenetic interrogation of behavior. *Neuron* (2015) 86(4):936–46. doi:10.1016/j.neuron.2015.03.065
14. Singla N, Minkowitz H, Soergel D, Burt D, Skobieranda F. (432) Respiratory safety signal with oliceridine (TRV130), a novel μ receptor G protein pathway selective modulator (μ-GPS), vs morphine: a safety analysis of a phase 2b randomized clinical trial. *J Pain* (2016) 17(4S):S82. doi:10.1016/j.jpain.2016.01.409
15. Soergel DG, Subach RA, Burnham N, Lark MW, James IE, Sadler BM, et al. Biased agonism of the μ-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: a randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain* (2014) 155(9):1829–35. doi:10.1016/j.pain.2014.06.011
16. Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, et al. Structure-based discovery of opioid analgesics with reduced side effects. *Nature* (2016) 537(7619):185–90. doi:10.1038/nature19112
17. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev* (2009) 89(4):1379–412. doi:10.1152/physrev.00005.2009
18. Roberts AJ, Gold LH, Polis I, McDonald JS, Filliol D, Kieffer BL, et al. Increased ethanol self-administration in delta-opioid receptor knockout mice. *Alcohol Clin Exp Res* (2001) 25(9):1249–56. doi:10.1111/j.1530-0277.2001.tb02344.x
19. Jiang ZG, North RA. Pre- and postsynaptic inhibition by opioids in rat striatum. *J Neurosci* (1992) 12(1):356–61.
20. Scherrer G, Tryoen-Tóth P, Filliol D, Matifas A, Laustriat D, Cao YQ, et al. Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc Natl Acad Sci U S A* (2006) 103(25):9691–6. doi:10.1073/pnas.0603359103
21. Banghart MR, Neufeld SQ, Wong NC, Sabatini BL. Enkephalin disinhibits mu opioid receptor-rich striatal patches via delta opioid receptors. *Neuron* (2015) 88(6):1227–39. doi:10.1016/j.neuron.2015.11.010
22. Oude Ophuis RJ, Boender AJ, van Rozen AJ, Adan RA. Cannabinoid, melanocortin and opioid receptor expression on DRD1 and DRD2 subpopulations in rat striatum. *Front Neuroanat* (2014) 8:14. doi:10.3389/fnana.2014.00014
23. Atwood BK, Kupferschmidt DA, Lovinger DM. Opioids induce dissociable forms of long-term depression of excitatory inputs to the dorsal striatum. *Nat Neurosci* (2014) 17(4):540–8. doi:10.1038/nn.3652
24. Patton MH, Roberts BM, Lovinger DM, Mathur BN. Ethanol disinhibits dorsolateral striatal medium spiny neurons through activation of a presynaptic delta opioid receptor. *Neuropsychopharmacology* (2016) 41(7):1831–40. doi:10.1038/npp.2015.353
25. Nielsen CK, Simms JA, Li R, Mill D, Yi H, Feduccia AA, et al. Delta-opioid receptor function in the dorsal striatum plays a role in high levels of ethanol consumption in rats. *J Neurosci* (2012) 32(13):4540–52. doi:10.1523/JNEUROSCI.5345-11.2012
26. Zhou L, Bohn LM. Functional selectivity of GPCR signaling in animals. *Curr Opin Cell Biol* (2014) 27:102–8. doi:10.1016/j.ceb.2013.11.010
27. Kenakin T. Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* (2011) 336(2):296–302. doi:10.1124/jpet.110.173948
28. Chiang T, Sansuk K, van Rijn RM. Beta-arrestin 2 dependence of delta opioid receptor agonists is correlated with alcohol intake. *Br J Pharmacol* (2016) 173(2):323–43. doi:10.1111/bph.13374
29. van Rijn RM, Whistler JL. The delta(1) opioid receptor is a heterodimer that opposes the actions of the delta(2) receptor on alcohol intake. *Biol Psychiatry* (2009) 66(8):777–84. doi:10.1016/j.biopsych.2009.05.019
30. Whistler JL, Tsao P, von Zastrow M. A phosphorylation-regulated brake mechanism controls the initial endocytosis of opioid receptors but is not required for post-endocytic sorting to lysosomes. *J Biol Chem* (2001) 276(36):34331–8. doi:10.1074/jbc.M104627200
31. Paxinos G, Franklin K. *The Mouse Brain in Stereotaxic Coordinates*. 3rd ed. San Diego: Academic (2008).
32. Pan WX, Mao T, Dudman JT. Inputs to the dorsal striatum of the mouse reflect the parallel circuit architecture of the forebrain. *Front Neuroanat* (2010) 4:147. doi:10.3389/fnana.2010.00147
33. Zhu H, Pleil KE, Urban DJ, Moy SS, Kash TL, Roth BL. Chemogenetic inactivation of ventral hippocampal glutamatergic neurons disrupts consolidation of contextual fear memory. *Neuropsychopharmacology* (2014) 39(8):1880–92. doi:10.1038/npp.2014.35
34. Parnaudeau S, Taylor K, Bolkan SS, Ward RD, Balsam PD, Kellendonk C. Mediodorsal thalamus hypofunction impairs flexible goal-directed

behavior. *Biol Psychiatry* (2015) 77(5):445–53. doi:10.1016/j.biopsych.2014.03.020
35. Gomez JL, Bonaventura J, Lesniak W, Mathews WB, Sysa-Shah P, Rodriguez LA, et al. Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science* (2017) 357(6350):503–7. doi:10.1126/science.aan2475
36. Pradhan AA, Perroy J, Walwyn WM, Smith ML, Vicente-Sanchez A, Segura L, et al. Agonist-specific recruitment of arrestin isoforms differentially modify delta opioid receptor function. *J Neurosci* (2016) 36(12):3541–51. doi:10.1523/JNEUROSCI.4124-15.2016
37. Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, et al. In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* (2009) 4(5):e5425. doi:10.1371/journal.pone.0005425
38. van Rijn RM, Brissett DI, Whistler JL. Emergence of functional spinal delta opioid receptors after chronic ethanol exposure. *Biol Psychiatry* (2012) 71(3):232–8. doi:10.1016/j.biopsych.2011.07.015
39. Jutkiewicz EM, Rice KC, Traynor JR, Woods JH. Separation of the convulsions and antidepressant-like effects produced by the delta-opioid agonist SNC80 in rats. *Psychopharmacology (Berl)* (2005) 182(4):588–96. doi:10.1007/s00213-005-0138-9
40. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods* (2005) 2(12):905–9. doi:10.1038/nmeth819
41. Palmiter RD. Dopamine signaling in the dorsal striatum is essential for motivated behaviors: lessons from dopamine-deficient mice. *Ann N Y Acad Sci* (2008) 1129:35–46. doi:10.1196/annals.1417.003
42. Burnett CJ, Krashes MJ. Resolving behavioral output via chemogenetic designer receptors exclusively activated by designer drugs. *J Neurosci* (2016) 36(36):9268–82. doi:10.1523/JNEUROSCI.1333-16.2016
43. Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, et al. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *J Clin Invest* (2011) 121(4):1424–8. doi:10.1172/JCI46229
44. Pina MM, Young EA, Ryabinin AE, Cunningham CL. The bed nucleus of the stria terminalis regulates ethanol-seeking behavior in mice. *Neuropharmacology* (2015) 99:627–38. doi:10.1016/j.neuropharm.2015.08.033
45. Cassataro D, Bergfeldt D, Malekian C, Van Snellenberg JX, Thanos PK, Fishell G, et al. Reverse pharmacogenetic modulation of the nucleus accumbens reduces ethanol consumption in a limited access paradigm. *Neuropsychopharmacology* (2014) 39(2):283–90. doi:10.1038/npp.2013.184
46. Merritt KE, Bachtell RK. Initial d2 dopamine receptor sensitivity predicts cocaine sensitivity and reward in rats. *PLoS One* (2013) 8(11):e78258. doi:10.1371/journal.pone.0078258
47. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, et al. Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol* (2011) 80(3):367–77. doi:10.1124/mol.111.072801
48. Ito S, Mori T, Sawaguchi T. Dopamine-independent psychostimulant activity of a delta-agonist. *Behav Pharmacol* (2008) 19(2):113–9. doi:10.1097/FBP.0b013e3282f62c1b
49. Packard MG, McGaugh JL. Double dissociation of fornix and caudate nucleus lesions on acquisition of two water maze tasks: further evidence for multiple memory systems. *Behav Neurosci* (1992) 106(3):439–46. doi:10.1037/0735-7044.106.3.439
50. Packard MG, Hirsh R, White NM. Differential effects of fornix and caudate nucleus lesions on two radial maze tasks: evidence for multiple memory systems. *J Neurosci* (1989) 9(5):1465–72.
51. Dickinson A, Wood N, Smith JW. Alcohol seeking by rats: action or habit? *Q J Exp Psychol B* (2002) 55(4):331–48. doi:10.1080/0272499024400016
52. Packard MG, McGaugh JL. Inactivation of hippocampus or caudate nucleus with lidocaine differentially affects expression of place and response learning. *Neurobiol Learn Mem* (1996) 65(1):65–72. doi:10.1006/nlme.1996.0007
53. Quinn JJ, Pittenger C, Lee AS, Pierson JL, Taylor JR. Striatum-dependent habits are insensitive to both increases and decreases in reinforcer value in mice. *Eur J Neurosci* (2013) 37(6):1012–21. doi:10.1111/ejn.12106
54. Cuzon Carlson VC, Seabold GK, Helms CM, Garg N, Odagiri M, Rau AR, et al. Synaptic and morphological neuroadaptations in the putamen associated with long-term, relapsing alcohol drinking in primates. *Neuropsychopharmacology* (2011) 36(12):2513–28. doi:10.1038/npp.2011.140
55. Wilcox MV, Cuzon Carlson VC, Sherazee N, Sprow GM, Bock R, Thiele TE, et al. Repeated binge-like ethanol drinking alters ethanol drinking patterns and depresses striatal GABAergic transmission. *Neuropsychopharmacology* (2014) 39(3):579–94. doi:10.1038/npp.2013.230
56. Darcq E, Morisot N, Phamluong K, Warnault V, Jeanblanc J, Longo FM, et al. The neurotrophic factor receptor p75 in the rat dorsolateral striatum drives excessive alcohol drinking. *J Neurosci* (2016) 36(39):10116–27. doi:10.1523/JNEUROSCI.4597-14.2016
57. Jeanblanc J, He DY, Carnicella S, Kharazia V, Janak PH, Ron D. Endogenous BDNF in the dorsolateral striatum gates alcohol drinking. *J Neurosci* (2009) 29(43):13494–502. doi:10.1523/JNEUROSCI.2243-09.2009
58. Zhang W, Basile AS, Gomeza J, Volpicelli LA, Levey AI, Wess J. Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. *J Neurosci* (2002) 22(5):1709–17.
59. Hioki H, Kameda H, Nakamura H, Okunomiya T, Ohira K, Nakamura K, et al. Efficient gene transduction of neurons by lentivirus with enhanced neuron-specific promoters. *Gene Ther* (2007) 14(11):872–82. doi:10.1038/sj.gt.3302924
60. Aschauer DF, Kreuz S, Rumpel S. Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PLoS One* (2013) 8(9):e76310. doi:10.1371/journal.pone.0076310
61. Low K, Aebischer P, Schneider BL. Direct and retrograde transduction of nigral neurons with AAV6, 8, and 9 and intraneuronal persistence of viral particles. *Hum Gene Ther* (2013) 24(6):613–29. doi:10.1089/hum.2012.174
62. Wess J, Nakajima K, Jain S. Novel designer receptors to probe GPCR signaling and physiology. *Trends Pharmacol Sci* (2013) 34(7):385–92. doi:10.1016/j.tips.2013.04.006
63. Alvarez-Curto E, Prihandoko R, Tautermann CS, Zwier JM, Pediani JD, Lohse MJ, et al. Developing chemical genetic approaches to explore G protein-coupled receptor function: validation of the use of a receptor activated solely by synthetic ligand (RASSL). *Mol Pharmacol* (2011) 80(6):1033–46. doi:10.1124/mol.111.074674
64. Milan-Lobo L, Enquist J, van Rijn RM, Whistler JL. Anti-analgesic effect of the mu/delta opioid receptor heteromer revealed by ligand-biased antagonism. *PLoS One* (2013) 8(3):e58362. doi:10.1371/journal.pone.0058362
65. Bosse KE, Oginsky MF, Susick LL, Ramalingam S, Ferrario CR, Conti AC. Adenylyl cyclase 1 is required for ethanol-induced locomotor sensitization and associated increases in NMDA receptor phosphorylation and function in the dorsal medial striatum. *J Pharmacol Exp Ther* (2017) 363(2):148–55. doi:10.1124/jpet.117.242321
66. Stoppel LJ, Auerbach BD, Senter RK, Preza AR, Lefkowitz RJ, Bear MF. β-Arrestin2 couples metabotropic glutamate receptor 5 to neuronal protein synthesis and is a potential target to treat fragile X. *Cell Rep* (2017) 18(12):2807–14. doi:10.1016/j.celrep.2017.02.075
67. Pontrello CG, Sun MY, Lin A, Fiacco TA, DeFea KA, Ethell IM. Cofilin under control of β-arrestin-2 in NMDA-dependent dendritic spine plasticity, long-term depression (LTD), and learning. *Proc Natl Acad Sci U S A* (2012) 109(7):E442–51. doi:10.1073/pnas.1118803109
68. Eng AG, Kelver DA, Hedrick TP, Swanson GT. Transduction of group I mGluR-mediated synaptic plasticity by β-arrestin2 signalling. *Nat Commun* (2016) 7:13571. doi:10.1038/ncomms13571
69. Björk K, Rimondini R, Hansson AC, Terasmaa A, Hyytiä P, Heilig M, et al. Modulation of voluntary ethanol consumption by β-arrestin 2. *FASEB J* (2008) 22(7):2552–60. doi:10.1096/fj.07-102442
70. Li H, Tao Y, Ma L, Liu X, Ma L. β-Arrestin-2 inhibits preference for alcohol in mice and suppresses Akt signaling in the dorsal striatum. *Neurosci Bull* (2013) 29(5):531–40. doi:10.1007/s12264-013-1350-y
71. Zurkovsky L, Sedaghat K, Ahmed MR, Gurevich VV, Gurevich EV. Arrestin-2 and arrestin-3 differentially modulate locomotor responses and sensitization to amphetamine. *Neuropharmacology* (2017) 121:20–9. doi:10.1016/j.neuropharm.2017.04.021
72. Robins MT, Chiang T, Berry JN, Ko MJ, Ha JE, van Rijn RM. Behavioral characterization of β-arrestin 1 knockout mice in anxiety-like and alcohol behaviors. *Front Behav Neurosci* (2018) 12:54. doi:10.3389/fnbeh.2018.00054
73. Fan XL, Zhang JS, Zhang XQ, Yue W, Ma L. Differential regulation of beta-arrestin 1 and beta-arrestin 2 gene expression in rat brain by morphine. *Neuroscience* (2003) 117(2):383–9. doi:10.1016/S0306-4522(02)00930-2
74. Oakley RH, Revollo J, Cidlowski JA. Glucocorticoids regulate arrestin gene expression and redirect the signaling profile of G protein-coupled receptors. *Proc Natl Acad Sci U S A* (2012) 109(43):17591–6. doi:10.1073/pnas.1209411109

75. Lombardi MS, van den Tweel E, Kavelaars A, Groenendaal F, van Bel F, Heijnen CJ. Hypoxia/ischemia modulates G protein-coupled receptor kinase 2 and beta-arrestin-1 levels in the neonatal rat brain. *Stroke* (2004) 35(4):981–6. doi:10.1161/01.STR.0000121644.82596.7e
76. de la Cour C, Sørensen G, Wortwein G, Weikop P, Dencker D, Fink-Jensen A, et al. Enhanced self-administration of alcohol in muscarinic acetylcholine M4 receptor knockout mice. *Eur J Pharmacol* (2015) 746:1–5. doi:10.1016/j.ejphar.2014.10.050
77. Crabbe JC, Phillips TJ, Feller DJ, Hen R, Wenger CD, Lessov CN, et al. Elevated alcohol consumption in null mutant mice lacking 5-HT1B serotonin receptors. *Nat Genet* (1996) 14(1):98–101. doi:10.1038/ng0996-98
78. Luttrell LM, Gesty-Palmer D. Beyond desensitization: physiological relevance of arrestin-dependent signaling. *Pharmacol Rev* (2010) 62(2):305–30. doi:10.1124/pr.109.002436
79. Ibba F, Vinci S, Spiga S, Peana AT, Assaretti AR, Spina L, et al. Ethanol-induced extracellular signal regulated kinase: role of dopamine D1 receptors. *Alcohol Clin Exp Res* (2009) 33(5):858–67. doi:10.1111/j.1530-0277.2009.00907.x
80. Thorsell A, Tapocik JD, Liu K, Zook M, Bell L, Flanigan M, et al. A novel brain penetrant NPS receptor antagonist, NCGC00185684, blocks alcohol-induced ERK-phosphorylation in the central amygdala and decreases operant alcohol self-administration in rats. *J Neurosci* (2013) 33(24):10132–42. doi:10.1523/JNEUROSCI.4742-12.2013
81. van Rijn RM, Brissett DI, Whistler JL. Distinctive modulation of ethanol place preference by delta opioid receptor-selective agonists. *Drug Alcohol Depend* (2012) 122(1–2):156–9. doi:10.1016/j.drugalcdep.2011.09.024
82. van Rijn RM, Brissett DI, Whistler JL. Dual efficacy of delta opioid receptor selective ligands for ethanol drinking and anxiety. *J Pharmacol Exp Ther* (2010) 335(1):133–9. doi:10.1124/jpet.110.170969
83. Dyr W, McBride WJ, Lumeng L, Li TK, Murphy JM. Effects of D1 and D2 dopamine receptor agents on ethanol consumption in the high-alcohol-drinking (HAD) line of rats. *Alcohol* (1993) 10(3):207–12. doi:10.1016/0741-8329(93)90037-O
84. Logrip ML, Janak PH, Ron D. Blockade of ethanol reward by the kappa opioid receptor agonist U50,488H. *Alcohol* (2009) 43(5):359–65. doi:10.1016/j.alcohol.2009.05.001
85. Mizushima K, Miyamoto Y, Tsukahara F, Hirai M, Sakaki Y, Ito T. A novel G-protein-coupled receptor gene expressed in striatum. *Genomics* (2000) 69(3):314–21. doi:10.1006/geno.2000.6340

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