NOVEL PAIN THERAPEUTICS: FROM BASIC RESEARCH TO CLINICAL TRANSLATION AND REHABILITATION

EDITED BY: Giacinto Bagetta, Maria Tiziana Corasaniti, Tsukasa Sakurada, Damiana Scuteri and Paolo Tonin

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Topic Editors:
Giacinto Bagetta, University of Calabria, Italy
Maria Tiziana Corasaniti, University of Catanzaro, Italy
Tsukasa Sakurada, Daiichi University of Pharmacy, Japan
Damiana Scuteri, University of Calabria, Italy
Paolo Tonin, Sant'Anna Institute, Italy

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EDITORIAL
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Editorial: "Novel Pain Therapeutics: From Basic Research to Clinical Translation and Rehabilitation"

Damiana Scuteri[1,2], *Tsukasa Sakurada*[3], *Paolo Tonin*[2], *Maria Tiziana Corasaniti*[4,5] *and Giacinto Bagetta*[1]*

[1]Pharmacotechnology Documentation and Transfer Unit, Section of Preclinical and Translational Pharmacology, Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende, Italy, [2]Regional Center for Serious Brain Injuries, S. Anna Institute, Crotone, Italy, [3]Center for Supporting Pharmaceutical Education, Faculty of Pharmaceutical Sciences, Daiichi University of Pharmacy, Fukuoka, Japan, [4]Department of Health Sciences, University "Magna Graecia" of Catanzaro, Catanzaro, Italy, [5]School of Hospital Pharmacy, University "Magna Graecia" of Catanzaro, Catanzaro, Italy

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Editorial on the Research Topic

Novel Pain Therapeutics: From Basic Research to Clinical Translation and Rehabilitation

Chronic pain is a major public health problem representing a global leading cause of disability and disease burden with low back pain and headache disorders ranking top-ten as causes of disability-adjusted life-years (DALYs) throughout the whole lifetime from teenage to old age (Diseases and Injuries, 2020). In fact, some 30–50% of the world population and almost 20% of the Europeans actually suffer from chronic pain with devastating cost for the society in terms of disability and hospitalization (Breivik et al., 2006; Reid et al., 2011; van Hecke et al., 2013; Mills et al., 2019). Therefore, research aiming at understanding the mechanisms involved in chronic pain is the key to improve treatment and quality of life of patients. This is even more important at the time of COVID-19 that poses several limitations to the access to chronic pain treatment (Karos et al., 2020), enhancing its impact especially on aged, cognitively impaired and non communicative patients (Scuteri et al., 2020b). For a better understanding and management of pain there are four main aspects to be considered: 1) neurotransmission and anomalous plasticity; 2) neuron-glial interaction in central sensitization; 3) drugs and botanicals for pain therapy; 4) pharmacology, assessment and management of chronic pain in the fragile populations. Nociceptive primary afferent fibers release mainly glutamate, substance P and calcitonin gene-related peptide (CGRP); modulation of pain by these neurotransmitters is fundamental for translation into clinical practice. Accordingly, understanding of the role of CGRP neurotransmission represents a noteworthy example of advances in migraine prevention and treatment, previously relying only on triptans and symptomatic, non specific, analgesics (Scuteri et al., 2020a). The progress guaranteed by anti-CGRP monoclonal antibodies for migraine, and by eptinezumab, in particular, allowing faster action due to intravenous administration (Scuteri et al., 2019b), is due to the dissection of CGRP signaling mechanisms (Scuteri et al., 2019a). The modulation of the endogenous pain inhibitory system is implicated in the action of pain-relieving drugs (Ossipov et al., 2010). Metabotropic glutamate receptors can promote chronic pain through sensitization of the central nucleus of the amygdala (Ossipov et al., 2010). Thus, glutamatergic transmission is involved in the ascending but also in the descending pathways modulation due to presynaptic glutamate metabotropic autoreceptors like mGlu2/3. The latter are involved in the mechanisms of antinociception of drugs and botanicals (Scuteri et al., 2019d). In fact, the essential oil of bergamot is able to modulate synaptic level of

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Nicholas M Barnes, University of Birmingham, United Kingdom

***Correspondence:**
Giacinto Bagetta
giacinto.bagetta@unical.it

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glutamate (Morrone et al., 2007) and this contributes to its analgesic activity (Scuteri et al., 2019c), which is enriched by anxiolytic properties devoid of sedation (Rombola et al., 2019). Interestingly, the analgesic effect occurs also when it is administered via the inhalatory route (Scuteri et al., 2018a), though it is independent on olfactory stimulation and mediated by systemic absorption of active principles (Hamamura et al.). Trazodone has been demonstrated to restore acutely the aberrant plasticity to which mGlu autoreceptors are subjected after spinal nerve ligation (Cisani et al.). Furthermore, glutamate is involved in the antinociceptive effect of apelin, the endogenous ligand for the putative receptor protein related to the type 1 angiotensin receptor (APJ) orphan G-protein-coupled receptor (Lv et al.). The activation of N-methyl-D-aspartate (NMDA) receptor elicits cholecystokinin-8 (CCK-8) pronociceptive effects (Hayashi et al.). Transient Receptor Potential (TRP) Vanilloid 1 and Ankyrin 1 (TRPV1, TRPA1) channels represent important targets for analgesia at level of the nociceptive primary sensory neurons (Horváth et al.). The neuropeptide oxytocin is endowed with preemptive analgesic properties in a validated postoperative pain model (Espinosa De Los Monteros-Zuniga et al.). Galanin receptor agonists have been demonstrated to have potential analgesic properties with action on protein kinase C (Li et al.). Opioid receptors occurring on nociceptor terminals play a pivotal role in pain modulation. For instance, in neuropathic pain deltorphin II can inhibit the response of C fibers in a concentration-dependent and delta opioid receptors-mediated manner (Joukal et al.). Moreover, methadone has been found to be effective in morphine-resistant hyperalgesia and to restore morphine antinociception in the inflammatory model of the complete Freund's adjuvant (CFA) (Watanabe et al.). The latter is interesting also in view of the lack of efficacy and the onset of serious side effects for chronic administration of opioids in the treatment of osteoarthritis. The role of the inflammasome in pain is fundamental and the interaction between immune and nervous system contributes to the development of chronic pain. Also, the modulation of microglia can reduce allodynia in a sex-dependent manner (Saika et al.). Oxidative stress and nuclear factor erythroid derived-2-related factor 2 (Nrf2) pathway is notably implicated in neuropathic pain (Zhao et al.). Other targets for pain treatment are the small-conductance Ca^{2+}-activated K^{+} channels for visceral hypersensitivity (Ji et al.). In particular, the study of visceral hypersensitivity is of the utmost importance since it can predispose to visceral chronic pain and it can be caused by maternal separation responsible for elevated firing frequency of corticotropin-releasing factor neurons (Huang et al.). Among the non pharmacologic approaches, acupuncture has been studied in primary dysmenorrhea and its efficacy can be predicted by pain-related functional connectivity patterns through machine learning multivariate pattern analysis (Yu et al.); the endocannabinoid system seems to be implicated in electroacupuncture-induced analgesia (MacDonald and Chen). Hence, the use of novel technological tools, e.g., machine learning, can improve the comprehension of the machinery of chronic pain. A better understanding of the above mechanisms can lead to discover the properties of already known and widely used analgesics, like acetaminophen, which still holds some surprises, e.g., the action of its metabolite N-acylphenolamine on brain TRPV1 and cannabinoid 1 receptors with consequent central analgesia (Ohashi and Kohno). These findings highlight the need for more basic as well as clinical studies on commonly used painkillers. One of the most remarkable is the case of opioids, often inappropriately used in fragile populations of the elderly commonly suffering from diseases that impair their communication skills. The treatment of chronic pain *per se* represents an area of strong therapeutic inappropriateness resulting in real-world limited access to care for fragile patients, e.g., demented aged people (Scuteri et al., 2017; Scuteri et al., 2018b; Scuteri et al., 2021). This is mirrored by the scarce use of the existing adequate observational pain scales and by the lack of standardization and guidelines for clinical practice in dementia (Gimenez-Llort et al.). This issue is very serious also for patients suffering from post-stroke pain. In fact, what emerges from the systematic review and meta-analysis of current literature is that the use of opioids in these patients is not specifically studied in the latter population with clinical trials designed for this purpose and using suitable pain assessment tools (Scuteri et al.). The assessment and the treatment of pain after spinal cord injury is still an unmet need (Finnerup, 2013; Yasko and Mains, 2018). The correct management of pain in patients who have been subjected to a severe brain injury with disorders of consciousness is fundamental to allow the best therapeutic approach and the planning of the most effective rehabilitation strategy (Riganello et al.).

DATA AVAILABILITY STATEMENT

All the datasets generated/analyzed for this study are included in the article.

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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Chemogenetic Regulation of CX3CR1-Expressing Microglia Using Gi-DREADD Exerts Sex-Dependent Anti-Allodynic Effects in Mouse Models of Neuropathic Pain

Fumihiro Saika[1], *Shinsuke Matsuzaki*[1], *Daichi Kobayashi*[1,2], *Yuya Ideguchi*[1], *Tomoe Y. Nakamura*[1], *Shiroh Kishioka*[1,3] *and Norikazu Kiguchi*[1]*

[1] Department of Pharmacology, Wakayama Medical University, Wakayama, Japan, [2] Department of Immunology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan, [3] Faculty of Wakayama Health Care Sciences, Takarazuka University of Medical and Health Care, Wakayama, Japan

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Edited by:
Giacinto Bagetta, University of Calabria, Italy

Reviewed by:
Livio Luongo, University of Campania Luigi Vanvitelli, Italy
Paola Sacerdote, University of Milan, Italy

***Correspondence:**
Norikazu Kiguchi
kiguchi@wakayama-med.ac.jp

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Despite growing evidence suggesting that spinal microglia play an important role in the molecular mechanism underlying experimental neuropathic pain (NP) in male rodents, evidence regarding the sex-dependent role of these microglia in NP is insufficient. In this study, we evaluated the effects of microglial regulation on NP using Gi-designer receptors exclusively activated by designer drugs (Gi-DREADD) driven by the microglia-specific *Cx3cr1* promoter. For the Cre-dependent expression of human Gi-coupled M4 muscarinic receptors (hM4Di) in CX3C chemokine receptor 1-expressing ($CX3CR1^+$) cells, R26-LSL-hM4Di-DREADD mice were crossed with CX3CR1-Cre mice. Mouse models of NP were generated by partial sciatic nerve ligation (PSL) and treatment with anti-cancer agent paclitaxel (PTX) or oxaliplatin (OXA), and mechanical allodynia was evaluated using the von Frey test. Immunohistochemistry revealed that hM4Di was specifically expressed on $Iba1^+$ microglia, but not on astrocytes or neurons in the spinal dorsal horn of CX3CR1-hM4Di mice. PSL-induced mechanical allodynia was significantly attenuated by systemic (intraperitoneal, i.p.) administration of 10 mg/kg of clozapine N-oxide (CNO), a hM4Di-selective ligand, in male CX3CR1-hM4Di mice. The mechanical threshold in naive CX3CR1-hM4Di mice was not altered by i.p. administration of CNO. Consistently, local (intrathecal, i.t.) administration of CNO (20 nmol) significantly relieved PSL-induced mechanical allodynia in male CX3CR1-hM4Di mice. However, neither i.p. nor i.t. administration of CNO affected PSL-induced mechanical allodynia in female CX3CR1-hM4Di mice. Both i.p. and i.t. administration of CNO relieved PTX-induced mechanical allodynia in male CX3CR1-hM4Di mice, and a limited effect of i.p. CNO was observed in female CX3CR1-hM4Di mice. Unlike PTX-induced allodynia, OXA-induced mechanical allodynia was slightly improved, but not significantly relieved, by i.p. administration of CNO in both male and female CX3CR1-hM4Di mice. These results suggest that spinal microglia can be regulated by Gi-DREADD and support the notion that

CX3CR1+ spinal microglia play sex-dependent roles in nerve injury-induced NP; however, their roles may vary among different models of NP.

Keywords: allodynia, chronic pain, oxaliplatin, paclitaxel, spinal cord

INTRODUCTION

Chronic pain is a serious problem that not only afflicts patients but also represents a substantial economic burden to the international community (Smith and Torrance, 2012). Neuropathic pain (NP), resulting from a lesion in the peripheral or central nervous system (PNS or CNS), is characterized by pain sensation in response to innoxious stimuli (allodynia) and increased sensitivity to pain (hyperalgesia) (Jensen and Finnerup, 2014). Moreover, treatment with anti-cancer agents [such as paclitaxel (PTX) and oxaliplatin (OXA)] has also been shown to induce NP (Colvin, 2019). Chemotherapy-induced neuropathic pain (CINP) markedly worsens the quality of life of cancer patients and often leads to chemotherapy discontinuation (Hershman et al., 2014). It is estimated that 7%–8% of the general population suffers from NP (van Hecke et al., 2014); however, the molecular and cellular mechanisms underlying this condition are poorly understood. Hence, there is a strong need for better insights into such mechanisms and the development of effective therapeutics based on these mechanisms.

Accumulating evidence suggests that an interaction between the immune system and the nervous system, including the brain, spinal cord, dorsal root ganglia, and peripheral nerves, largely contributes to the pathogenesis of NP (Ji et al., 2016). In the PNS, bone marrow-derived macrophages clearly accumulated in the damaged nerves and drive long-lasting inflammation underlying NP (Scholz and Woolf, 2007; Ji et al., 2016; Kiguchi et al., 2017). On the other hand, among the variety of spinal glial cells, microglia, the resident macrophages of the CNS responsible for its innate immunity, play a critical role in the development and maintenance of NP (Chen et al., 2018; Inoue and Tsuda, 2018). Several researchers have shown that spinal microglia become activated and secrete various inflammatory mediators, such as cytokines and chemokines, that sensitize pain-processing neurons in the spinal dorsal horn (SDH) (Milligan and Watkins, 2009; Ji et al., 2016). However, recent reports have demonstrated that spinal microglia are important in NP in male, but not in female, mice, suggesting sex-dependent roles for microglia in NP (Sorge et al., 2015; Rosen et al., 2017). Further investigations focusing on sex differences in NP pathogenesis are warranted to uncover the underlying mechanisms and develop effective therapeutics.

Designer receptors exclusively activated by designer drugs (DREADD) are genetically modified G-protein-coupled receptors (GPCRs). This technology represents a useful chemogenetic strategy, allowing researchers to gain control of Gi- or Gq-signaling pathways, remotely and noninvasively (Urban and Roth, 2015). The DREADD technology has been frequently used to regulate the activity of various types of neurons. Through application of selective ligands, clozapine-N-oxide (CNO) can stimulate or inhibit certain populations of glutamatergic, GABAergic or dopaminergic neurons (Dell'Anno et al., 2014; Koga et al., 2017; Zhang et al., 2020). In addition, the DREADD system has been used to regulate cellular activities of myeloid cells, breast cancer cells, and glial cells (Urban and Roth, 2015). Furthermore, Grace et al. have demonstrated that chemogenetic inhibition of spinal microglia *via* human Gi-coupled M4 muscarinic receptors (hM4Di) attenuates mechanical allodynia following peripheral nerve injury, while chemogenetic activation of spinal microglia *via* human Gq-coupled M3 muscarinic receptors (hM3Dq) induces mechanical allodynia in naïve rats using viral gene transfer (Grace et al., 2018). Nevertheless, state-dependent effects of Gi- or Gq-DREADD on intracellular signaling in microglia are still unclear, and evidence regarding the sex-dependent role of microglia in NP is insufficient because of the diversity of the experimental models used to study the condition.

In this study, we evaluated the sex-dependent effects of microglial regulation on NP caused by partial sciatic nerve ligation (PSL) and CINP, using Gi-DREADD driven by the microglia-specific *Cx3cr1* promoter (CX3CR1-hM4Di), in mice.

MATERIALS AND METHODS

Mice

All animal experiments were approved by the Animal Research Committee of Wakayama Medical University and were carried out in accordance with the in-house guidelines for care and use of laboratory animals of Wakayama Medical University and the Ethical Guidelines of the International Association for the Study of Pain. R26-LSL-hM4Di-DREADD mice [B6N.129-Gt(ROSA)26Sor$^{tm1(CAG-CHRM4*,-mCitrine)Ute}$/J; stock #026219] (Zhu et al., 2016) and CX3CR1-Cre transgenic (Tg) mice [Tg(Cx3cr1-cre)MW126Gsat/Mmucd; stock #036395] were purchased from the Jackson Laboratory and Mutant Mouse Resource & Research Centers (MMRRC), respectively. R26-LSL-hM4Di-DREADD mice were maintained as heterozygous or homozygous genotype. For the Cre-dependent expression of the Gi-DREADD system in the *Rosa26* locus in CX3CR1-expressing (CX3CR1$^+$) cells, R26-LSL-hM4Di-DREADD mice were crossed with CX3CR1-Cre mice. Subsequently, 6–12-week-old mice heterozygous for ROSA26 and CX3CR1-Cre were used for the experiments. All mice were housed in groups of 5–6 in plastic cages at controlled temperature (23°C–24°C) and humidity (60%–70%), and the environment was maintained on a 12-h dark/light cycle, with free access to standard food and water.

Drug Administration

Paclitaxel (TAXOL® Injection; Bristol-Myers Squibb Company, New York, NY, USA) and oxaliplatin (ELPLAT® i.v. infusion

solution; Yakult Honsha Co., Ltd, Tokyo, Japan) were diluted in 5% glucose solution. Clozapine N-oxide (CNO: Enzo Life Sciences, Farmingdale, NY, USA) was dissolved in sterile water and diluted as needed. CNO was administered intraperitoneally (i.p.) at a volume of 0.1 ml/10 g body weight to awake mice or intrathecally (i.t.) at a volume of 5 µl to isoflurane-anesthetized mice, as previously described (Kiguchi et al., 2020). Under isoflurane anesthesia, mice were secured by a firm grip on the pelvic girdle, and drugs were injected by lumbar puncture between the L5 and L6 vertebrae using a 30-gauge needle fitted with Hamilton microsyringe.

Neuropathic Pain Models

Partial Sciatic Nerve Ligation (PSL) Model

The mice were subjected to PSL as previously described (Seltzer et al., 1990; Kiguchi et al., 2018). Briefly, under isoflurane anesthesia, the left common sciatic nerve (SCN) of each mouse was exposed at the mid-thigh level through a small skin incision on one side, hereafter indicated as ipsilateral. Approximately one-third of the SCN thickness was tightly ligated with a silk suture (No. 1; Natsume Seisakusho, Tokyo, Japan); then, the muscle and skin layers were closed with sutures and the surgical area was sterilized with povidone–iodine. The untreated right limb is indicated as contralateral.

Chemotherapy-Induced Neuropathic Pain (CINP) Models

Paclitaxel (4 mg/kg/day), oxaliplatin (5 mg/kg/day), or vehicle (Veh; 5% glucose solution) was administered i.p. to the mice at a volume of 0.1 ml/10 g body weight four times every second day (on days 0, 2, 4, and 6).

Immunohistochemistry

The mice were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with ice-cold phosphate-buffered saline (PBS), followed by 4% (w/v) paraformaldehyde/phosphate buffer solution. Then, the lumbar spinal cord (L4–5) or the SCN was dissected, post-fixed in 4% paraformaldehyde/phosphate buffer solution, and put overnight in a 30% (w/v) sucrose/PBS solution at 4°C for cryoprotection. The tissue was then embedded in freezing optimal cutting temperature compound (Sakura, Tokyo, Japan). Subsequently, the specimens were longitudinally cut into 30 µm (spinal cord)- or 15 µm (SCN)-thick sections with a cryostat (Leica Microsystems, Wetzlar, Germany). The sections were treated with PBS containing 0.1% Triton X-100 (PBST) for 1 h and then blocked with Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) at 15°C–25°C for 5–10 min. They were then incubated with primary antibodies against hemagglutinin (HA) epitope-tag (mouse monoclonal, 1:250; BioLegend, San Diego, CA, USA), Iba1 (for microglia; rabbit polyclonal, 1:500; Wako, Japan), GFAP (for astrocytes; rabbit polyclonal, 1:500; Proteintech, Rosemont, IL, USA), NeuN (for neurons; rabbit monoclonal, 1:500; Millipore, Billerica, MA, USA), and F4/80 (for macrophages; rat monoclonal, 1:200; Cederlane, Burlington, Canada) in Blocking One Histo/PBST at 4°C overnight. Subsequently, the sections were rinsed in PBST and incubated with fluorescence-conjugated secondary antibodies (1:200; Abcam, Cambridge, UK) at 15°C–25°C for 2 h. They were then rinsed in PBS and incubated with Hoechst 33342 (1:1000; Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature in the dark. Finally, the sections were washed with PBS (10 min), mounted on glass slides, and covered with a cover slip with PermaFluor (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence images were detected using a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Behavioral Testing

von Frey test: To evaluate mechanical allodynia, the 50% paw withdrawal threshold was determined through the von Frey test, in accordance with a previously described method (Chaplan et al., 1994; Saika et al., 2019). Briefly, the mice were individually placed on a metal mesh (5 × 5 mm) grid floor and covered with an opaque acrylic box. Before the test, the mice were habituated to the experimental environment for at least 3–4 h. On the test day, after adaptation for 2-3 h, calibrated von Frey filaments (North Coast Medical, Inc., Gilroy, CA, USA) were applied to the middle of the plantar surface of the hind paw through the bottom of the mesh floor. The filament set used in this study consisted of nine calibrated von Frey filaments—0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g. In the paradigm of the up–down method, the test always started with the application of 0.4 g filaments. Quick withdrawal, shaking, biting or licking of the stimulated paw were regarded as positive paw withdrawal response. In the absence of a paw withdrawal response to the selected force, the next stronger stimulus was applied. In the presence of paw withdrawal, the next weaker stimulus was chosen. In accordance with Chaplan et al.'s procedure, after the response threshold was first crossed (the two responses straddling the threshold), four additional stimuli were applied. Based on the responses to the von Frey filaments series, the 50% paw withdrawal threshold was calculated according to the method described by Dixon (Dixon, 1980).

Rotarod test: To assess motor function, the Rotarod apparatus (Panlab, Barcelona, Spain) was used. A few days before the test, the mice were pre-trained in order to habituate them to the rod, rotating at various speeds (5, 10, and 15 rpm). The mice were placed on the rod facing the opposite direction to the rotation and ambulated until they fell from the rod or the maximum observation time had elapsed. The maximum observation time was 180 s. For all experiments, the latency and rotational velocity at which the animal fell from the Rotarod were recorded. Following training for a few days, the mice rested for 1 day and were then tested (three trials per day) on the rod rotating at various speeds (10, 15, 20 rpm), with 10-15 min interval between each trial. The mean time spent on the Rotarod apparatus was measured on each velocity (Pre-test). After the Pre-test phase, the mice received a single i.p. administration of CNO (10 mg/kg). The test was repeated 24 h after administration (Post-test).

Statistical Analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using Student's t-test, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or two-way ANOVA followed by

Bonferroni's multiple comparison test, as appropriate. P-values less than 0.05 were considered statistically significant.

RESULTS

hM4Di Expression in Spinal Microglia of CX3CR1-hM4Di Mice

First, we evaluated the effects of chemogenetic modulation of CX3C chemokine receptor 1-expressing (CX3CR1$^+$) cells on PSL-induced NP using Cre-dependent Gi-DREADD. For the Cre-dependent expression of hM4Di in CX3CR1$^+$ cells, R26-LSL-hM4Di-DREADD (Control-hM4Di) mice were crossed with CX3CR1-Cre mice (**Figure 1A**). Following Cre-mediated removal of an upstream floxed-STOP cassette, the expression of HA-tagged hM4Di was observed using an antibody against HA by immunohistochemistry. Iba1$^+$ microglia were markedly increased in the ipsilateral compared to the contralateral side of the SDH in both male Control-hM4Di and CX3CR1-Cre/R26-LSL-hM4Di-DREADD (CX3CR1-hM4Di) mice on day 7 after PSL. HA-hM4Di was highly expressed in the SDH of CX3CR1-hM4Di mice, but not in the SDH of Control-hM4Di mice (**Figure 1B**). In CX3CR1-hM4Di mice, HA-hM4Di overlapped with Iba1; however, it was not localized in GFAP$^+$ astrocytes or NeuN$^+$

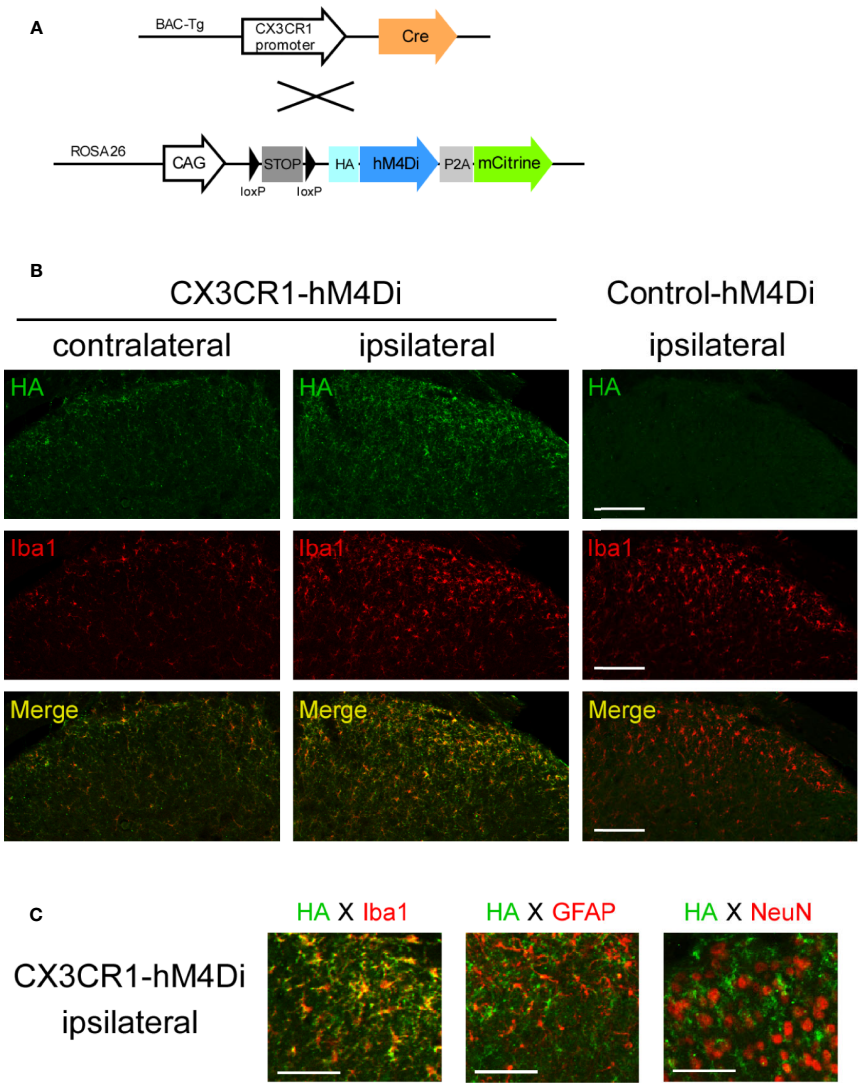

FIGURE 1 | hM4Di expression in spinal microglia of CX3CR1-hM4Di mice. **(A)** Scheme of Cre-dependent expression of hM4Di in CX3CR1$^+$ cells by crossing CX3CR1-Cre (Tg) mice with R26-LSL-hM4Di-DREADD mice (Control-hM4Di). Following Cre-mediated removal of an upstream floxed-STOP cassette, the HA-tagged hM4Di and mCitrine were expressed in CX3CR1$^+$ cells. **(B, C)** The mice were subjected to partial sciatic nerve ligation (PSL), and the lumbar spinal cord (L4-5) was dissected on day 7 after PSL. Expression of HA-tagged hM4Di in the spinal dorsal horn (SDH) was visualized by immunohistochemistry. **(B)** Expression of HA-tagged hM4Di in the SDH of CX3CR1-hM4Di mice. Scale bars = 100 μm. **(C)** Localization of HA-hM4Di in Iba1$^+$ microglia, but not GFAP$^+$ astrocytes or NeuN$^+$ neurons. Scale bars = 50 μm.

neurons (**Figure 1C**). Additionally, F4/80+ macrophages were clearly accumulated in the injured SCN in both male Control-hM4Di and CX3CR1-hM4Di mice on day 7 after PSL. HA-hM4Di was also expressed in macrophages of CX3CR1-hM4Di mice, but not in macrophages of Control-hM4Di mice (**Figure S1**).

Relief of Mechanical Allodynia After PSL by Systemic CNO in Male, but Not in Female CX3CR1-hM4Di Mice

To determine whether induction of Gi-DREADD in CX3CR1+ cells affects PSL-induced mechanical allodynia in male mice, different doses of CNO (0.1-10 mg/kg, i.p.) were administered daily, from days 7 to 11 after PSL, and pain assessment was performed the day after each administration. PSL-induced mechanical allodynia was significantly attenuated by 10 mg/kg of CNO (**Figure 2A**), but neither CNO (10 mg/kg, i.p.) nor Veh altered the 50% withdrawal threshold in naive male CX3CR1-hM4Di mice (**Figure 2B**). PSL-induced mechanical allodynia was significantly prevented by single administration of CNO (10 mg/kg, i.p.) compared to administration of Veh in male CX3CR1-hM4Di mice, but not in male Control-hM4Di mice (**Figure 2C**). In both male CX3CR1-hM4Di and Control-hM4Di mice, CNO (10 mg/kg, i.p.) did not affect the time on the Rotarod at 10, 15, and 20 rpm on day 1 after administration, indicating no

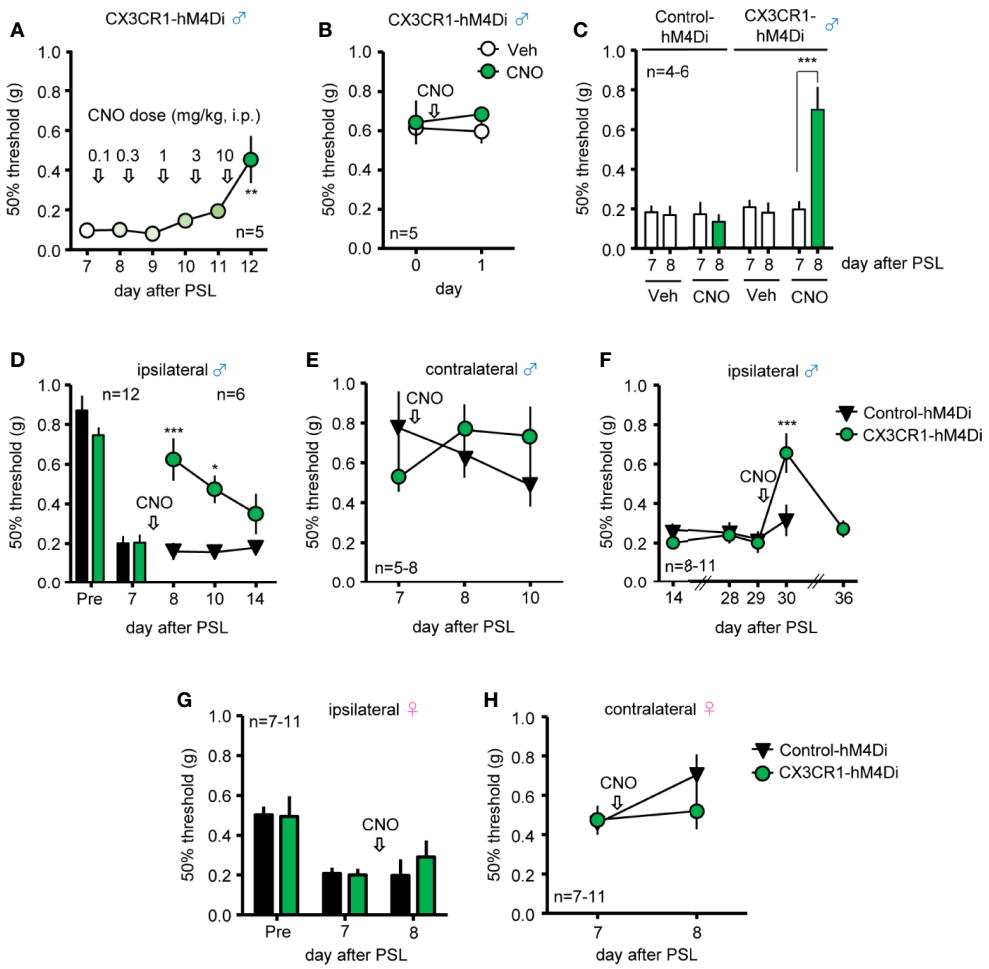

FIGURE 2 | Relief of mechanical allodynia after PSL by systemic CNO in male CX3CR1-hM4Di mice. The mice were subjected to PSL, and CNO was intraperitoneally (i.p.) administered as follows: **(A)** Increasing doses of CNO (0.1-10 mg/kg, i.p.) were administered daily from days 7 to 11 after PSL, and pain assessment was performed the next day after each administration. **(B–H)** CNO was i.p. administered after pain assessment as indicated by arrows. The 50% paw withdrawal threshold was assessed by the up–down method using the von Frey test. **(A)** Effects of different doses of CNO (0.1-10 mg/kg) on mechanical allodynia after PSL were evaluated 24 h after each CNO administration (before next doses) in male CX3CR1-hM4Di mice. **(B)** No effect of CNO (10 mg/kg) on 50% paw withdrawal threshold in naïve male CX3CR1-hM4Di mice. **(C)** Relieving effect of CNO (10 mg/kg) on PSL-induced mechanical allodynia in male CX3CR1-hM4Di mice. **(D, E)** Effect of CNO (10 mg/kg) on the 50% paw withdrawal threshold in the ipsilateral and contralateral side of male CX3CR1-hM4Di and Control-hM4Di mice after PSL. **(F)** Prolonged relieving effect of CNO (10 mg/kg) on mechanical allodynia in male CX3CR1-hM4Di and Control-hM4Di mice after PSL. **(G, H)** Effect of CNO (10 mg/kg) on the 50% paw withdrawal threshold in the ipsilateral and contralateral side of female CX3CR1-hM4Di and Control-hM4Di mice after PSL. Data are presented as mean ± S.E.M. n = 5-11. p***< 0.001, p** < 0.01, p* < 0.05 vs day 7 or day 29 before CNO, or Control-hM4Di.

motor dysfunction due to CNO (10 mg/kg) (**Figure S2**). Single administration of CNO (10 mg/kg, i.p.) on day 7 after PSL transiently relieved mechanical allodynia (days 8-10), but the anti-allodynic effect disappeared within 7 days after CNO administration (day 14) in the ipsilateral side of male CX3CR1-hM4Di mice (**Figure 2D**). The 50% withdrawal threshold showed no significant difference in the contralateral side (**Figure 2E**). Moreover, even on day 28 after PSL, PSL-induced mechanical allodynia in the ipsilateral paw was significantly suppressed by CNO (10 mg/kg, i.p.) in male CX3CR1-hM4Di, but not in male Control-hM4Di mice (**Figure 2F**). However, CNO (10 mg/kg, i.p.) had no significant effect on the 50% withdrawal threshold in the ipsilateral or contralateral side of female CX3CR1-hM4Di mice (**Figures 2G, H**).

Relief of Mechanical Allodynia After PSL by Intrathecal CNO in Male, but Not in Female CX3CR1-hM4Di Mice

Next, to clarify the effects of Gi-DREADD in spinal CX3CR1$^+$ microglia under PSL-induced mechanical allodynia, CNO (20 nmol) or Veh was i.t. administered in both CX3CR1-hM4Di and Control-hM4Di mice. Single administration of CNO (20 nmol, i.t.) on day 7 after PSL significantly relieved PSL-induced mechanical allodynia compared to administration of Veh (days 8-10) in male CX3CR1-hM4Di but not Control-hM4Di mice; however, the anti-allodynic effect of i.t. CNO disappeared 7 days after CNO administration (day 14) in male CX3CR1-hM4Di mice (**Figures 3A, B**). In contrast, i.t. administration of CNO had no effect on PSL-induced mechanical allodynia in female CX3CR1-hM4Di mice (**Figure 3C**).

Effects of CNO on PTX- or OXA-Induced CINP in CX3CR1-hM4Di Mice

Finally, we investigated whether induction of Gi-DREADD in CX3CR1$^+$ cells can relieve CINP. Repetitive i.p. administration of PTX in male CX3CR1-hM4Di and Control-hM4Di mice caused mechanical allodynia on day 7 compared to administration of Veh (5% glucose solution) (**Figure 4A**). Single administration of CNO (10 mg/kg, i.p.) on day 7 relieved mechanical allodynia by PTX on the following day (day 8), and the anti-allodynic effect persisted for 7 days after CNO administration (until day 14) in male CX3CR1-hM4Di, but not in Control-hM4Di mice (**Figures 4A, B**). Moreover, single administration of CNO (20 nmol, i.t.) on day 7 significantly relieved PTX-induced mechanical allodynia as well (**Figure 4C**). In female CX3CR1-hM4Di mice also, single administration of CNO (10 mg/kg, i.p.) on day 7 attenuated PTX-induced mechanical allodynia on the following day (day 8), but this effect disappeared 7 days after CNO administration (**Figure 4D**). Unlike the PTX-induced effect, OXA-induced mechanical allodynia was slightly improved, but not significantly relieved, by single administration of CNO (10 mg/kg, i.p.) in both male and female CX3CR1-hM4Di mice (**Figures 4E, F**).

DISCUSSION

In this study, we provided several novel findings. CX3CR1$^+$ microglia could be regulated by Gi-DREADD in mice, and PSL-induced mechanical allodynia was relieved by CX3CR1-Gi-DREADD, regardless of time after injury, in male but not in female mice. Moreover, the relieving effect of CX3CR1-Gi-DREADD on PTX-, but not OXA-induced allodynia, was dominantly observed in male compared to female mice. These lines of evidence illustrate model- and sex-dependent roles of spinal microglia in the pathophysiology of NP.

Among different microglial promoters, the *Cx3cr1* promoter system is frequently used to implement microglia-specific gene manipulation in mice. To date, different CX3CR1-Cre lines have been developed and widely used in a number of studies, focusing on the roles of microglia in the CNS (Parkhurst et al., 2013; Peng et al., 2016). However, it is also reported that some CX3CR1-Cre lines have a possibility for leakage of Cre expression into other cells, such as neurons and astrocytes (Hwang et al., 2017; Zhang et al., 2018). Zhao et al. demonstrated that CX3CR1 expression

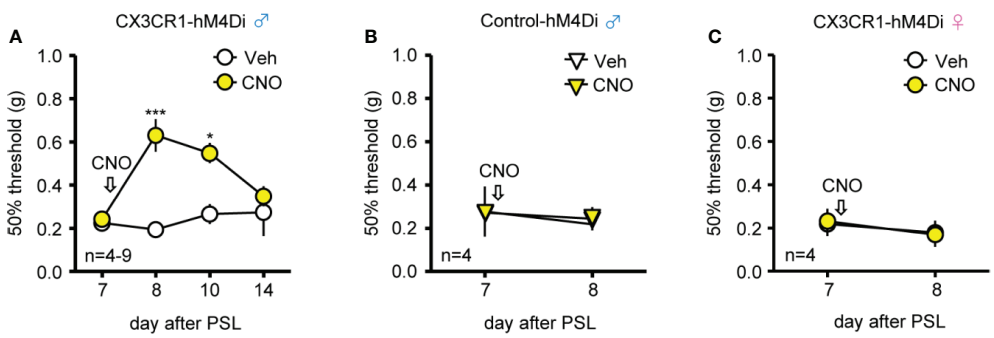

FIGURE 3 | Relief of mechanical allodynia after PSL by intrathecal CNO in male CX3CR1-hM4Di mice. The mice were subjected to PSL, and CNO (20 nmol) or Veh was intrathecally (i.t.) administered after pain assessment as indicated by arrows. The 50% paw withdrawal threshold was assessed by the up–down method using the von Frey test. **(A, B)** Effect of CNO on the 50% paw withdrawal threshold in the ipsilateral side of male CX3CR1-hM4Di and Control-hM4Di mice after PSL. **(C)** No effect of CNO on mechanical allodynia in the ipsilateral side of female CX3CR1-hM4Di mice after PSL. Data are presented as mean ± S.E.M. n = 4-9. p***< 0.001, p* < 0.05 vs Veh.

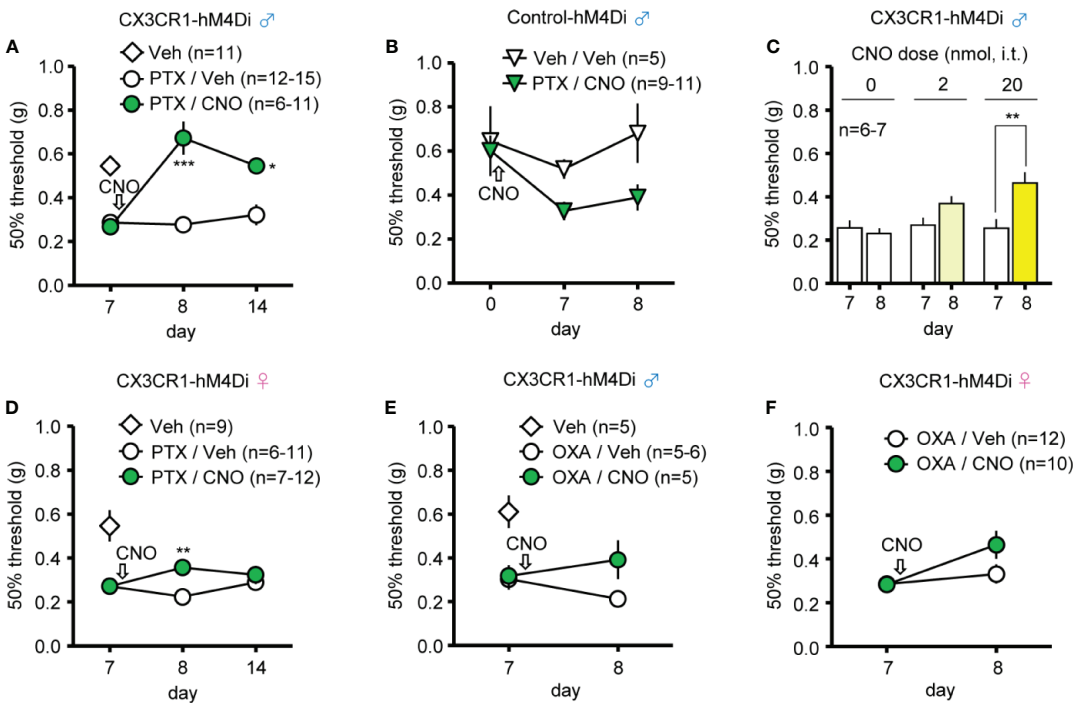

FIGURE 4 | CNO effect on chemotherapy-induced neuropathic pain (CINP) caused by paclitaxel (PTX) and oxaliplatin (OXA) in CX3CR1-hM4Di mice. PTX (4 mg/kg) or OXA (5 mg/kg) was i.p. administered to mice four times every second day (on days 0, 2, 4, and 6), and CNO or Veh was administered after pain assessment as indicated by arrows. The 50% paw withdrawal threshold was assessed by the up–down method using the von Frey test. **(A, B)** Effect of CNO (10 mg/kg, i.p.) on the 50% paw withdrawal threshold in male CX3CR1-hM4Di and Control-hM4Di mice after PTX administration. **(C)** Dose-dependent effect of CNO (2 or 20 nmol, i.t.) on PTX-induced mechanical allodynia in male CX3CR1-hM4Di mice. **(D)** Effect of CNO (10 mg/kg, i.p.) on PTX-induced mechanical allodynia in female CX3CR1-hM4Di mice. **(E, F)** Effect of CNO (10 mg/kg, i.p.) on OXA-induced mechanical allodynia in male and female CX3CR1-hM4Di mice. Data are presented as mean ± S.E.M. n = 5-12. $p^{***} < 0.001$, $p^{**} < 0.01$, $p^{*} < 0.05$ vs day 7 before CNO, or Veh.

was neither observed in neurons nor in astrocytes in CX3CR1-Cre mice obtained from MMRRC (#036395) and concluded that the line was microglia-specific (Zhao et al., 2019). In agreement with this, our data also showed that hM4Di was expressed by Iba1$^+$ microglia, but not GFAP$^+$ astrocytes and NeuN$^+$ neurons in the SDH of CX3CR1-hM4Di mice. On the other hand, hM4Di expression was also observed in F4/80$^+$ macrophages in the PNS of CX3CR1-hM4Di mice. Given that macrophages and microglia share several common systems and molecules including CX3CR1 (Greter et al., 2015), it is expected that hM4Di is also expressed in not only spinal microglia but also peripheral macrophages.

CNO has been used as a selective ligand to induce Gi- and Gq-DREADDs in neurons. When CNO binds to hM4Di, a canonical Gi-protein-dependent pathway causes membrane hyperpolarization (Armbruster et al., 2007). Even though the molecular mechanisms of Gi-DREADD have been well documented in neurons (Urban and Roth, 2015), the effects of Gi-DREADD in non-neuronal cells are poorly understood. Regarding microglia, only two groups of researchers demonstrated that induction of Gi-DREADD may attenuate the activation of microglia with inflammatory phenotypes (Grace et al., 2018; Coleman et al., 2020). Nevertheless, detailed mechanisms and state-dependent effects of Gi- or Gq-DREADD on intracellular signaling in microglia are still unclear. It is important that we also confirmed that hM4Di-signaling in CX3CR1$^+$ microglia can exert an inhibitory regulation through the Gi-DREADD system, resulting in NP relief in mice. Molecular-based mechanisms of microglial regulation by Gi- and Gq-DREADD need to be further investigated in the future.

Gomez et al. reported that systemically-administered CNO is converted to clozapine, which produces non-specific suppressive effects on locomotor activity in mice (Gomez et al., 2017); this suggests that it is important to carefully investigate the effects of CNO *in vivo*. Therefore, we compared the effect of CNO on CX3CR1-hM4Di mice with that on Control-hM4Di mice in all experiments. Unlike i.t. CNO administration (20 nmol), i.p. CNO injection (1 mg/kg) slightly decreased the locomotor activity of the mice, and higher doses of i.p. CNO (3-10 mg/kg) caused sedation over several hours after administration in both male and female CX3CR1-hM4Di mice. Given that CNO (10 mg/kg, i.p.) did not cause such sedative effects in Control-hM4Di mice, the induction of Gi-signaling in CX3CR1$^+$ cells may elicit unfavorable behavioral effects during acute periods in both sexes. Nevertheless, we did not observe motor dysfunction 24 h after CNO administration (**Figure S2**) in CX3CR1-hM4Di mice, and the mechanical threshold of naïve CX3CR1-hM4Di mice was not altered by i.p. CNO injection. Therefore, to evaluate the selective roles of Gi-DREADD on CX3CR1$^+$ cells on

mechanical allodynia, behavioral testing was conducted 24 h after CNO administration, regardless of the delivery route.

It is well known that microglial activation in the SDH is linked to several types of NP in rodents (Chen et al., 2018; Inoue and Tsuda, 2018). In case of peripheral nerve injury, the damaged neurons send excitatory signals to the spinal microglia *via* chemical substances, such as ATP and chemokines; these, in turn, cause microglial proliferation and upregulation of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, resulting in NP (Milligan and Watkins, 2009; Zhao et al., 2017). Given that activated microglia largely affect neuronal and astrocytic activity under NP conditions, it is pivotal to determine disease- and sex-specific roles of microglia in NP. A large body of evidence indicates that inhibition of microglial activation by small molecule compounds, such as minocycline (Raghavendra et al., 2003; Ledeboer et al., 2005; Mei et al., 2011) and propentofylline (Tawfik et al., 2007), can prevent inflammatory responses and NP. Furthermore, also ablation of activated microglia by pharmacological or genetic approaches demonstrated that these cells significantly contribute to the NP condition (Sorge et al., 2015; Peng et al., 2016). However, in terms of specificity or time dependency, the current evidence is insufficient to uncover the complicated roles of microglia in NP. Importantly, our results clearly showed that both systemic (i.p.) and local (i.t.) administration of CNO significantly relieved PSL-induced mechanical allodynia in male CX3CR1-hM4Di mice, which is consistent with the results of previous studies (Sorge et al., 2015).

Sex difference in pain is clinically often observed, and the proportion of male patients suffering from chronic pain is lower than that of female patients (Mogil, 2012). However, to date, male animals have traditionally been used in the majority of laboratory pain studies. Mechanical allodynia develops similarly in both male and female mice after nerve injury, whereas there are significant differences in NP mechanisms between male and female (Boccella et al., 2019; Del Rivero et al., 2019; Inyang et al., 2019). In particular, the neuro-immune interaction seems to be one of the causes for sex difference in pathological pain (Rosen et al., 2017). Sorge et al. demonstrated that i.t. administration of glial inhibitors or microglia-targeting toxins exerted significant inhibitory effects on mechanical allodynia following nerve injury in male, but not in female mice, suggesting male-selective roles of microglia in NP (Sorge et al., 2015). Moreover, inhibitors of several microglia-related proteins, such as Toll-like receptor 4, brain-derived neurotrophic factor (BDNF), and purinergic P2X4 receptor (P2X4R) also produced sex-dependent effects on NP (Sorge et al., 2015). Notably, P2X4R is upregulated on microglia after nerve injury in male, but not in female mice, and the activation of P2X4R causes synthesis and release of BDNF through p38-mitogen-activated protein kinase, suggesting that P2X4R might be responsible for the sex differences in NP (Trang et al., 2009; Mapplebeck et al., 2016). In contrast to P2X4R, expression levels of CX3CR1 in the SDH after nerve injury were similar between in male and female mice (Taves et al., 2016), suggesting that Gi-DREADD might be commonly induced in both sexes. In consistent with these findings, we found that induction of Gi-DREADD in CX3CR1$^+$ cells, by either i.p or i.t. CNO administration, exerted relieving effects on PSL-induced mechanical allodynia in male CX3CR1-hM4Di but not female mice, supporting the notion that microglial activation plays a pivotal role in the pathogenesis of NP only in male mice.

Since lumbar puncture i.t.-administered agents are known to distribute in the dorsal root ganglion (DRG) region (Kawasaki et al., 2008; Alessandri-Haber et al., 2009), it is difficult to rule out the possibility that CNO also affected other cells at this level. Based on the critical role of the macrophages infiltrating the peripheral nerves in NP (Thacker et al., 2007; Kiguchi et al., 2017; Saika et al., 2019), Gi-DREADD may also be induced in CX3CR1$^+$ macrophages after i.p. or i.t. CNO administration. Recently, Yu et al. demonstrated that the depletion of DRG macrophages prevented nerve injury-induced mechanical allodynia not only in male but also in female mice (Yu et al., 2020). Given the sex-independent role of DRG macrophages, we hypothesized that the relieving effects of CX3CR1-Gi-DREADD on NP in male mice were mainly exerted *via* inhibitory regulation of CX3CR1$^+$ microglia. Nevertheless, as several reports suggest sex-specific roles of peripheral immune cells (Luo et al., 2019), further studies are required to clarify common molecular mechanisms involved in NP.

CINP is the dose-limiting side effect of anti-cancer agents, such as PTX and OXA, and remains a challenging clinical problem. Previous reports clarified that PTX treatment induced microglial activation in the SDH, leading to NP that is attenuated by minocycline (Burgos et al., 2012; Pevida et al., 2013). Consistently, we found that i.p. or i.t. CNO administration reversed PTX-induced mechanical allodynia in male CX3CR1-hM4Di mice. Although CX3CR1-Gi-DREADD slightly improved mechanical allodynia also in female mice, the degree of improvement in this case was clearly lower than that in male mice. These results indicate that CX3CR1$^+$ microglia play an important role in PTX-induced mechanical allodynia as well, at least in male mice. Unlike in the case of PTX, CX3CR1-Gi-DREADD did not show any significant preventive effect on OXA-induced mechanical allodynia in both sexes. Based on several cases in the literature regarding the mechanisms of OXA-induced NP (Di Cesare Mannelli et al., 2013; Robinson et al., 2014; Kerckhove et al., 2017), spinal microglia may not significantly contribute to OXA-induced mechanical allodynia. Given that astrocytes widely contributed to different types of CINP, common regulatory mechanisms in the CNS may also exist between PTX- and OXA-induced NP (Sisignano et al., 2014). Although there are controversial reports regarding the involvement of spinal microglia in PTX- or OXA-induced NP, our results emphasize the model- and sex-specific contribution of CX3CR1$^+$ microglia to this condition; this is also supported by evidence obtained using microglial inhibitors, such as minocycline and propentofylline.

Collectively, we clearly demonstrated that chemogenetic regulation of CX3CR1$^+$ microglia using Gi-DREADD exerted sex-dependent (i.e., male-selective) relieving effects on nerve injury-induced NP in mice. In contrast, CX3CR1-Gi-DREADD also prevented CINP caused by PTX, but not OXA, in male mice,

suggesting that the contribution of spinal microglia to CINP may be agent dependent. Given that several gaps in the pathophysiology of NP exist because of the effect of sex and the different models used to study the condition, pharmacology-based comparative studies using microglia-specific tools are warranted to uncover the comprehensive mechanisms of NP.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Committee of Wakayama Medical University.

AUTHOR CONTRIBUTIONS

Participated in research design: FS and NK. Conducted experiments: FS, DK, YI, and NK. Performed data analysis: FS, SM, TN, SK, and NK. Wrote or contributed to writing the manuscript: FS, TN, SK, and NK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00925/full#supplementary-material

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Acute Low Dose of Trazodone Recovers Glutamate Release Efficiency and mGlu2/3 Autoreceptor Impairments in the Spinal Cord of Rats Suffering From Chronic Sciatic Ligation

*Francesca Cisani[1], Alessandra Roggeri[1], Guendalina Olivero[1], Beatrice Garrone[2], Serena Tongiani[2], Francesco Paolo Di Giorgio[2] and Anna Pittaluga[1,3]**

[1] Department of Pharmacy, DIFAR, Pharmacology and Toxicology Section and Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy, [2] Angelini RR&D (Research, Regulatory & Development), Angelini Pharma S.p.A., Rome, Italy, [3] IRCCS Ospedale Policlinico San Martino, Genova, Italy

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***Correspondence:**
Anna Pittaluga
pittalug@difar.unige.it

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We investigated whether chronic sciatic ligation modifies the glutamate release in spinal cord nerve endings (synaptosomes) as well as the expression and the function of presynaptic release-regulating mGlu2/3 autoreceptors and 5-HT_{2A} heteroreceptors in these particles. Synaptosomes were from the spinal cord of animals suffering from the sciatic ligation that developed on day 6 post-surgery a significant decrease of the force inducing paw-withdrawal in the lesioned paw. The exocytosis of glutamate (quantified as release of preloaded [^{3}H]D-aspartate, [^{3}H]D-Asp) elicited by a mild depolarizing stimulus (15 mM KCl) was significantly increased in synaptosomes from injured rats when compared to controls (uninjured rats). The mGlu2/3 agonist LY379268 (1000 pM) significantly inhibited the 15 mM KCl-evoked [^{3}H]D-Asp overflow from control synaptosomes, but not in terminals isolated from injured animals. Differently, a low concentration (10 nM) of (±) DOI, unable to modify the 15 mM KCl-evoked [^{3}H]D-Asp overflow in control spinal cord synaptosomes, significantly reduced the glutamate exocytosis in nerve endings isolated from the injured rats. Acute oral trazodone (TZD, 0.3 mg/kg on day 7 post-surgery) efficiently recovered glutamate exocytosis as well as the efficiency of LY379268 in inhibiting this event in spinal cord synaptosomes from injured animals. The sciatic ligation significantly reduced the expression of mGlu2/3, but not of 5-HT_{2A}, receptor proteins in spinal cord synaptosomal lysates. Acute TZD recovered this parameter. Our results support the use of 5-HT_{2A} antagonists for restoring altered spinal cord glutamate plasticity in rats suffering from sciatic ligation.

Keywords: synaptosomes, mGlu2/3 receptor, 5-HT_{2A} receptor, glutamate exocytosis, trazodone, spinal cord, chronic sciatic ligation, neuropathic pain

INTRODUCTION

Metabotropic glutamate (mGlu) receptors are fine tuners of the chemical transmission in the central nervous system (CNS) and represent the target of drugs proposed for the cure of neurological disorders. Naïve mGlu receptors exist as homo-dimers, as intra- (i.e. the mGlu1/mGlu5 and the mGlu2/3 receptor dimers, Longordo et al., 2006; Di Prisco et al., 2016) or inter-group heteromeric complexes (the mGlu2/mGlu4 heterodimers, Doumazane et al., 2010, the mGlu5/mGlu3 complex, Di Menna et al., 2018), or oligomerize with non-glutamatergic G protein coupled receptors to form inter-family heteromeric assemblies. It is the case of the 5-HT_{2A}/mGlu2/3 receptor-receptor complex in the cortex and in the spinal cord of mammals, of the mGlu1/$GABA_B$ receptor-receptor interaction in GABAergic and glutamatergic cortical terminals and of the mGlu5/A2A receptor-receptor association in the striatum (Díaz-Cabiale et al., 2002; Moreno et al., 2011; Moreno et al., 2012; Delille et al., 2012; Vergassola et al., 2018).

The allosteric properties of the interaction linking different receptors expressed within the same cells were first proposed by Agnati and colleagues in 1980 and nowadays represents a major topic to define the physio-pathological events controlling chemical transmission in CNS.

Homo- or hetero-oligomerization implies the colocalization and the physical association of receptors (i.e. the mGlu2/mGlu4 complex, Doumazane et al., 2010; mGlu2/3 – 5-HT_{2A}, Moreno et al., 2012; Delille et al., 2013; Olivero et al., 2018) and functional outcomes depend on i) the reciprocal role of the receptors involved, ii) the transducing pathways they associate to, and iii) the endogenous transmitters acting at each component of the receptor complex. The intimate association of two receptor proteins to form heterodimers also implies that drugs acting at one receptor affect stereo-chemically the coupled receptor and could alter its insertion in plasmamembranes.

As far as the mGlu2/3 and the 5-HT_{2A} heterodimers are concerned, this receptor complex was first shown to exist in the cortex of mammals. Here, these receptors were reported to interact in an antagonist-like fashion, since the blockade of one receptor reinforced the signalling elicited by the other one (Moreno et al., 2011; Delille et al., 2012). This observation led to propose that the antipsychotic activity of mGlu2/3 agonists could in part rely on their ability to functionally antagonize the colocalized 5-HT_{2A} receptors and, conversely, that pathological alterations of the mGlu2/3–5-HT_{2A} receptor–receptor coupling could account for developing schizophrenia (Marek et al., 2001; González-Maeso et al., 2008; Moreno et al., 2016).

mGlu2/3 autoreceptors and 5-HT_{2A} heteroreceptors also exist in spinal cord glutamatergic nerve endings, at the presynaptic level, where they hetero-dimerize in an antagonist-like fashion to control glutamate exocytosis. Due to the relevance of presynaptic release-regulating autoreceptors in the modulation of synaptic strength at glutamatergic synapses, the mGlu2/3 – 5-HT_{2A} cross talk might represents an innovative target for drugs that modulates the efficiency of fast synaptic transmission in the spinal cord. In particular, we proposed that 5-HT_{2A} antagonists might act as "Indirect Positive Allosteric Modulators" (IPAMs) of the mGlu2/3 receptors, since blockade of the presynaptic release-regulating 5-HT_{2A} receptors would indirectly strengthen the functional outcomes of the colocalized presynaptic release-regulating glutamate receptors (Olivero et al., 2018). To verify the hypothesis Olivero and colleagues (2018) tested the impact of few 5-HT_{2A} antagonists, including trazodone (TZD) on the expression and the functions of mGlu2/3 autoreceptors. TZD was developed as antidepressant and anxiolytic drug (Stahl, 2009), but so far its pharmacological profile is not fully elucidated. The drug inhibits the serotonin transporters and blocks the 5-HT_2 receptors (the 5-HT_{2A} and 5-HT_{2C} receptor subtypes), but also exerts antagonistic effects against 5-HT_{1A} receptors, α -adrenergic receptors, and H-histaminergic receptors (reviewed by Fagiolini et al., 2012). By blocking 5-HT_{2A} heteroreceptors, "*in vitro*" TZD was found to reinforce the mGlu2/3 inhibitory effects on glutamate exocytosis (Olivero et al., 2018).

Data in the literature suggest that glutamate exocytosis, as well as mGlu2/3 receptor expression and functions are altered in animal suffering from the chronic sciatic ligation (Dubner and Ruda, 1992; Dickenson et al., 1997). The present study aims at investigating whether the expression and the functions of the presynaptic release-regulating mGlu2/3 and 5-HT_{2A} receptors in spinal cord glutamatergic nerve endings (we refer to as synaptosomes) are altered in animal suffering from the chronic sciatic ligation and if oral acute trazodone could impact mGlu2/3 functions recovering the altered glutamate exocytosis.

MATERIALS AND METHODS

Animals

Experiments were performed on male CD®IGS rats weighing 190 to 250 g (Charles River, Italy). The animals were housed at Porsolt (Le Genest-Saint-Isle, France) in groups of 5 rats in macrolon cages until surgery with free access to food and water under a 12/12 h light/dark cycle (light cycle: 7:00 AM to 7:00 PM.

Chronic Constriction Injury, Quantification of Tactile Allodynia, and Trazodone Treatment

The surgery was carried out at Porsolt. Under anaesthesia (combination of ketamine and medetomidine), the sciatic nerve of the left hind paw was exposed at the level of the middle of the thigh by dissection through biceps femoris. The nerve was freed of adhering tissue, and four ligatures were loosely tied around it with approximately 1 mm spacing. After recovery, on day 6 post-surgery, rats were subjected to a pre-test using tactile stimulation of both hindpaws to verify the presence of neuropathic pain. Only rats responding on the lesioned paw to force between 0% and 30% of the force inducing withdrawal of

Abbreviations: [3H]D-Asp, [3H]D-aspartate; CNS, central nervous system; IPAMs, Indirect Positive Allosteric Modulators; mGlu2/3 receptor, metabotropic glutamate receptor type 2/3; 5-HT2A, serotonin type 2A; TZD, trazodone.

the non-lesioned paw were included in the experiments. These animals are indicated throughout the text as injured rats, while the control rats are naïve animals. Tactile allodynia was evaluated using the electronic von Frey test (Bioseb, EVF2). The animals were placed under an inverted acrylicplastic box (18 x 11.5 x 14 cm) on a gridfloor. The tip of an electronic von Frey probe was then applied with increasing force first below the non-lesioned and then below the lesioned hindpaw. The force required to induce paw-withdrawal was automatically recorded. This procedure was carried out three times, and the mean force per paw was calculated.

When indicated, on day 7 post-surgery, injured rats were orally treated with trazodone (TZD) at the dose of 0.3 mg/kg (dispersed in 0.5% methylcellulose in distilled water) and sacrificed 1 h after the treatment. The spinal cords (cerebral level to L2 level) were collected and frozen at −80°C in buffered sucrose (0.32 M, pH 7.4, buffered with TRIS 0.01 M). The frozen tissues were sent to DIFAR, Section of Pharmacology and Toxicology to carry out functional and biochemical studies in isolated synaptosomes (Hardy et al., 1983; Dunkley et al., 1988; Franklin and Taglialatela, 2016).

All experimental procedures were approved by Porsolt's internal ethical review committee and are in accordance with French Government and NIH guidelines.

Preparation of Synaptosomes

Purified synaptosomes were isolated from the frozen spinal cords of control (naive), injured (animal suffering from sciatic ligation), vehicle-treated injured, and the TZD-treated injured rats as previously described (Musante et al., 2011). Synaptosomes were then resuspended in a physiological solution with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.4.

Experiments of Transmitter Release

Synaptosomes were incubated for 15 min at 37°C in a rotary water bath in the presence of [^{3}H]D-aspartate ([^{3}H]D-Asp, f.c.: 50 nM). Identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel thermostated chambers in a superfusion system (Raiteri et al., 1974; Summa et al., 2013; Ugo Basile, Gemonio, Varese, Italy).

Synaptosomes were transiently (90 s) exposed, at t = 39 min, to high KCl containing medium (Di Prisco et al., 2012) in the absence or in the presence of agonists. Fractions were collected as follow: two 3-min fractions (basal release), one before (t = 36–39 min) and one after (t = 45–48 min) a 6-min fraction (t = 39–45 min; evoked release). Fractions collected and superfused synaptosomes were measured for radioactivity.

The amount of radioactivity released into each superfusate fraction was expressed as percentage of the total radioactivity. The KCl-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (induced release, b2). Within the text, the effect of agonists/antagonists is also expressed as percentage of the KCl-induced overflow of tritium observed in the absence of receptor agonists and antagonists (percent of control).

Immunoblotting

Rat spinal cord purified synaptosomes were lysed in modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors) and quantified for protein content. Samples were boiled for 5 min at 95°C in SDS-PAGE loading buffer and then separated by SDS-7.5% PAGE (20–30 µg/lane) and transferred onto PVDF membranes. Membranes were incubated for 1 h at room temperature in Tris-buffered saline-Tween (t-TBS: 0.02 M Tris, 0.15 M NaCl, and 0.05% Tween 20), containing 5% (w/v) non-fat dried milk and then probed with rabbit anti-mGlu2/3 (1:2000), rabbit anti-5-HT_{2A} (1:500) and rabbit anti-GAPDH (1:10000) antibodies overnight at 4°C. The anti-mGlu2/3 antibody recognizes an aminoacidic sequence of the NH_2 terminus common to both the mGlu2 and the mGlu3 receptor proteins, while the anti–5-HT2A antibody recognizes the NH_2 terminus (amino acids 22–41) of the 5-HT_{2A} receptor protein. After extensive washes in t-TBS, membranes were incubated for 1h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:20000). Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec, Cambridge, UK).

Calculations and Statistical Analysis

Multiple comparisons were performed with analysis of variance (ANOVA) followed Tukey's multiple-comparisons test; direct comparisons were executed by Student's t-test. Data were considered significant for $P < 0.05$ at least.

Chemicals

[2,3-^{3}H]D-Asp (specific activity 11.3 Ci/mmol) was from Perkin Elmer (Boston, MA, USA). LY379268 was purchased from Tocris Bioscience (Bristol, UK). (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride ((±)DOI), trazodone, horseradish peroxidase-conjugated anti-mouse, and anti-rabbit secondary antibodies were from Sigma (Milan, Italy). Luminata Forte Western blotting was purchased from Millipore (Temecula, CA, USA). Rabbit anti-GAPDH antibody was from Abcam (Cambridge, UK), rabbit anti-mGlu2/3 antibody was from Novus Biologicals (Littleton CO, USA), rabbit anti–5-HT_{2A} antibody was from Immunostar (Hudson, WI, USA).

RESULTS

Impact of the Sciatic Ligation on the [^{3}H] D-Aspartate Exocytosis From Spinal Cord Synaptosomes

The release of glutamate from synaptosomes isolated from the spinal cord of rats suffering from chronic sciatic ligation (injured rats) and of control (uninjured) animals was analysed. Injured animals are those rats that developed on day 6 post-surgery a

significant decrease of the paw-withdrawal force in the lesioned side as compared with the non-lesioned paw (−81.0 ± 0.5%, $p < 0.001$, n = 32 for each group, **Figure 1A**).

Glutamate exocytosis was quantified as 15 mM KCl-evoked overflow of preloaded [^{3}H]D-aspartate ([^{3}H]D-Asp), a non-metabolizable glutamate analogue routinely used as a marker of the endogenous aminoacid in release studies (Grilli et al., 2004; Di Prisco et al., 2012; Di Prisco et al., 2016). The tritium overflow elicited by the depolarizing stimulus from spinal cord synaptosomes of injured rats was significantly higher than that from spinal cord synaptosomes of control rats (+62.6 ± 9.4, results expressed as percent of increase, $p<0.05$, n = 6, **Figure 1B**).

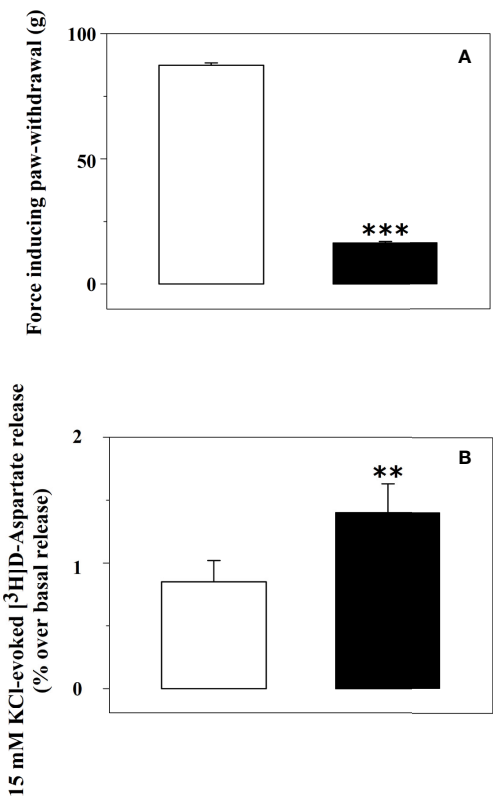

FIGURE 1 | Tactile allodynia correlates with impaired glutamate exocytosis from synaptosomes isolated from the spinal cord of injured rats. **(A)** Effect of chronic constriction injury on tactile allodynia. Control (uninjured, empty bar) and injured (black bar) rats were analysed for Tactile allodynia in the von Frey test. Results represent the tactile thresholds expressed as grams on day 6 following sciatic nerve ligation. Data are mean ± SEM of 32 rats for each group. ***$p< 0.001$ versus control group (Student's *t*-test). **(B)** 15 mM KCl-evoked release of [^{3}H]D-aspartate from synaptosomes isolated from the spinal cord of control rats and of animals suffering from sciatic ligation. Synaptosomes from control (uninjured, empty bar) and from injured (black bar) rats were label with [^{3}H]D-aspartate ([^{3}H]D-asp) and exposed in superfusion to 15 mM KCl-enriched medium to trigger exocytosis. The depolarization-evoked release is evaluated as induced overflow, and it represents the amount of tritium released upon the spontaneous release. Results are expressed as mean ± SEM of 8 experiments run in triplicate (three superfusion chambers for each experimental condition). **$p< 0.01$ versus control (Student's *t*-test).

Impact of the Sciatic Ligation on the Presynaptic mGlu2/3 Autoreceptors Controlling [^{3}H]D-Aspartate Exocytosis in Spinal Cord Synaptosomes

The exocytosis of [^{3}H]D-Asp from spinal cord synaptosomes is controlled presynaptically by release-regulating mGlu2/3 autoreceptors (Di Prisco et al., 2016; Olivero et al., 2018). The mGlu2/3 agonist LY379268 (10 and 1000 pM) inhibits in a concentration-dependent fashion the tritium exocytosis in control spinal cord synaptosomes (10 pM, −29.5 ± 6.4, n.s.; 1000 pM, −52.4 ± 7.9, $p<0.05$, n = 5, results expressed as percent of change). The agonist, however, lost efficacy in controlling glutamate exocytosis in spinal cord synaptosomes from animals suffering from the sciatic ligation (**Figure 2**). Particularly, LY379268 (10 and 1000 pM) failed to affect the 15 mM KCl-evoked [^{3}H]D-Asp overflow in these terminals (10 pM, −13.4 ± 6.3 n.s.; 1000 pM, −9.0 ± 12.7, n.s., n = 5, results expressed as percent of change).

Impact of the Sciatic Ligation on the Presynaptic 5-HT$_{2A}$ Heteroreceptors Controlling [^{3}H]D-Aspartate Exocytosis in Spinal Cord Synaptosomes

Rat spinal cord synaptosomes also possess inhibitory, presynaptic, release-regulating 5-HT$_{2A}$ heteroreceptors controlling glutamate exocytosis (Olivero et al., 2018). **Figure 3** shows that 100 nM (±) DOI, a selective 5-HT$_{2A}$ agonist, significantly reduces the 15 mM KCl-evoked release of [^{3}H]D-Asp in spinal cord synaptosomes from control rats (−37.5 ± 5.2, $p<0.05$, n = 6, results expressed as percent of change), being inactive when added at a lower (10 nM) concentration (+ 3.4 ± 5.9, n.s., n = 6, results expressed as percent of change).

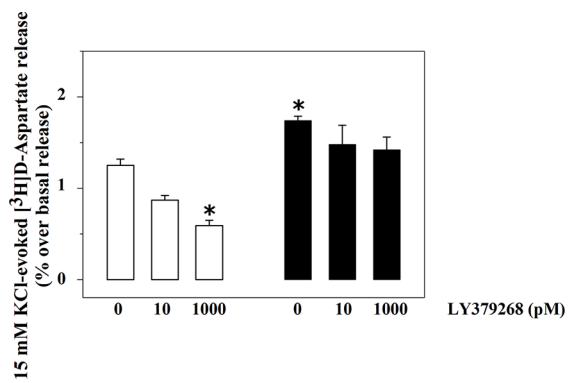

FIGURE 2 | Effects of LY379268 on the 15 mM KCl-evoked release of [^{3}H]D-aspartate exocytosis from synaptosomes isolated from the spinal cord of injured rats. Spinal cord synaptosomes were prepared from rats suffering from sciatic ligation (black bar) and from control uninjured (empty bar) rats. Synaptosomes were exposed in superfusion to the 15 mM KCl-enriched medium in the absence or in the presence of LY379268 (concentrations as indicated). Results are expressed as mean ± SEM of 8 experiments run in triplicate. *$p< 0.05$ versus controls (two-way ANOVA and Tukey's multiple-comparisons test).

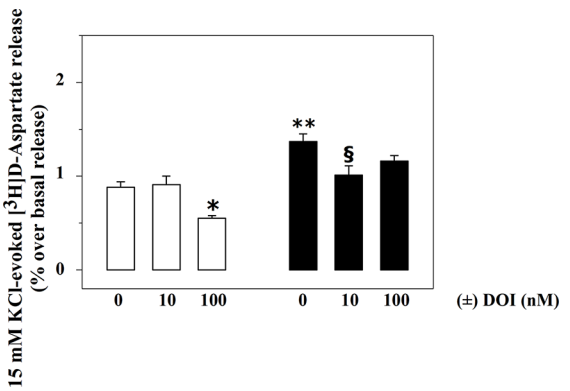

FIGURE 3 | Effects of (±) DOI on 15 mM KCl-evoked release of [^{3}H]D-aspartate exocytosis from synaptosomes isolated from the spinal cord of injured rats. Synaptosomes from control (empty bar) and injured (black bar) rats were exposed in superfusion to 15 mM KCl-enriched medium in the absence or in the presence of (±) DOI (concentrations as indicated) as previously described. Results are expressed as mean ± SEM of 5 experiments run in triplicate. *p< 0.05 versus control; **p< 0.01 versus control; §p< 0.05 versus injured rats (two-way ANOVA and Tukey's multiple-comparisons test).

Differently, 100 nM (±) DOI slightly, although not significantly, affected the 15 mM KCl-evoked release of [^{3}H]D-Asp from the spinal cord synaptosomes of injured rats (−14.6 ± 5.7, n.s., n = 6, results expressed as percent of change). The agonist, however, significantly inhibited tritium overflow when added at 10 nM (−27.9 ± 4.1, p<0.05, n = 6, results expressed as percent of change).

Impact of Trazodone Treatment on the mGlu2/3 Autoreceptors and 5-HT$_{2A}$ Heteroreceptors in Spinal Cord Synaptosomes From Animals Suffering From Sciatic Ligation

In healthy condition, in spinal cord nerve endings, presynaptic release-regulating 5-HT$_{2A}$ heteroreceptors couple in an antagonist-like fashion the release-regulating presynaptic mGlu2/3 autoreceptors (Olivero et al., 2018). We asked whether blockade of the 5-HT$_{2A}$ heteroreceptors could recover the reduced efficiency of spinal mGlu2/3 autoreceptors in injured rats. As far as the 5-HT$_{2A}$ antagonists are concerned, we focussed on the orally active 5-HT$_{2A}$ antagonist TZD, due to the wide literature describing its pharmacodynamic and pharmacokinetic profile (Cheng et al., 1999; Luparini et al., 2004; Ghanbari et al., 2010).

Injured rats were randomly subdivided into two groups, one orally administered TZD (0.3 mg/kg, TZD-treated injured rats) and the other one vehicle (0.5% methylcellulose, vehicle-treated injured rats). The glutamate exocytosis from spinal cord synaptosomes isolated from TZD-treated injured rats was significantly lower than that from vehicle-treated injured rats (**Figures 4A, B**). Vehicle administration did not modify on its own the 15 mM KCl-evoked release of tritium from control rats (not shown).

LY379268 (10–1,000 pM) failed to affect significantly the 15 mM KCl-evoked release of [^{3}H]D-Asp in spinal cord

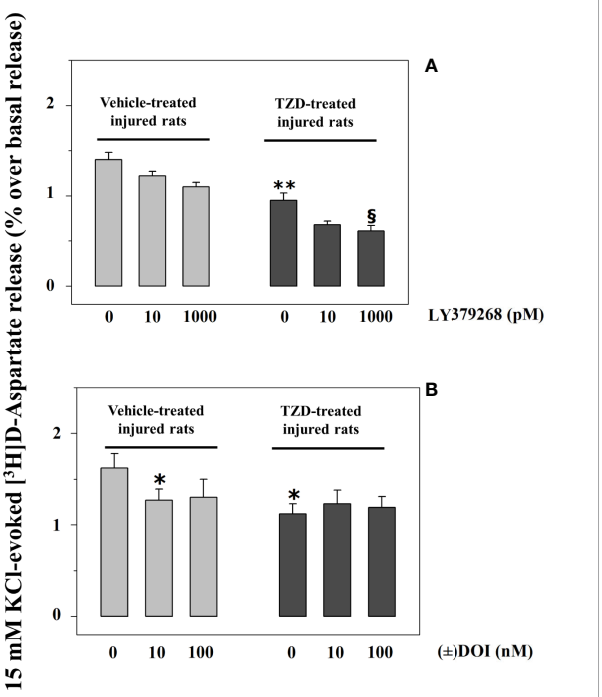

FIGURE 4 | Acute trazodone modifies functionally the presynaptic release-regulating mGlu2/3 and 5-HT$_{2A}$ receptors in synaptosomes isolated from the spinal cord of injured rats. Synaptosomes from TZD-untreated injured animals (vehicle-treated, light grey bar) and from TZD-treated injured rats (dark grey bar) were exposed in superfusion to the 15 mM KCl-enriched medium in the absence or in the presence of LY379268 (A) or of (±) DOI (B, concentrations as indicated). Results are expressed as mean ± SEM of 6 **(A)** and 5 **(B)** experiments respectively run in triplicate. *p< 0.05 versus vehicle-treated synaptosomes; **p< 0.01 versus vehicle-treated synaptosomes; §p< 0.05 versus TZD-treated rats synaptosomes (two-way ANOVA and Tukey's multiple-comparisons test).

synaptosomes from vehicle-treated injured rats (compare **Figure 2** and **Figure 4A**). Differently, 1000 pM LY379268 significantly inhibited the [^{3}H]D-Asp exocytosis from spinal cord synaptosomes isolated from TZD-treated injured rats, while 10 pM LY379268 slightly, although not significantly, reduced it (**Figure 4A**).

Experiments were also dedicated to test the impact of (±) DOI on glutamate exocytosis from TZD-treated spinal cord synaptosomes. The oral TZD administration reduced the 15 mM KCl-evoked release of tritium from spinal cord synaptosomes from injured rats when compared to vehicle-treated animals. The glutamate exocytosis from these terminals, however, was not affected by the 5-HT$_{2A}$ agonist (**Figure 4B**).

Impact of the Chronic Sciatic Ligation on the Expression of mGlu2/3 and 5-HT$_{2A}$ Receptor Proteins in Spinal Cord Synaptosomes

Immunochemical studies were carried out to quantify the expression of the mGlu2/3 and the 5-HT$_{2A}$ receptor proteins

in the lysates of spinal cord synaptosomes from injured rats. The anti-mGlu2/3 antibody recognized a band of ∼220 kDa in both the spinal cord synaptosomal lysates from control and injured rats, but not at ∼100 kDa that represents the expected weight of the receptor subunit, consistent with the existence of the mGlu2/3 dimeric form of the receptor in these terminals (**Figure 5A**). Differently, the anti–5-HT_{2A} antibody unveiled a component in the synaptosomal lysate with a mass (∼ 75 kDa) corresponding to the monomeric form of the receptor (**Figure 5C**). GAPDH was used as an internal control (**Figures 5A, C**). The receptor protein signals were expressed as mGlu2/3 ÷ GAPDH or 5-HT_{2A} ÷ GAPDH ratio (**Figures 5B, D**). The results showed a significant reduction of the mGlu2/3 ÷ GAPDH ratio value in injured animals (−27.6 ± 5.6%, results expressed as percent of change, $p < 0.05$; n = 5, **Figure 5B**) in 5 lysates out of the 9 synaptosomal preparations analyzed (**Figure 5B**) when compared to control, while an increase (+30.3 ± 9.8, results expressed as percent of change, $p< 0.05$, not shown) was observed in 3 lysates out of the 9 and no change (+ 4.2, result expressed as percent of change, not shown) in 1 synaptosomal lysate. Differently, the 5-HT_{2A} ÷ GAPDH ratio value in injured animals was comparable to that in control rats in all the preparations analyzed (**Figure 5D**).

Impact of Trazodone Treatment on the mGlu2/3 Receptor Proteins in Spinal Cord Synaptosomes From Injured Rats

We analysed the expression of mGlu2/3 receptor proteins in TZD-treated injured spinal cord synaptosomal lysates when compared to vehicle-treated injured animals. A representative blot is reported in **Figure 6A** and the mean values of the mGlu2/3 ÷ GAPDH ratio for both the vehicle-treated and the TZD-treated injured rats are reported in **Figure 6B**. The results from 10 lysates out of 12 showed a significant increase of the mGlu2/3 ÷ GAPDH ratio when compared to controls (+48.8 ± 10.1, $p< 0.05$; results expressed as percent of change).

DISCUSSION

Spinal cord hyper-glutamatergicity contributes to the development of central hyperalgesia in animals suffering from the chronic constriction of the sciatic nerve. The altered glutamatergic transmission might depend on several concomitant maladaptive events, including changes in the efficiency of transmitter exocytosis from nerve terminals (and

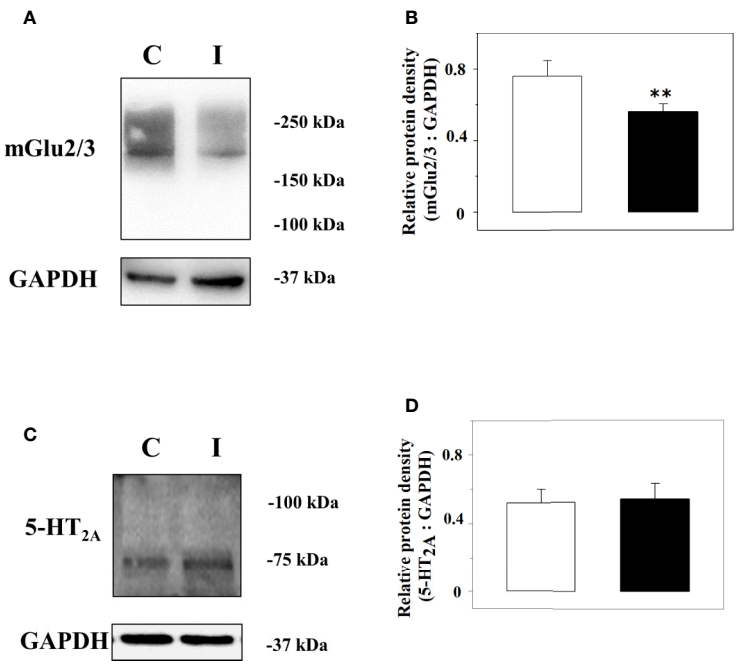

FIGURE 5 | Western Blot analysis of the mGlu2/3 and the 5-HT_{2A} receptor proteins in synaptosomal lysates from the spinal cord of control and injured rats. Left panels: Representative western blot showing modifications in mGlu2/3 **(A)** and of the 5-HT_{2A} **(C)** receptor proteins in spinal cord synaptosomal lysates from rats suffering from sciatic ligation (injured, I) and from control uninjured (control, **C**) rats. The blot is representative of five (mGlu2/3) and 7 (5-HT_{2A}) analyses carried out in different days. Right panels: quantification of the mGlu2/3 ÷ GAPDH ratio **(B)** and of the 5-HT_{2A} ÷ GAPDH ratio **(D)** in synaptosomes isolated from controls (white bar) and injured (black bar) animals. Results are expressed as mean ± SEM. **p<0.01 vs control, (Student's *t*-test).

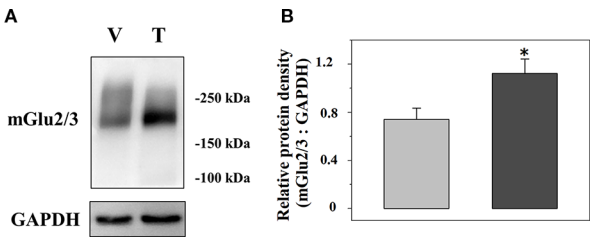

FIGURE 6 | Acute trazodone modifies the expression of the presynaptic release-regulating mGlu2/3 autoreceptors in synaptosomes isolated from the spinal cord of injured rats. Western Blot analysis **(A)** and quantification **(B)** of the mGlu2/3 receptor proteins expression in synaptosomal lysates from TZD-treated injured rats (T) when compared to vehicle-treated injured rats (V). The GAPDH protein was used as an internal control. The blot is representative of five blots. **(B)** TZD-treated injured animals (dark grey bar) and vehicle-treated injured rats (light grey bar). Results are expressed as the mean of the mGlu2/3 ÷ GAPDH ratio. *p<0.05 vs control, (Student's *t*-test).

astrocytes/glial cells as well) and altered expression/functions of the presynaptic release-regulating autoreceptors.

As far as the glutamate release efficiency is concerned, its dysregulation was suggested by evidence in the literature demonstrating impaired glutamate content and exocytosis from isolated nerve endings of the spinal cord of lesioned animals (Somers and Clemente, 2002; Morioka et al., 2015; Wang et al., 2015) and it is here confirmed by the results showing increased glutamate exocytosis from the synaptosomes isolated from the spinal cord of rats suffering from the sciatic ligation.

In this context, checking the efficiency of presynaptic inhibitory glutamatergic release-regulating autoreceptors became mandatory when considering that the loss of efficiency of these receptors could further worsen the pathological framework.

Among glutamate receptors, the mGlu belonging to the group II function as inhibitory release-regulating autoreceptors in the CNS of mammals, including the spinal cord (Gerber et al., 2000; Di Prisco et al., 2012; Di Prisco et al., 2016). These receptors preferentially locate nearby the site of transmitter exocytosis and are activated by glutamate exceeding the physiological level and diffusing beyond the synaptic active zone (Olivero et al., 2019; Pittaluga, 2019).

A large body of evidence published in the last two decades supports the main role of mGlu2/3 receptors as regulators of glutamate release from primary afferent fibres in the dorsal horn of the spinal cord (Gerber et al., 2000) as well as main players in analgesia (Simmons et al., 2002; Chiechio et al., 2009; Chiechio et al., 2010; Bernabucci et al., 2012; Chiechio and Nicoletti, 2012). The data so far available concerning their expression in the spinal cord of animals suffering from neuropathic pain, however, are conflicting. A significant reduction of the mGlu2/3 receptor expression was detected in and around the lesion site in spinal cord injured rats, although it was uncertain in which cell types the changes occurred, i.e. neuronal or glial ones (Mills et al., 2001). Differently, significant changes in the expression of mGlu2/3 receptor proteins in the lumbar segment of the spinal cord of animals suffering from the monolateral chronic sciatic constriction did not emerge soon after the induction but several (3 to 7) days after the lesion (Chiechio et al., 2002).

Based on the assumption that the increased glutamate release could depend on the reduced efficiency of the presynaptic autoreceptors in controlling the vesicular exocytosis, we analysed the efficiency of LY379268 in inhibiting glutamate overflow from spinal cord synaptosomes of lesioned rats 7 days after the sciatic ligation. In line with the hypothesis, our functional results showed a significant loss of efficacy of the agonist in controlling glutamate exocytosis that in a large percentage of the animals was paralleled by a reduced insertion of the receptor protein in synaptosomal plasmamembranes.

Besides genetic and epigenetic mechanisms of control of the mGlu2/3 receptors expression (Chiechio et al., 2009), we recently demonstrated that their insertion in synaptosomal plasmamembranes as well as their releasing activity is controlled by mechanisms of "metamodulation" (Olivero et al., 2019). The term "metamodulation" refers to the mechanism(s) of control of synaptic plasticity based on the functional crosstalk linking two receptors colocalized on the same nerve endings (Sebastião and Ribeiro, 2015). As far as the glutamatergic spinal cord synaptosomes are concerned, we demonstrated that the mGlu2/3 autoreceptors colocalize and functionally cross-talk in glutamatergic nerve endings with the 5-HT_{2A} heteroreceptors. Particularly, the receptor-receptor interaction assures the serotonergic-induced, antagonist-like regulation of the mGlu2/3 receptors controlling glutamate exocytosis (Olivero et al., 2018).

The role of serotonin and 5-HT_{2A} receptors in controlling pain perception has been matter of study and the involvement of the descending serotonergic pathway and of the 5-HT_{2A} receptors in the spinal cord sensitization is supported by data in the literature. In 1980, Proudfit demonstrated that lesions of the raphe magnus resulted in decreased nociceptive thresholds and attenuation of morphine-induced analgesia (Proudfit, 1980). In 2007, Okamoto and colleagues highlighted the role of 5-HT_{2A} receptors in the control of nociceptive neural activities (Okamoto et al., 2007). More recently, Liu and colleagues (2010) proposed that changes in the descending inhibitory 5-HT system occur upon spinal nerve injury and participates to central sensitization and pain perception. Furthermore, Lopez-Alvarez and colleagues (2018) reported the altered expression of the 5-HT_{2A} receptors in the spinal cord dorsal horn following sciatic nerve transection in rats 2 weeks after injury. Finally, data exists in the literature describing the efficacy of the 5-HT_{2A} antagonist TZD to attenuating pain perception in rats suffering from chronic constriction (Okuda et al., 2003) and in humans suffering from diabetic neuropathy (Wilson, 1999).

An interesting result of the present study is that, opposite to LY379268, the 5-HT_{2A} agonist (±) DOI does not lose efficacy in controlling presynaptically glutamate exocytosis in spinal cord synaptosomes from injured rats. Rather, in these rats, its potency is "apparently" increased, since a concentration one-fold lower than that effective in healthy conditions caused significant inhibition of glutamate outflow. Since evident changes in the 5-HT_{2A} receptors protein expression in the synaptosomal lysates

did not emerge, we speculated that the functional adaptation of the presynaptic serotonergic heteroreceptors could be explained by assuming that the 5-HT_{2A}-mGlu2/3 balance was impaired in the spinal cord of injured rat, and, particularly, that the serotonergic tone became predominant in the receptor-receptor cross-talk, silencing the colocalized mGlu2/3 autoreceptors. The hypothesis is supported by the release studies that unveiled a loss of function of the presynaptic mGlu2/3 autoreceptors concomitant to the gain of function of the 5-HT_{2A} heteroreceptors.

Well in line with the hypothesis, we found that the acute oral administration of TZD, i.e. a treatment that would allow a systemic blockade of the 5-HT_{2A} receptors, including those located in the spinal cord glutamatergic nerve endings, recovered almost all the molecular impairments observed in injured rats. In particular, the acute oral administration of TZD i) reduced the glutamate exocytosis from spinal cord terminals, ii) silenced the presynaptic release-regulating 5-HT_{2A} heteroreceptors, iii) recovered the efficiency of the presynaptic mGlu2/3 autoreceptors. All these events could be tentatively explained by assuming that the "*in vivo*" antagonism of the 5-HT_{2A} heteroreceptors was "memorized and retained" by glutamatergic nerve endings, emerging in "ex vivo, *in vitro*" release studies as loss of function of the presynaptic 5-HT_{2A} heteroreceptors and concomitant gain of function of the coupled mGlu2/3 release-regulating autoreceptors (see for a recent review Pittaluga, 2019).

An intriguing observation is that the mGlu2/3 receptor proteins in spinal cord synaptosomal lysates from TZD-treated injured rats were significantly increased when compared to control. In an attempt to find a rationale for the changes in the receptor protein content, one might hypothesize that the acute (1 h) oral administration of a low dose (0.3 mg/kg) of TZD could have modified the expression of the mGlu2/3 receptor proteins in spinal cord glutamatergic nerve endings. An alternative hypothesis, however, considers the possibility that the blockade of the 5-HT_{2A} counterpart within the intra-group heteromeric complex could stabilize mGlu2/3 receptors in plasmamembranes, slowing their internalization and degradation in nerve terminals. Despite the timing of the TZD treatment seems best in line with the second hypothesis, the data so far available are insufficient to propose a mechanism accounting for this effect.

To conclude, the findings described in this study confirm that mGlu2/3 autoreceptors in spinal cord glutamatergic nerve endings of rats suffering from the sciatic ligation undergo selective functional adaptations that minimize their role as modulators of glutamate transmission. Furthermore, they demonstrate that the early spinal glutamatergic maladaptation in lesioned animals can be recovered by administering acutely 5-HT_{2A} antagonists. Interestingly, the analgesic effect is observed following the administration of a dose (0.3 mg/kg) of TZD that is two order of magnitude lower than those (20 to 80 mg/kg) found to ameliorate the thermal hyperalgesia in rats suffering from sciatic ligation (Okuda et al., 2003). The apparent discrepancy might rely on differences in the drug administration protocols (TZD was administered at day 7 post injury in the present study and at day 15 post injury in the study of Okuda and colleagues) as well as in the test applied to quantify pain (tactile vs thermal allodynia). Further investigations are needed to correctly address this point.

It is proposed that responses to nociceptive stimuli can be transformed into memories if they cause long-lasting, activity-dependent changes in synaptic strength. Our findings suggest that, among the molecular events accounting for the early maladaptation, impaired mGlu2/3 – 5-HT_{2A} metamodulation could be relevant to the sensitization of nociceptive dorsal horn neurons. Drugs that could restore the pathological unbalance between the two receptors can recover the physiological neuromodulation of the spinal glutamate transmission, reducing pain perception.

Reinforcing the functioning of the mGlu2/3 receptors is recognized as a successful approach to treat the development of pain and, accordingly, different classes of therapeutics have been proposed to this aim, including substances that modulate epigenetically the expression of the group II receptor protein (Chiechio et al., 2002; Chiechio et al., 2009; Chiechio et al., 2010; Bernabucci et al., 2012; Chiechio and Nicoletti, 2012; Zammataro et al., 2014). Although further studies are needed to definitively prove the efficacy of the 5-HT_{2A} antagonists for the cure of spinal pain, our results support their use as alternative therapeutic approach to modulate the mGlu2/3-mediated signalling in pathological conditions associated to neuropathic pain.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ supplementary material.

ETHICS STATEMENT

The experimental procedures in animals were reviewed and approved by Porsolt's internal ethical review committee and are in accordance with French Government and NIH guidelines.

AUTHOR CONTRIBUTIONS

AP designed the experiments, supervised the execution of the research activity and the statistical analysis, and wrote the manuscript. FC, AR, and GO performed release experiments and western blot analysis. BG, ST, and FG made the tissues available, supported the scientific data analysis and discussion, and revised the manuscript. FC, AR, GO, BG, ST, and FG approved the final version of the manuscript and agree to be accountable for all the aspects of the work.

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Peripheral Deltorphin II Inhibits Nociceptors Following Nerve Injury

Marek Joukal[1], *Lucy Vulchanova*[2], *Cecilia Huffman*[2], *Petr Dubový*[1] *and Christopher N. Honda*[2]*

[1] Department of Anatomy, Cellular and Molecular Neurobiology Research Group, Faculty of Medicine, Masaryk University, Brno, Czechia, [2] Department of Neuroscience, University of Minnesota, Minneapolis, MN, United States

Clinical and preclinical studies have revealed that local administration of opioid agonists into peripheral tissue attenuates inflammatory pain. However, few studies have examined whether peripherally restricted opioids are effective in reducing mechanical allodynia and hyperalgesia that usually follows nerve injury. The aim of the present study was to determine whether the mechanical responsiveness of C-fiber mechanical nociceptors innervating skin under neuropathic pain conditions is depressed by direct activation of delta opioid receptors (DORs) on their peripheral terminals. A murine model of peripheral neuropathic pain was induced with a spared nerve (tibial) injury, in which mice survived 7 or 28 days after surgery before electrophysiological testing began. Control groups comprised naïve and sham-operated animals. An ex vivo preparation of mouse plantar skin with attached tibial nerve was used to examine electrophysiologically the effects of the selective DOR agonist, deltorphin II, on the response properties of individual cutaneous C-fiber nociceptors. In contrast to naïve and sham-operated animals, deltorphin II induced an inhibition of the mechanical responsiveness of C-fiber mechanical nociceptors innervating skin under neuropathic conditions. The effects of deltorphin II were concentration-dependent and prevented by pretreatment with naltrindole indicating DOR-mediated inhibitory effects of deltorphin II. Our results provide the first direct evidence for expression of functional DORs on mechanical nociceptors innervating skin in an animal model of neuropathic pain.

Keywords: nociceptors, spared nerve injury, delta opioid receptors, neuropathic pain, deltorphin II

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***Correspondence:**
Christopher N. Honda
cnhonda@umn.edu

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INTRODUCTION

Peripheral neuropathic pain, manifested as spontaneous pain, hyperalgesia, and allodynia, can result from many forms of nerve damage (Woolf and Mannion, 1999; Jensen et al., 2001). Current therapeutic approaches reduce, but do not eliminate, the hyperalgesia and allodynia. Systemically delivered opioids have very modest effects on neuropathic pain (Bian et al., 1999; Przewłocki and Przewłocka, 2001), usually requiring much higher doses for adequate relief (Portenoy and Hagen, 1990). Unfortunately, effective central analgesic actions of opioids are usually accompanied by untoward centrally mediated effects, such as sedation and respiratory depression, as well as peripheral effects, such as gastrointestinal disturbance and nausea. Moreover, prolonged opioid use can lead to tolerance and dependence. In view of the current opioid epidemic (Skolnick, 2018),

there is an especially relevant need to seek out therapeutic alternatives to centrally acting opioids, such as manipulation of an endogenous peripheral opioid analgesia system (Stein, 2018). In the peripheral nervous system, opioid receptors are synthesized in somata of primary sensory neurons in dorsal root ganglia (DRG) then distributed centrally to axon terminals in superficial layers of spinal dorsal horn and peripherally to processes of small-caliber fibers (Coggeshall et al., 1997). During peripheral inflammation, synthesis of opioid receptors in DRG is upregulated, their axonal transport in peripheral nerves is enhanced, and peripheral density of receptors is elevated (Hassan et al., 1993; Mousa et al., 2001). Delta opioid receptors (DORs) have been immunohistochemically localized to axons innervating healthy skin (Coggeshall et al., 1997; Wenk and Honda, 1999), yet their functional competence under naïve conditions has been difficult to demonstrate.

It is well-established however, that peripherally restricted opioids are very effective under conditions of inflammation. Peripheral opioids attenuate behavioral hyperalgesia in models of inflammatory pain (Joris et al., 1987; Stein et al., 1988; Stein et al., 1989; Stein and Zöllner, 2009), and these effects are dose-dependent and antagonist-reversible (Ferreira and Nakamura, 1979; Levine and Taiwo, 1989; Joris et al., 1990; Barber and Gottschlich, 1992). In electrophysiological studies, locally applied morphine has been shown to inhibit spontaneous activity (Russell et al., 1987) as well as mechanical and thermal responses of nociceptors innervating inflamed skin while having no effects in healthy skin (Wenk et al., 2006). In addition, direct application of DOR agonists to receptive fields of nociceptors resulted in robust inhibition in inflamed, but not healthy skin (Brederson and Honda, 2015).

Less is known about the functional status or efficacy of peripheral opioid analgesic systems following nerve injury. In rodent models of neuropathic pain, peripheral delivery of mu opioid (Guan et al., 2008) and DOR agonists attenuates behavioral hyperalgesia (Obara et al., 2004; Kabli and Cahill, 2007; Obara et al., 2009). Kabli and Cahill (2007) also demonstrated concurrent increased expression of DORs in peripheral nerve and DRG following nerve injury. However, Obara et al. (2009) observed decreased levels of mRNA for DORs in spinal cord and DRG under similar conditions of nerve injury. In electrophysiological studies, peripheral delivery of mu opioid receptor agonists reduced the excitability of nociceptors following nerve injury (Schmidt et al., 2012). The existence of functional DORs on nociceptors under neuropathic conditions, and whether their activation contributes to attenuation of hyperalgesia and allodynia is unclear.

Therefore, the goal of the present study was to determine whether the mechanical responsiveness of nociceptors was attenuated by direct and localized activation of DORs on their peripheral terminals following spared tibial nerve injury (SNIt). Electrophysiological recordings in an isolated preparation of mouse hind paw skin with attached tibial nerve was used to examine effects of the selective DOR agonist, deltorphin II, on the responsiveness of C-fiber mechanical nociceptors innervating skin under experimental (SNIt) and control (naïve and sham-operated) conditions. We report that application of deltorphin II decreased evoked activity of skin nociceptors in the SNIt neuropathic pain model compared to control animals. This effect was prevented by co-application of naltrindole, a DOR-selective antagonist. These results provide direct evidence for existence of functional DORs on peripheral axon terminals of mechanical nociceptors innervating skin under conditions of neuropathic pain.

MATERIALS AND METHODS

Animals and Surgical Procedures

All work with animals adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and was approved by the Institutional Animal Care and Use Committee at the University of Minnesota in accordance with American Veterinary Medical Association guidelines. Experiments were performed on 31 adult (25–35 g; 4-6 weeks) male outbred ICR/CD-1 mice (Envigo, Indianapolis, IN, USA). We induced spared nerve (tibial) injury (SNIt) as a model of peripheral nerve injury-induced neuropathic pain. The SNIt model of sparing the tibial nerve produces robust and consistent behavioral signs of neuropathic pain including reduction of nociceptive threshold (Shields et al., 2003). All surgical procedures were sterile and performed under deep isoflurane anesthesia. After incision of skin and muscle of the right hind limb, the sciatic nerve was exposed, and sural and common peroneal nerves were tightly ligated with 6-0 silk suture. Next, the ligated sciatic nerve branches were transected distal to the ligature, and approximately 2 mm of each distal nerve stump was removed. The retracted muscles were closed with absorbable suture (Ethicon) and the skin incision was closed with wound clips. In sham-operated animals, the right sciatic nerve was exposed, but no ligations or lesions were performed. The SNIt and sham-operated animals survived for 7 (sham, n = 4; SNIt, n = 8) and 28 days (sham, n = 4; SNIt, n = 9).

Behavioral Mechanical Sensitivity

Mechanical sensitivity of the right hind paw (ipsilateral) was tested using Von Frey nylon monofilaments (Stoelting, Wood Dale, IL). Experimental and control animals were placed on a wire mesh grid under glass enclosures and allowed to acclimate for 30 min before behavioral testing. Tips of monofilaments were then pressed to the mid-plantar surface of the hind paw with enough bending force to cause the mouse to withdraw its paw from the tip, typically with a flinching behavior. Mechanical withdrawal thresholds were determined using the up-down method according to Chaplan et al. (1994) in sham-operated (n = 8) and SNIt mice (n = 8) 7 and 28 days after surgery. Data are reported as the mean percent of baseline withdrawal threshold ((postoperative threshold/baseline threshold) × 100) ± standard deviation (SD).

Isolated Skin–Nerve Preparation

An isolated skin-nerve preparation (Reeh, 1986) was used for combined electrophysiological and pharmacological study of

single afferent fibers innervating plantar skin of right (ipsilateral) hind paws. Experimental (SNIt) and control (naïve and sham-operated) animals were deeply anesthetized with isoflurane. The glabrous skin of the hind paw was dissected and excised together with the attached tibial nerve and the medial and lateral plantar nerves. The skin–nerve preparation was immediately transferred to a chamber continuously perfused (15–20 ml per minute) with warmed (26 ± 2°C) oxygen-saturated synthetic interstitial fluid [SIF; (Bretag, 1969)] containing (in mM) 123 NaCl; 3.5 KCl, 0.7 $MgSO_4$, 2.0 $CaCl_2$, 9.5 Na gluconate, 1.7 NaH_2PO_4, 5.5 glucose, 7.5 sucrose, 10.0 Hepes (pH 7.45 ± 0.05 mOsm, 290 ± 0.05 mOsm). Warmed and oxygenated SIF was used in all subsequent procedures. The preparation was then oriented corium side up and anchored with insect pins before being further dissected to clear the skin and nerve of all tendons, muscles, and vasculature. The cut end of the tibial nerve was threaded through a small aperture into an adjacent small recording chamber and placed on the surface of a mirrored dissection platform. The main chamber was continuously perfused with SIF, and the recording chamber was filled with SIF below, and oil above the mirror.

General Electrophysiological Procedures

A compound action potential (neurogram) was recorded at the beginning of most experiments. A monopolar microelectrode (insulated except at tip) was placed on the main trunk of the medial or lateral plantar nerve for electrical stimulation. A large bundle of fibers was first divided from the main tibial nerve and lifted onto a fine gold wire electrode for extracellular recording. The recording electrode was suspended in the oil layer of the recording chamber and referenced to the bath with a silver/silver chloride electrode. Stimulating current was delivered with increasing intensity until each waveform component (Aαβ, Aδ, and C) of the compound action potential could be evoked and differentiated. The rate of conduction was calculated for each waveform and expressed as meters per second. Subsequently recorded single fibers were classified by conduction velocity based on the conduction velocities of the neurogram waveforms. When a particular waveform component could not be evoked for a given experiment, or if no compound action potential was recorded for an experiment, single units were classified according to the mean conduction rate of waveforms from compound action potentials recorded in all experiments. Electrical signals were differentially amplified (DAM50, World Precision Instruments, Austin, TX), filtered, and routed in parallel to an oscilloscope and computerized data acquisition system.

Isolation of Single Units

Initially, small bundles of nerve fibers were teased from the nerve trunk and placed on the recording electrode to observe activity from multiple axons. The corium surface of the skin was then gently probed with a blunt glass rod to identify the general area of skin innervated by the small bundle. Next, electrical search stimuli were delivered to the nerve trunk through a microelectrode to elicit single fiber activity as progressively smaller filaments were isolated and placed on the recording electrode. Once single unit activity could be isolated, a second, roving, stimulating electrode was progressively traced along the plantar nerve branches until the receptive field could be electrically identified. Conduction velocity of individual axons was determined by electrical stimulation of the center of the receptive field and expressed as meters per second. Individual units were classified based on conduction velocity ranges obtained from whole-nerve compound action potential recordings made at the beginning of most experiments.

Functional Characterization of Afferent Fibers

Because of sampling bias inherent in the search protocol (described above) and the limited ability to search with thermal stimuli, all afferent fibers encountered were mechanoreceptors. The mechanical threshold for each single unit was determined using a series of calibrated von Frey nylon monofilaments applied to the corium surface of the skin. Threshold was defined as the lowest bending force that consistently evoked an action potential response 50% of the time. Fibers were classified as nociceptors if they exhibited slowly adapting responses to sustained mechanical stimulation, and their firing rate increased monotonically with increasing force of stimulation. After functional characterization of each fiber, a small cylinder (5 mm diameter) was sealed over the receptive field with petroleum jelly and filled with SIF. The cylinder served as a reservoir for subsequent mechanical and thermal testing as well as drug delivery. The thermal responsiveness of fibers was qualitatively assessed by filling the cylinder sequentially with cold (5°C), warm, then hot (45°C) SIF. Thermal stimulation was used to complete the functional characterization of mechanical nociceptors, but changes in thermal responses were not evaluated during drug testing.

Quantification of Mechanical Responses

After functional characterization, each afferent fiber was quantitatively tested for responses to mechanical stimulation before and after exposure to drug or vehicle. Mechanical stimulation was delivered by a von Frey filament with suprathreshold bending force that was mounted in a micromanipulator and lowered onto the receptive field encircled by the cylinder. Testing trials consisted of three 5-s periods of mechanical stimulation, each preceded by 10 seconds without stimulation. The response for each stimulation period was determined by subtracting the number of spikes in the preceding 5 seconds from each 5 second period of stimulation. The mean number of spikes of the three periods of stimulation represented the "response measure" for each trial. Data are recorded either as numbers of spikes (response measure) or spikes per second (Hz, firing rate). The stimulation onset and offset times were signaled to the online data acquisition program (see below).

Spontaneous Activity

Un-evoked neuronal activity was recorded for at least 30 seconds before quantitative mechanical testing. Units were classified as having spontaneous activity if their firing rates were ≥ 0.1 Hz in the absence of any intentional stimulus.

Preparation of Drugs

Stock solutions of deltorphin II (100 μM; Phoenix Pharmaceuticals, Belmont, CA) were made in water and stored at 4°C. Working concentrations of the ligand were diluted in SIF, as needed. Naltrindole hydrochloride (Tocris, Ellisville, MO) was reconstituted in water to a stock solution of 100 μM and was stored at −20°C. Each drug solution was warmed to room temperature and saturated with oxygen prior to use.

Peripheral Delivery of Drug and Mechanical Testing

After functional characterization of each fiber, a small cylinder (5 mm diameter) was placed over the receptive field to serve as a reservoir for drug delivery. After the baseline (before drug) mechanical stimulation trial, the drug reservoir was emptied of SIF with a suction pipette and replaced with oxygenated deltorphin II, deltorphin II plus naltrindole, or vehicle (SIF) for 2 min. After 2 min of drug exposure, a second mechanical stimulus trial was performed before rinsing of cylinder with fresh SIF. Mechanical responses were retested every 15 to 30 min after drug washout to test for recovery. Recovery from drug application was defined as at least a 50% return towards baseline mechanical response. Units that did not recover were excluded from this study. Based on a previous electrophysiological study in inflamed skin (Brederson and Honda, 2015) and in preliminary spared nerve experiments, 300nM was used as the test concentration for deltorphin II. Concentration-response relationships were determined using cumulative ascending dosing without intervening rinses with SIF. Data are reported as the mean percent of baseline firing ((post-drug response/baseline response) × 100) ± standard deviation (SD) for each unit unless otherwise noted.

Data Collection and Analysis

Compound action potentials, teased fiber recordings, and stimulus delivery times were collected with Spike 2 software and Power1401 interface (CED, Cambridge, England). Data analysis and spike discrimination were performed online and offline. STATISTICA 5.5 software (StatSoft, Tulsa, OK, USA) was used for statistical analysis and generation of graphs. Statistical analyses are described as necessary for each section of *Results*. Unless specified otherwise, values are expressed as means ± SD.

RESULTS

Compound Action Potentials

Compound action potential recordings were made from the tibial nerve at the start of each experiment. Isolated single units were classified into fiber type based on comparison of their conduction velocities to those of compound action potential waveforms for that experiment. There was no difference in conduction velocities of compound action potential waveforms found between naïve, sham, and SNIt groups of animals. Combining all groups, the mean values for ranges of conduction velocities (± SD) of compound action potential waveforms were: Aαβ (n = 30) 22.7 to 10.5 m/s; Aδ (n =,29) 5.3 to 2.3 m/s; C (n = 29) 1.5 to 0.3 m/s.

Functional Classification of Primary Afferent Units

A total of 42 single units isolated from the tibial nerves of 31 mice (experimental and control) were included in this study. All fibers conducted within the C-fiber range with a mean conduction velocity of 0.72 m/s ± 0.46. Receptive fields were evenly distributed across the plantar aspect of the foot, and no attempt was made to measure or otherwise quantify receptive field size. All fibers included in this study were functionally classified as nociceptors based on their stimulus–response relationships, and they were assigned to one of the following categories based on their response properties: C-mechanical nociceptors (CM), C-mechanoheat nociceptors (CMH), C-mechanocold nociceptors (CMC) and C-mechanoheat-cold nociceptors (CMHC) (**Table 1**).

Effects of Spared Nerve Tibial Injury on Mechanical Sensitivity

Behavioral testing revealed significant decreases in mechanical withdrawal thresholds for both SNIt and sham-operated animals at 7 and 28 days survival times, when compared to naïve animals. Moreover, the decrease in thresholds for SNIt animals was significantly greater than sham-operated animals at the same time points ($P<0.05$, Mann-Whitney *U* test) (**Figure 1**). **Table 2** summarizes comparisons of properties of individual nociceptors from control and nerve-injury groups. Conduction velocities and rates of spontaneous activity (axon firing in the absence of intentional stimulation) were not affected by sham or experimental nerve injury. Mechanical thresholds of individual fibers from sham-operated animals did not differ from those in the naïve group. However, after nerve injury, median mechanical thresholds of individual nociceptors in 7- and 28-day SNIt animals were significantly lower than both naïve and sham-operated animals ($P<0.05$, Kruskal-Wallis one-way ANOVA).

Effects of Spared Nerve Tibial Injury on Sensitivity to Deltorphin II

Sensitivity of individual nociceptors to deltorphin II was tested in experimental and control animals. **Figure 2A** illustrates the lack

TABLE 1 | Distribution of functional categories of nociceptors among control and experimental groups.

	Naive	Sham-7D	Sham-28D	SNI-7D	SNI-28D
CMN	5 (83)	4 (80)	6 (60)	6 (46)	7 (78)
CMH	1 (17)	0	2 (20)	6 (54)	1 (11)
CMHC	0	1 (20)	0	0	1 (11)
CMC	0	0	2 (20)	0	0
Total	6	5	10	12	9

All units were classified as C fibers based on conduction velocities then classified based on response properties. C-mechanical nociceptors (CMN), C-mechanoheat nociceptors (CMH), C-mechanocold nociceptors (CMC) and C-mechanoheat and cold nociceptors (CMHC). Values are numbers of individual units with percentages of column totals in parentheses.

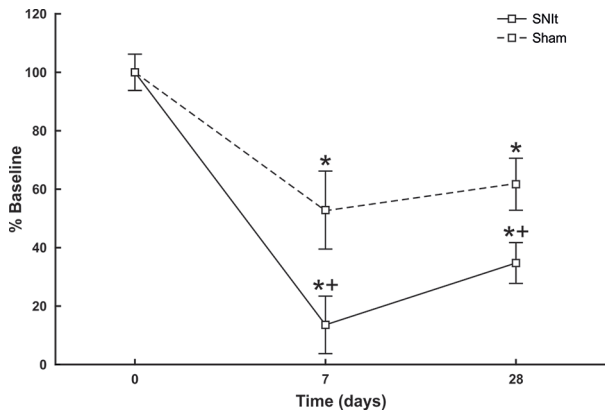

FIGURE 1 | Behavioral assessment of SNIt model. Changes in withdrawal thresholds to mechanical stimulation were determined in sham-operated (n = 8) and SNIt mice (n = 8) 7 and 28 days after surgery. Sham surgery resulted in a significant decrease in withdrawal threshold at both survival times, and SNIt caused greater decreases in threshold compared to sham-operated animals. Changes in mechanical thresholds are reported as percent of pre-surgery baseline ± standard deviation of mean. *Significant difference when compared with baseline (Mann-Whitney U-test; P< 0.05). + indicates significant difference when compared with sham-operated animals (Mann-Whitney U-test; P< 0.05).

TABLE 2 | Comparisons of properties of nociceptors in control and experimental groups.

	CV (m/s)	Spontaneous Activity (Hz)	Mechanical Threshold (mN)
Naive	0.63 ± 0.45	0.28 ± 0.12	10.35 (5.6, 23.1)
Sham-7D	0.63 ± 0.40	0.22 ± 0.18	10.00 (8.0, 26.9)
Sham-28D	0.75 ± 0.40	0.10 ± 0.15	14.55 (9.0, 23.1)
SNIt-7D	0.64 ± 0.51	0.18 ± 0.07	*6.15 (4.6, 13.4)
SNIt-28D	0.91 ± 0.54	0.15 ± 0.05	*4.08 (4.07, 10.0)

*Conduction velocity (CV) and spontaneous activity (SA) are expressed as mean ± S.D., whereas mechanical thresholds (MT) are expressed as median and interquartile range. * indicates significant difference in mechanical threshold compared to fibers from naïve and corresponding sham groups (Kruskal-Wallis one way ANOVA, P < 0.05).*

of effect of deltorphin II in control animals on the responses of individual fibers to mechanical stimulation after drug application. The mean percent baseline responses after drug application in naïve animals (120.9 ± 18.2) and in sham operated animals 7 days (103.2 ± 16.0) and 28 days (108.9 ± 13.7) after surgery were not significantly different (P > 0.05, Mann-Whitney *U* test). In contrast, peripheral application of deltorphin II produced robust inhibition of responses to noxious mechanical stimulation in nociceptors of SNIt-operated mice (**Figure 2B**). The mean percent baseline response following 300 nM deltorphin II in SNIt operated mice was 31.9 ± 24.5% after 7 days and 33.5 ± 2.8% after 28 days. Fibers recovered from inhibition in less than 1 h (range, 15–60 h) after drug rinse and washout. A representative example of the effect of deltorphin II on an individual nociceptor from a 28 days SNIt animal is shown in **Figure 3**.

Deltorphin II Concentration-Response Relationship

Concentration response relationships were determined in a subset (n = 4) of nociceptors (SNI 28 days) that all responded with inhibition to 300 nM concentration of deltorphin II. After recovery from the initial 300 nM test, a series of increasing concentrations of deltorphin II (10, 30, 100, 300, and 1,000 nM) was applied to receptive field of the same fiber for 2 min each. A baseline mechanical stimulus trial was first performed with fresh SIF. The SIF was next replaced with 10 nM deltorphin II solution for 2 min before a second mechanical stimulus trial was performed in the presence of deltorphin II. The reservoir was then emptied, and the next higher concentration of drug was added, and after a 2-min incubation, another mechanical stimulus trial was performed. This protocol was repeated for the remaining concentrations of deltorphin II before the drug was washed out and recovery was tested. All fibers included in construction of the dose response curve recovered in 15 to 35 min. Mechanical responses were expressed as percent of the initial baseline trial response. Percent response inhibition was calculated, and a concentration response curve was constructed (**Figure 4**).

Inhibitory Effects of Deltorphin II Are Prevented by Naltrindole

To test whether the inhibitory effects of deltorphin II were mediated by DOR, equimolar concentrations of deltorphin II (300 nM) and the selective DOR antagonist, naltrindole (300 nM), were co-applied to nociceptors 7 days (n = 3) and 28 days (n = 2) after SNIt surgery. Responses to mechanical stimulation were first inhibited by deltorphin II alone to 19.4 ± 15.1% of baseline at 7 days and to 12.0 ± 1.2% of baseline at 28 days after SNIt (P<0.05, paired t-test). Following washout and recovery from deltorphin II (alone) a new baseline mechanical trial was performed for each nociceptor before co-delivery of deltorphin II and naltrindole. **Figure 5** shows that naltridole reversed the inhibitory effects of deltorphin II at 7 days (105.7 ± 12.9% of baseline) and 28 days (107.0 ± 2.7% baseline) after SNIt (P > 0.05, paired t-test).

DISCUSSION

The objective of the present study was to determine if functional DORs are expressed on the peripheral processes of cutaneous nociceptors in a rodent model of neuropathic pain resulting from a nerve injury with spared tibial nerve (SNIt). We tested this idea by assessing responses to mechanical stimulation in the presence of deltorphin II administered locally in naive, sham- and SNIt-operated mice. Application of deltorphin II directly to the receptive fields of afferent fibers innervating skin of SNIt animals suppressed responses to noxious mechanical stimulation in all units studied. Deltorphin II effects were concentration-dependent and prevented by pretreatment with naltrindole indicating that deltorphin II effects were DOR-mediated. These data provide direct electrophysiological

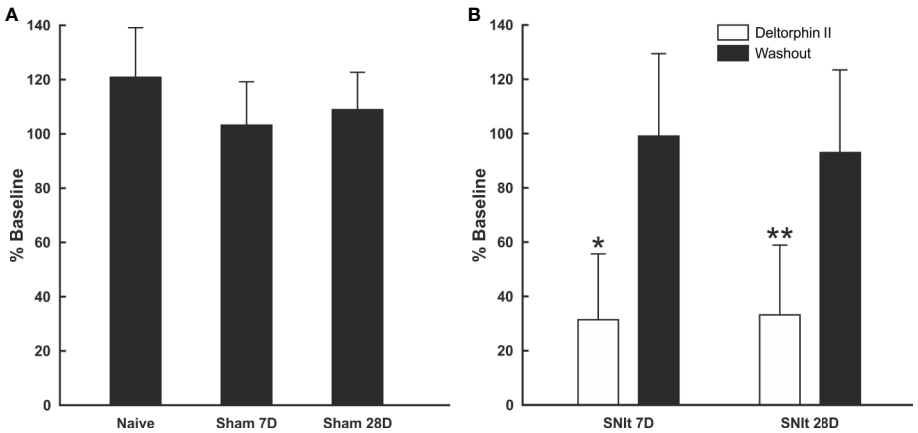

FIGURE 2 | Responses of afferent fibers from control and nerve-injured animals after exposure to deltorphin II. **(A)** Deltorphin II did not reduce responses of nociceptors to mechanical stimulation in naïve or sham-operated animals ($P > 0.05$, Mann-Whitney U-test). Response measures for each condition are presented as the mean percent of baseline response (± SD) to mechanical stimulation after application of 300 nM deltorphin II in naïve (120.9 ± 18.2; n = 5) and sham-operated animals surviving 7 days (103.2 ± 16.0; n = 6) and 28 days (108.9 ± 13.7; n = 10) after surgery. **(B)** Deltorphin II reduced responses of afferent fibers to mechanical stimulation after peripheral nerve injury (*$P < 0.05$, **$P < 0.01$; Mann-Whitney U-test). Response measures for each condition are presented as the mean percent baseline response (± SD) to mechanical stimulation after application of 300 nM deltorphin II in SNIt operated mice surviving 7 days (31.92 ± 24.46; n = 12) and 28 days (33.52 ± 25.84; n = 9) after surgery.

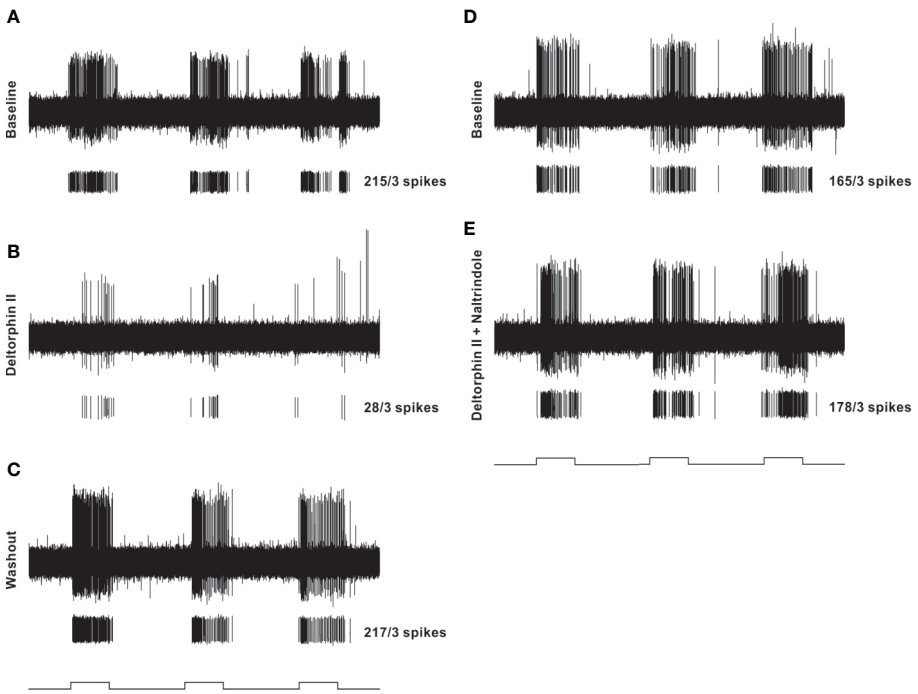

FIGURE 3 | Representative example of testing protocol and responses of an individual nociceptor before and after localized administration of test drugs that were restricted to a cylinder centered over the receptive field. In each panel **(A–E)**, the top trace is a raw recording from a C-fiber mechanical nociceptor (CV 0.61 m/s; mechanical threshold 4.1 mN). Each lower trace contains individual spikes sorted in software from the raw trace along with the total number of spikes counted during the three stimulation periods. The test mechanical stimulus was 38 mN, and stimulus timing (5 s, each separated by 10 secs) is indicated at the bottom of each column. The left column shows baseline response before drug **(A)**, response 2 min after 300 nM deltorphin II **(B)**, and response after 20 min of washout **(C)**. After washout, a new baseline was established **(D)** before application of 300 nM deltorphin II plus 300 nM naltrindole **(E)**.

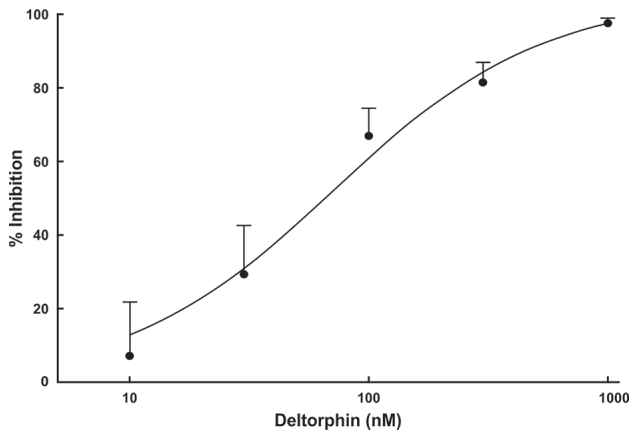

FIGURE 4 | Deltorphin II concentration-response relationship. Sequential application of deltorphin II (10, 30, 100, 300, 1,000 nM) directly to the receptive fields of individual C-fiber nociceptors (n = 4) innervating skin in SNIt animals 28 days after surgery reduced responses to mechanical stimulus (38 mN) in a concentration-dependent manner. Data points represent mean percent inhibition of response in presence of drug, relative to baseline response of each unit (± SEM). Estimated EC_{50} value is 73.5 nM. Regression lines and estimated EC_{50} value were calculated using STATISTICA 5.5.

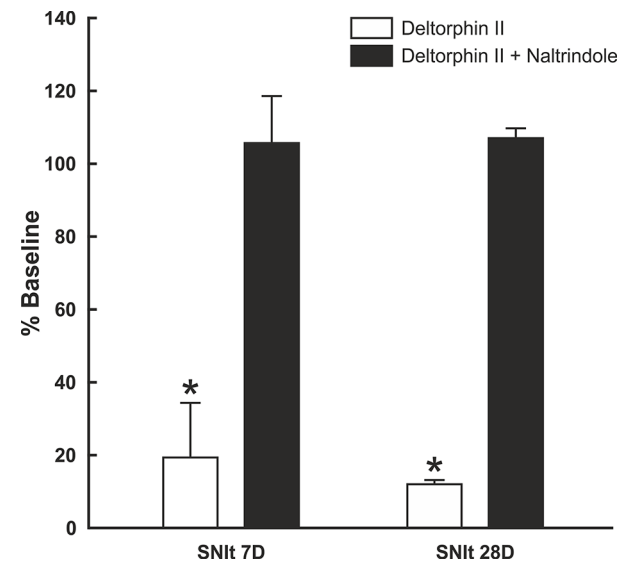

FIGURE 5 | Inhibitory effects of deltorphin II were prevented by naltrindole. Nociceptors from mice 7 days (n = 3) and 28 days (n = 2) after SNIt were first tested with 300 nM deltorphin II. After washout and recovery, they were re-tested with equimolar (300 nM) concentrations of deltorphin II plus naltrindole. Mean percent baseline response (± SD) was decreased to 19.4 ± 15.1% (SNIt 7D) and 12.0 ± 1.2% (SNIt 28D) when deltorphin II was applied alone, compared to 105.7 ± 12.9% (SNIt 7D) and 107.0 ± 2.7% (SNIt 28D) of baseline when deltorphin II was co-applied with naltrindole. *Significant difference compared to baseline ($P < 0.05$, paired t-test).

evidence for functional DORs on the peripheral terminals of somatic afferent neurons under neuropathic conditions.

Spared (Tibial) Nerve Injury Induced Behavioral Allodynia and a Corresponding Decrease in Thresholds of Mechanical Nociceptors

Spared nerve injury (SNI) is a model of partial-limb denervation with minimal variability of nerve damage. The plantar area of the hindlimb is innervated by three branches of the sciatic nerve, the common fibular, the tibial, and the sural nerves (Swett and Woolf, 1985). In contrast to other models of peripheral nerve injury, SNI models produce a consistent degree of axonal damage. Even if only two nerve branches out of three are axotomized, there will be changes in soma found in all DRGs associated with the sciatic nerve. Moreover, there is considerable co-mingling of cell bodies of injured and uninjured neurons in corresponding DRG (Klusáková and Dubový, 2009). The classic SNI model described by Decosterd and Woolf (2000) was based on transection of the tibial and the common fibular nerves, leaving the sural nerve intact.

In the present experiments we utilized a modification of SNI, in which the tibial nerve was left uninjured. The SNIt model in mice produces robust behavioral mechanical allodynia accompanied by consistent anatomic changes in IB4-binding afferent terminals in the spinal cord (Shields et al., 2003). Wallerian degeneration causes electrophysiological changes in injured nerve axons (Chaudhry and Cornblath, 1992), so an important feature of the SNIt model is that it permitted recording from uninjured fibers some distance from degenerating axons. The present behavioral tests showed clearly that SNIt caused significant decreases of mechanical withdrawal threshold 7 and 28 days after injury compared to sham-operated animals. However, sham-operated animals also displayed a smaller decrease in mechanical withdrawal thresholds that persisted at 28 days. It is unclear why the results from the present behavioral studies do not agree completely with results from other laboratories. It is possible that there were unknown issues in the group of behavioral animals related to testing protocols or surgical technique. It is important to note that the decrease in behavioral mechanical sensitivity was not paralleled by a decreased mechanical sensitivity in individual nociceptors isolated from sham-operated animals. Importantly, electrophysiological study of tibial nerve afferent axons in SNIt-operated animals at the same time points revealed a significant decrease in mechanical threshold of nociceptors. Our observation of a lowering of mechanical threshold in nociceptors following spared tibial nerve injury that is not accompanied by changes in spontaneous activity is similar to reports from a study of spared sural nerve injury (Smith et al., 2013). We were not able to test consistently for the electrophysiological correlate of behavioral hyperalgesia (increased neuronal activity in response to identical noxious stimulation). Nor, did we detect a change in the incidence or rate of spontaneous activity following SNIt. The SNIt model of neuropathic pain has proven particularly valuable

for combined electrophysiological and behavioral study following nerve injury because the tibial nerve is not directly involved with the nerve injury, and the innervation territory of the tibial nerve on the plantar surface of the hindlimb is amenable to behavioral testing.

Deltorphin II Inhibits Responses of C-Fiber Nociceptors to Mechanical Stimulation After Nerve Injury

Many earlier studies evaluating effects of opioid receptor agonists in neuropathic pain models were based on their systemic administration. However, fewer studies have examined the effects of peripherally restricted opioids. Experiments of local administration of morphine, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin – DAMGO, endomorphine 1, endomorphine 2, and peripheral κ-opioid receptor agonist asimadoline revealed anti-allodynic and anti-hyperalgesic effects in neuropathic pain models (Walker et al., 1999; Pertovaara and Wei, 2001; Truong et al., 2003; Obara et al., 2004). It has also been shown that peripherally restricted opioids, in the form of small molecules, are effective under similar conditions (Obara et al., 2007).

The present results demonstrate that nerve injury induces the expression of functional DOR on skin nociceptors. Responses to mechanical stimulation of all studied nociceptors in SNIt-operated animals was decreased by more than 60% from baseline after deltorphin II application. Deltorphin II effects on C mechanical nociceptors were dose-dependent and prevented by application of naltrindole, providing direct pharmacological evidence that the inhibitory effects of deltorphin II were DOR-mediated. Our results are in agreement with those of Kabli and Cahill (2007) who described effects of deltorphin II intraplantar injection on attenuation of behavioral hyperalgesia and mechanical allodynia after chronic constriction injury.

Functional DOR Are Present on C Fibers Under Neuropathic Conditions, but Not in Naive or Sham-Operated Animals

Few studies have focused on changes of DOR in the peripheral nervous system following nerve injury. Some have shown that DOR expression decreased in corresponding DRG neurons after nerve injury (Zhang et al., 1997; Obara et al., 2009). However, Kabli and Cahill (2007) found up-regulation of DOR in DRG neurons after chronic constriction injury. Nevertheless, we found that nociceptors are very sensitive to DOR agonists 7 and 28 days after nerve injury, whereas nociceptors from naive and sham-operated animals do not appear to express functional DOR.

It is unclear how DOR become functionally competent on C-fiber nociceptors. One possibility is that partial nerve injury induces an increase density and sprouting of uninjured axons in their native uninjured dermatomes, i.e. in the skin area showing allodynia (Duraku et al., 2012; Duraku et al., 2013; Kuner and Flor, 2017). Nerve sprouts of regenerating C-fibers develop an early chemosensitivity to various substances including, e.g. bradykinin, histamine, serotonin, and capsaicin (Leah et al., 1988; Zimmermann, 2001). Another possible mechanism could be the paracrine secretion of proinflammatory cytokines and chemokines from injured neurons (Scholz and Woolf, 2007; Bai et al., 2016) that might evoke changes in expression of functional DOR in non-injured neurons. Finally, it is possible that a class of DOR-sensitive mechanically insensitive afferent (MIA) C-fibers becomes activated by tissue injury, inflammation or nerve injury (Meyer et al., 1991). Since MIA form a substantial proportion of the nociceptor population, the acquisition of mechanosensitivity after nerve injury may determine their role in development and maintenance of hyperalgesic and hypersensitive states (Gold and Gebhart, 2010).

The current findings provide new insight into intrinsic peripheral mechanisms of opioid analgesia that become enabled or engaged following damage to the peripheral nervous system. They highlight the importance of development of novel exogenous opioid agonists that can be delivered at low concentrations directly to peripheral targets or of agonists that can be delivered systemically but lack access to the central nervous system. Moreover, these findings suggest the more general need to develop therapeutic strategies that take advantage of an existing peripheral analgesic system including manipulation of levels of functional opioid receptors on peripheral afferent terminals, recruitment of peripheral opioid-producing cells, and synthesis and release of endogenous opioids.

CONCLUSIONS

The results of this study provide direct evidence for the expression of functionally competent DOR on the peripheral processes of nociceptors innervating skin under neuropathic conditions. We have shown that the selective DOR agonist deltorphin II decreased responsivity of cutaneous nociceptors to mechanical stimulation after nerve injury. In contrast, mechanical nociceptors innervating normal skin are not sensitive to deltorphin II, and they do not appear to express functional DORs. Furthermore, our data suggest potential clinical utility of peripherally restricted DOR agonists for the treatment of neuropathic pain.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Minnesota.

AUTHOR CONTRIBUTIONS

MJ: Conceptualization, investigation, writing-original/review, funding. LV: supervision, methodology, writing-review/edit,

funding. CH: Investigation. PD: Conceptualization, writing-review. CNH: Conceptualization, methodology, supervision, writing-review/edit, funding.

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Behavioral Effects of Continuously Administered Bergamot Essential Oil on Mice With Partial Sciatic Nerve Ligation

Kengo Hamamura[1†], Soh Katsuyama[2], Takaaki Komatsu[3], Damiana Scuteri[4†], Giacinto Bagetta[4†], Kosuke Aritake[1] and Tsukasa Sakurada[5]*

[1] Laboratory of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Daiichi University of Pharmacy, Fukuoka, Japan, [2] Center for Clinical Pharmacology and Pharmaceutics, Nihon Pharmaceutical University, Saitama, Japan, [3] Drug Analysis Laboratory, Faculty of Pharmaceutical Sciences, Daiichi University of Pharmacy, Fukuoka, Japan, [4] Preclinical and Translational Pharmacology, Department of Pharmacy, Health Science and Nutrition, University of Calabria, Cosenza, Italy, [5] Center for Supporting Pharmaceutical Education, Faculty of Pharmaceutical Sciences, Daiichi University of Pharmacy, Fukuoka, Japan

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***Correspondence:**
Tsukasa Sakurada
tsukasa@daiichi-cps.ac.jp

†ORCID:
Kengo Hamamura
orcid.org/0000-0001-9146-8554
Damiana Scuteri
orcid.org/0000-0001-5846-7058
Giacinto Bagetta
orcid.org/0000-0001-8540-6218

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Neuropathic pain is an intractable chronic pain condition that is mainly caused by allodynia. We had previously reported that intra-plantar administration of bergamot essential oil (BEO) containing an aromatic compound significantly suppressed partial sciatic nerve ligation (PSNL)-induced mechanical allodynia *via* opioid mu receptors in mice. However, it has also been reported that the inhalation of BEO reduced formalin-induced nociceptive responses. Therefore, we aimed to elucidate whether the analgesic action of BEO is mediated by olfactory stimulation through volatile components. In the current study, BEO was continuously administered with an osmotic pump during PSNL surgery, and the effects on mice behavior were examined pharmacologically using a double activity monitoring system, which can detect two-dimensional planar motion in a cage with an infrared beam sensor as well as active motion with a running wheel. Here, we report that the two-dimensional planar activity significantly increased in mice with PSNL in the light phase (from 8 o'clock to 20 o'clock) but not in the dark phase (from 20 o'clock to 8 o'clock) from the second day after surgery. However, this increase was not observed when BEO was continuously administered. The effect of BEO on the two-dimensional planar counts in mice with PSNL was antagonized by naloxone hydrochloride. Regarding the running wheel activity, the number of rotations decreased by PSNL in the dark phase from the 8th day after surgery. However, this was not apparent with BEO use. The effect of BEO on the number of rotations was also antagonized by naloxone hydrochloride. Furthermore, inhalation of BEO in PSNL mice did not affect mechanical allodynia or the two-dimensional planar motion or running wheel activities. These findings indicate that BEO exhibits an analgesic action, which is mediated by opioid receptors and not by the olfactory system.

Keywords: bergamot essential oil, neuropathic pain, partial sciatic nerve ligation, osmotic pump, double activity monitoring system®

INTRODUCTION

Neuropathic pain is a chronic condition that occurs after nerve compression due to cancer, diabetes, herpes virus infection, and autoimmune diseases (Woolf and Mannion, 1999). Currently, millions of patients worldwide endure neuropathic pain (Tsuda et al., 2005). One troubling and characteristic symptom of neuropathic pain is hypersensitivity to usually harmless stimuli, a condition known as "tactile allodynia," which is often resistant to NSAIDs and opioids (Backonja and Glanzman, 2003). Various models have been devised to reproduce disease-like conditions in rodents, such as diabetic neuropathy, chemotherapy-induced neuropathic pain, antiretroviral drug-induced neuropathy, and spinal and peripheral nerve damage. In recent years, it has become clear that chronic pain can affect cognitive behavior in animal models just as it does in humans (Guida et al., 2020). Therefore, the establishment of treatments for neuropathic pain is an important issue in terms of reducing anxiety and depression.

Aromatherapy refers to therapies that use essential oil or plant-derived fragrances to prevent illnesses, improve mental and physical health and relaxation, and relieve stress. Among the essential oils, bergamot essential oil (BEO) is obtained by cold pressing the epicarp and part of the mesocarp of the bergamot fruit (*Citrus bergamia* Risso et Poiteau) (Moufida and Marzouk, 2003). BEO is listed in Farmacopea Ufficiale Italiana (1991~) and is used in the pharmaceutical industry, mainly in dentistry, ophthalmology, and dermatology. Recently, several reports showed that the use of aromatherapy massage with various essential oils including BEO could relieve anxiety (Wilkinson et al., 2007; Seyyed-Rasooli et al., 2016), depressions (Wilkinson et al., 2007), and the perception of pain (Nasiri et al., 2016; Seyyed-Rasooli et al., 2016; Gok Metin et al., 2017).

Regarding the pain area, we had reported that the capsaicin-induced nociceptive response was significantly reduced by the intra-plantar injection of BEO in mice (Kuwahata et al., 2009). Next, we had reported that the opioid receptor antagonist naloxone hydrochloride significantly reversed the inhibitory effects of BEO on the capsaicin-induced behavioral response (Katsuyama et al., 2011). Next, we had performed a 2% formalin test as another nociceptive pain model mice. Plantar subcutaneous injection of 2% formalin caused biphasic (phases I and II) nociceptive behavior consisting of licking and biting. We reported that plantar subcutaneous injection of BEO reduced both the first and late phases of the formalin-induced licking and biting responses (Katsuyama et al., 2015). This inhibitory effect of BEO on the formalin-induced behavioral response was also antagonized by naloxone hydrochloride (Katsuyama et al., 2015). Therefore, BEO can be efficacious in nociceptive pain, and help to suppress partial sciatic nerve ligation (PSNL) mouse-induced allodynia (Komatsu et al., 2018). The inhibitory effect of BEO on PSNL-induced allodynia is also antagonized by naloxone methiodide (Komatsu et al., 2018).

BEO consists of volatile fractions (93–96% of the total) of monoterpene and sesquiterpene hydrocarbons (such as limonene) and oxygenated derivatives (such as linalool) and non-volatile fractions (4–7% of the total) of waxes, polymethoxylated flavones, coumarins, and psoralens, such as bergapten (5-methoxypsoralen) and bergamottine (5-geranyloxypsoralen) (Mondello et al., 1993; Dugo et al., 2000). Regarding the pharmacological action of scents, BEO inhalation was found to produce anxiolytic-like behavior (Brederson et al., 2013). More recently, BEO inhalation has been reported to reduce the behavioral signs of formalin-induced nociception in a dose-dependent manner (Scuteri et al., 2018). However, it was unclear whether the compound contained in BEO or its volatile component was important for anti-allodynic action. Therefore, we aimed to elucidate whether the analgesic action of BEO is mediated by olfactory stimulation by volatile components. In this study, we used PSNL mice, a neuropathic pain model, and we set up two experimental systems to examine behavior pharmacologically using a double activity monitoring system®, which can detect two-dimensional planar motion in a cage with an infrared beam sensor, as well as active motion with a running wheel. First, BEO was continuously administered subcutaneously with an osmotic pump to eliminate the effects of scent as much as possible. Second, we investigated the effects of BEO inhalation on pain-related activities.

MATERIALS AND METHODS

Animals

Four-week-old, male *ddY*-strain mice, weighing an average of 24 g (Japan SLC, Inc., Hamamatsu, Japan) were housed in groups (from 6 to 10 per cage) in a light-controlled room (illuminated from 8 o'clock to 20 o'clock) at 24 ± 1°C and 60 ± 10% humidity with free access to food (LabDiet 5L37, Japan SLC, Inc.) and water. Mice were acclimatized to the lighting conditions for 1 week. All experiments were performed following the approval of the Ethics Committee for Animal Experiments at Daiichi University of Pharmacy (Examination number: H30-006, approval number: 29004) and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Zimmermann, 1983). Every effort was made to minimize the number of animals used and any suffering in the experiments.

Partial Sciatic Nerve Ligation (PSNL)

The neuropathic pain model was created by ligating part of the sciatic nerve of 5-week-old male *ddY*-strain mice. Under anesthesia with isoflurane (2.0%, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), the sciatic nerve in the upper right thigh was exposed, and about half of the sciatic nerve was strongly ligated using 4-0 silk thread (Kusunose et al., 2010). Mice in which the sciatic nerve was not ligated formed the sham-operated group.

Double Activity Monitoring System®

Behavior was analyzed using a double activity monitoring system® (ShinFactory, Fukuoka, Japan) (**Figure 1A**), which can detect two-dimensional planar motion in a cage with an infrared beam sensor as well as active motion with a running wheel every 15 min. The planar activity was measured using an animal movement analyzing system (ACTIMO-100,

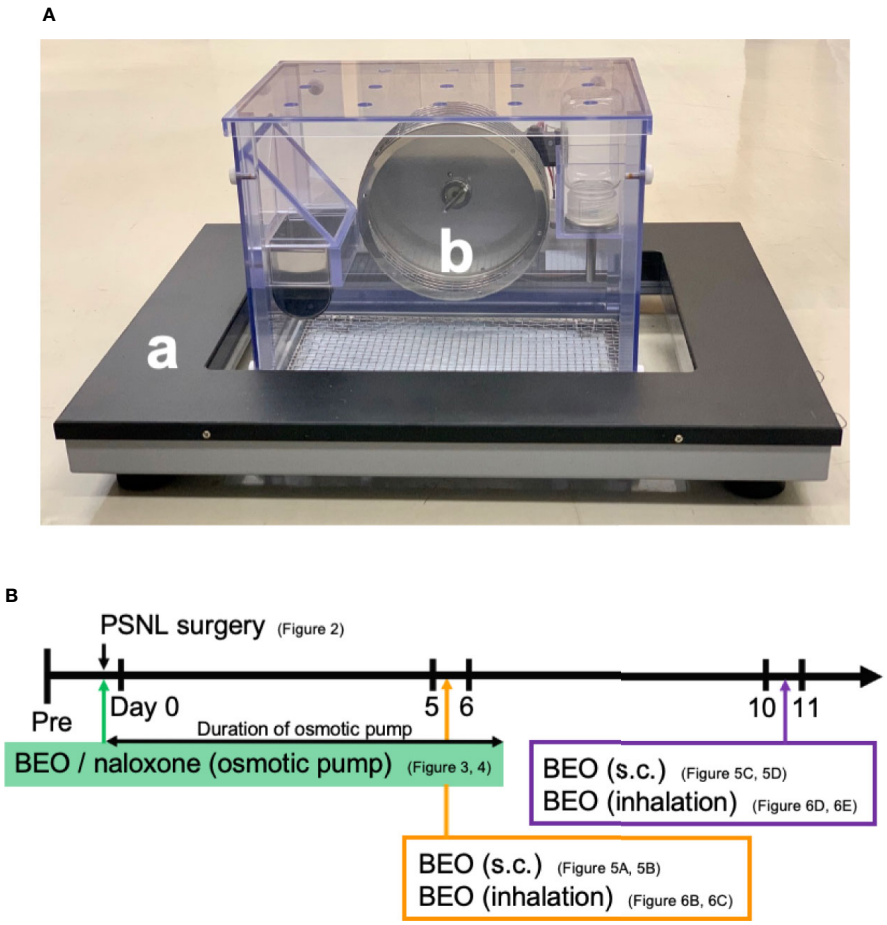

FIGURE 1 | (A) Behavior was analyzed using a double activity monitoring system®. (a) The planar activity was measured using an animal movement analyzing system. (b) The running wheel activity was measured using a cage with a rotating basket. **(B)** The scheme of experiments. Each experiment was conducted independently.

ShinFactory), which consists of a rectangular enclosure (30 × 20 cm) with a side wall equipped with photo sensors at 1.7 cm intervals. The running wheel activity was measured using a cage with a rotating basket (ACTIMO-RWM, ShinFactory). As for the results, the time zone with a white background on the horizontal axis is the light period from 8 o'clock to 20 o'clock, and with the gray background is the dark period from 20 o'clock to the next day's 8 o'clock. The behavior measurement started the day before the PSNL operation, and the operation day was set to Day 0. The mice were then observed for 14 days. The theoretical duration of the osmotic pump, which was 1 week, is shown with an orange background.

Assessment of Mechanical Allodynia by Von Frey Test

To assess mechanical allodynia, the mice were individually placed in a plastic animal chamber (internal dimensions 90 × 90 × 140 mm, Ugo Basile, Gemonio, Italy) on a stainless-steel mesh floor and habituated for 0.5 h to allow for acclimatization to the new environment. Calibrated von Frey filaments (pressure stimulus 0.40 g, Natsume Seisakusho Co., Ltd., Tokyo, Japan) were then applied to the right plantar surfaces of the hind paws of the mice. The paw withdrawal threshold was evaluated using the up-down method (Seltzer et al., 1990; Kusunose et al., 2010).

Drug Administration Using an Osmotic Pump

BEO was kindly provided from "Capua Company1880 S.r.l.," Campo Calabro, Reggio Calabria (Italy). According to the chromatographic analysis provided in the certificate of analysis, this batch of BEO contained: D-limonene (39.60%), linalyl acetate (31.09%), and linalool (9.55%). Jojoba wax (vehicle of BEO) was provided by "Company Farmalabor," Canosa of Puglia (Italy). Naloxone hydrochloride (Sigma-Aldrich, MO, USA) was dissolved in physiological saline (0.9% aqueous solution of sodium chloride, Nacalai Tesque, Kyoto, Japan). An Alzet® model 1007D mini osmotic pump (100 µl, 0.5 µl/h, 1 week sustained type, Durect Corporation Cupertino, CA, USA) containing either BEO (100 µl of stock solution) alone, BEO (100 µl of stock solution) and naloxone hydrochloride (1 mg/100 µl),

or jojoba wax (100 µl of stock solution) alone were surgically implanted under the back of mice anesthetized with isoflurane at the same time as PSNL (**Figure 1B**). The skin incision was closed with surgical sutures. The mice were immediately returned to the behavioral cage.

BEO Administration by Inhalation

BEO (0.04, 0.4, 4, 40, 400 µl/cage) was diluted with triethyl citrate to a total volume of 400 µl, and 100 µl each was dropped onto four filter paper discs attached to the four corners of the glass cage (30 × 60 × 34 cm; 61.2 L). The cage was filled with the vaporized solution by diffusion for 60 min. PSNL mice were then placed in the glass cage and they inhaled this for 60 min. After that, mice were removed from the glass cages, and a behavioral analysis was performed. In addition, when examining the effect on two-dimensional planar motion, inhalation of BEO in PSNL mice was performed from 10 o'clock to 11 o'clock on the 5th postoperative day. When examining the effect on the running wheel activity, inhalation of BEO by PSNL mice was performed from 18 o'clock to 19 o'clock on the 11th postoperative day.

Immunohistochemistry

Olfactory bulb samples were fixed with 4% paraformaldehyde (powder, Nacalai Tesque). The fixed samples were dehydrated with 30% sucrose (Nacalai Tesque) and embedded in O.C.T. compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan). Sections (20 µm thick) were antigen-activated with Histo VT One (Nacalai Tesque) for 20 min at 80°C. The steps after blocking were performed according to the protocol of the M.O.M. immunodetection kit (FMK-2201; Vector Laboratories, CA, USA). Anti-c-fos antibody (Santa Cruz Biotechnology, CA, USA) was used as the primary antibody. The sections were mounted with mounting medium (Vectashield Hard Set Mounting Medium with DAPI; Vector Laboratories). Fluorescent images were obtained with a fluorescence microscope (BZ-X810; Keyence Corporation, Osaka, Japan).

Statistical Analysis

Results are presented as mean ± standard error of the mean (S.E.M). Statistical differences were analyzed using the Student's t-test for two-group comparisons, and a one-way analysis of variance with Tukey's test for multiple-group comparisons. Statistical analysis was performed with Excel Statistics (Social Survey Research Information Co., Ltd., Tokyo, Japan). A p-value of <0.05 was considered statistically significant.

RESULTS

Two-Dimensional Planar Behavior Counts in PSNL Mice Increased in the Light Phase

First, we investigated the behavioral phenotype of PSNL mice. In the two-dimensional planar activity, representative data showed that the number of counts in mice with PSNL increased only in the light phase (from 8 o'clock to 20 o'clock) but not in the dark phase (from 20 o'clock to next 8 o'clock) from the second day after surgery (**Figure 2A**). On the 7th postoperative day, when allodynia was the most intense (Kusunose et al., 2010), a

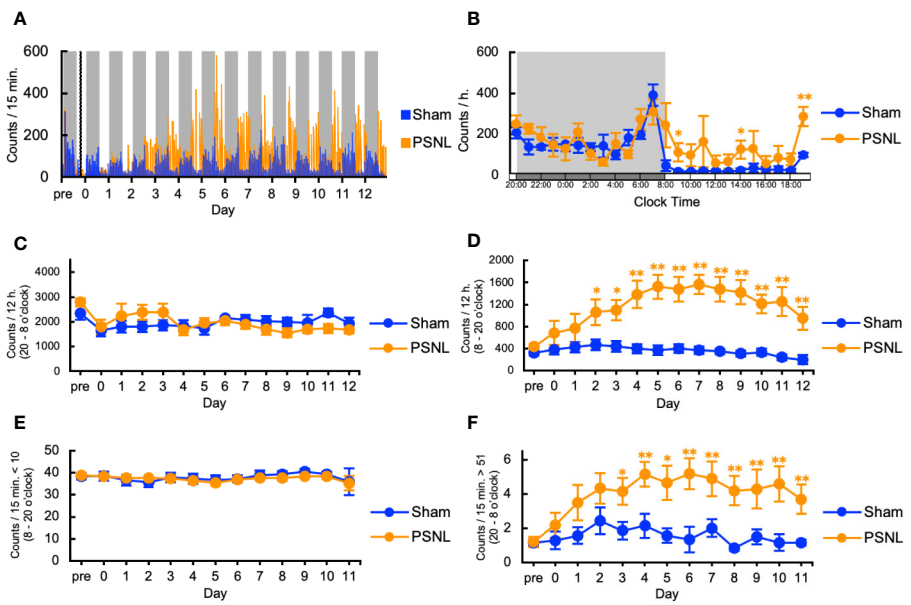

FIGURE 2 | Assessment of two-dimensional planar motion in partial sciatic nerve ligation (PSNL) mice. **(A)** Representative data of two-dimensional planar motion in PSNL mice every 15 min. **(B)** Circadian variation of two-dimensional planar behavior in PSNL mice on the 7th postoperative day. **(C, D)** Assessment of two-dimensional planar motion in PSNL mice during the dark period (20 o'clock–8 o'clock) **(C)**, and light period (8 o'clock–20 o'clock) **(D)**. **(E, F)** Assessment of two-dimensional planar immobility time (count per 15 min: 10 or less) **(E)**, and mobility time (count per 15 min: over 51) **(F)** in PSNL mice during the light period. **(B–F)** Values are the means ± S.E.M. (Sham: n = 7, PSNL: n = 9). **(B, D, F)** *P < 0.05; **P < 0.01, compared with the values at corresponding time points (Student's t-test).

comparison of the circadian changes in the two-dimensional planar activity revealed an increase at any time during the light period in PSNL mice (**Figure 2B**). Total counts of the two-dimensional planar activity during the dark phase did not change (**Figure 2C**). The total number of two-dimensional planar counts during the light phase of PSNL mice increased significantly from day 2 post-surgery, reaching its maximum at day 7 post-surgery (Sham: 369.83 ± 69.41, PSNL: 1,566.71 ± 175.23) (**Figure 2D**). To examine whether this was caused by a decrease in immobility time, a threshold value was set at 10 or less per 15 min, and the total number was calculated. As a result, immobility time in the light phase did not change between the PSNL and sham-operated groups (**Figure 2E**). The threshold was set to be more than 51 every 15 min to investigate whether it was due to an increase in the time. This showed that the number of counts significantly increased from day 3 post-surgery in PSNL mice and reached its maximum at day 6 post-surgery (Sham: 1.86 ± 0.51, PSNL: 5.20 ± 0.90) (**Figure 2F**). These results indicate that PSNL mice have a behavioral phenotype that changes a lot once they start to move during the light period.

Increased Counts by PSNL in the Light Phase Was Abolished by Continuously Administered BEO and Antagonized by Naloxone Hydrochloride

We investigated the effect of continuous administration of BEO using an osmotic pump on the two-dimensional planar activity in PSNL mice. Representative data showed that the increased counts produced by PSNL were abolished by continuously administered BEO in the light phase (**Figure 3A**). The effect of BEO on the two-dimensional planar counts in mice with PSNL was antagonized by naloxone hydrochloride (**Figure 3B**). Continuous administration of BEO and naloxone hydrochloride did not affect the total counts of two-dimensional planar activity during the dark phase (**Figure 3C**). The increase in the two-dimensional planar counts of PSNL-jojoba wax mice during the light phase was significantly decreased from day 3 post-surgery with continuous administration of BEO, and continued until the day 12 post-surgery at the end of the measurement (**Figure 3D**). The effect of BEO was significantly antagonized two days after co-administration with naloxone hydrochloride (**Figure 3D**). Immobility time (the threshold value was set at 10 or less per 15 min) in the light phase was not affected by continuous administration of BEO or naloxone hydrochloride (**Figure 3E**). On the other hand, when the threshold value was set to exceed 51 every 15 min, the increase in the two-dimensional planar counts of PSNL-jojoba wax mice during the light phase significantly decreased from day 2 post-surgery of continuous administration of BEO, and continued until the end day of the measurement (**Figure 3F**). The effect of BEO was significantly antagonized four days after co-administration with naloxone hydrochloride (**Figure 3F**).

Running Wheel Activity in PSNL Mice Decreased in the Dark Phase

We investigated the behavioral phenotype of PSNL mice on the running wheel activity. Representative data showed that the number

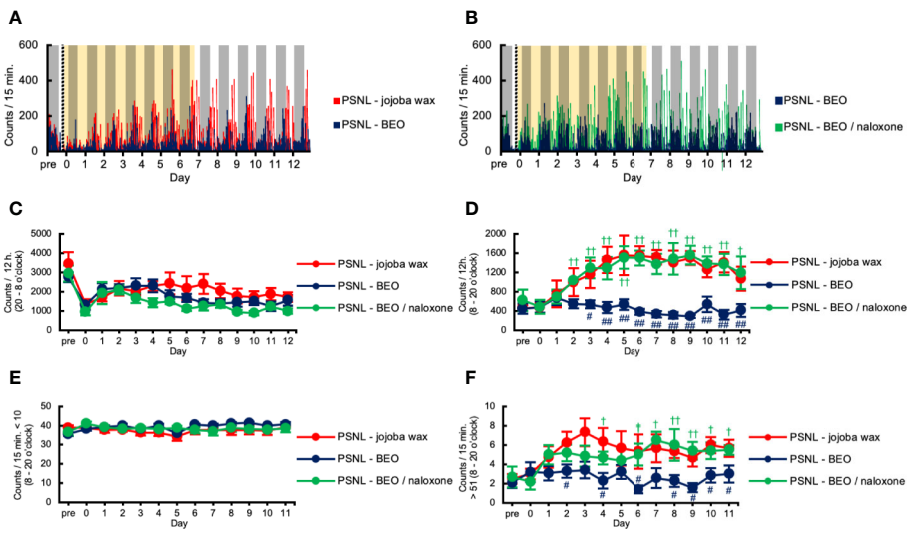

FIGURE 3 | Assessment of two-dimensional planar motion in PSNL mice with continuous subcutaneous administration of bergamot essential oil (BEO) and naloxone hydrochloride using an osmotic pump. **(A, B)** Representative data of two-dimensional planar motion in PSNL mice with continuous subcutaneous administration of jojoba-wax and BEO **(A)**, and BEO and BEO/naloxone **(B)** every 15 min using an osmotic pump. **(C, D)** Assessment of two-dimensional planar motion in PSNL mice with continuous subcutaneous administration of jojoba-wax, BEO, or BEO/naloxone using an osmotic pump during the dark period (20 o'clock–8 o'clock) **(C)** and light period (8 o'clock–20 o'clock) **(D)**. **(E, F)** Assessment of two-dimensional planar immobility time (count per 15 min: 10 or less) **(E)**, and mobility time (count per 15 min: over 51) **(F)** in PSNL mice with continuous subcutaneous administration of jojoba-wax, BEO and BEO/naloxone using an osmotic pump during the light period. **(C–F)** Values are the means ± S.E.M. (PSNL-jojoba wax: n = 6, PSNL-BEO: n = 9, PSNL-BEO/naloxone: n = 6). **(D, F)** $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, compared with PSNL-jojoba wax and PSNL-BEO at corresponding time points. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$, compared with PSNL-BEO and PSNL-BEO/naloxone at corresponding time points (one-way analysis of variance with Tukey's test).

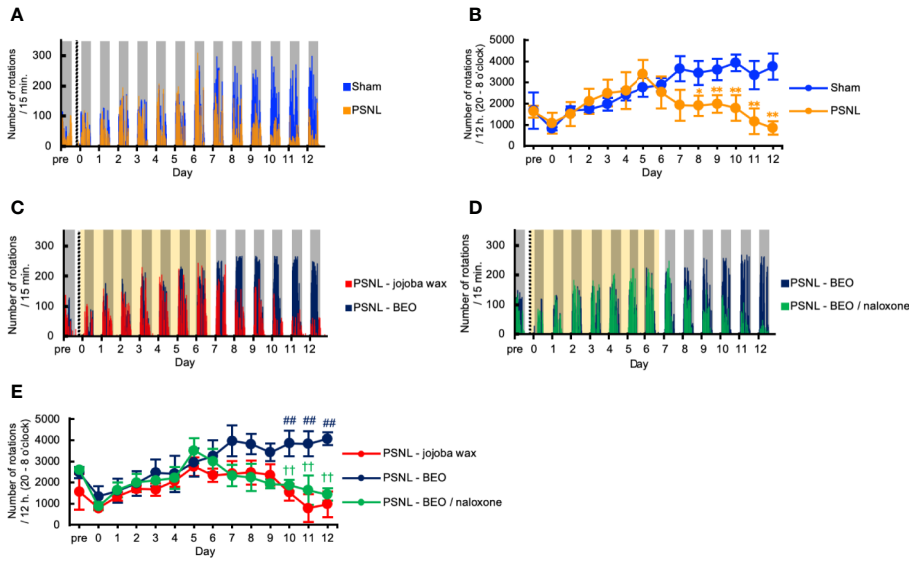

FIGURE 4 | Assessment of running wheel activity in PSNL mice with continuous subcutaneous administration of BEO and naloxone hydrochloride using an osmotic pump. **(A, C, D)** Representative data of running wheel activity in PSNL mice **(A)**, PSNL mice with continuous subcutaneous administration of jojoba-wax and BEO **(C)**, and BEO and BEO/naloxone **(D)** every 15 min using an osmotic pump. **(B, E)** Assessment of running wheel activity in PSNL mice **(B)**, or PSNL mice with continuous subcutaneous administration of jojoba-wax, BEO and BEO/naloxone using an osmotic pump **(E)** during the dark period (20 o'clock–8 o'clock). **(B, E)** Values are the means ± S.E.M. (Sham: n = 7, PSNL: n = 9, PSNL-jojoba wax: n = 6, PSNL-BEO: n = 9, PSNL-BEO/Naloxone: n = 6). **(B)** *P < 0.05; **P < 0.01, compared with Sham and PSNL at corresponding time points (Student's t-test). **(E)** $^{\#\#}$P < 0.01, compared with PSNL-jojoba wax and PSNL-BEO at corresponding time points. ††P < 0.01, compared with PSNL-BEO and PSNL-BEO/Naloxone at corresponding time points (one-way analysis of variance with Tukey's test).

of rotations in mice with PSNL was decreased in the dark phase from the 8th day after surgery (**Figure 4A**). On the other hand, in the light phase, both sham and PSNL groups rarely rotated the running wheel (**Figure 4A**). The total counts of rotations during the dark phase of PSNL mice decreased significantly from day 8 post-surgery reaching its largest differences at day 12 post-surgery, the final day of measurement (Sham: 3,743.00 ± 610.77, PSNL: 848.78 ± 306.06) (**Figure 4B**).

Decreased Number of Rotations by PSNL in the Dark Phase Was Abolished by Continuously Administered BEO and Antagonized by Naloxone Hydrochloride

We investigated the effect of the continuous administration of BEO using an osmotic pump on the running wheel activity in PSNL mice. Representative data showed that the decreased number of rotations produced by PSNL in the dark phase was abolished by continuously administered BEO (**Figure 4C**). The effect of BEO on the running wheel activity in mice with PSNL was antagonized by naloxone hydrochloride (**Figure 4D**). Even when the sample was increased, the number of rotations in mice with PSNL-jojoba wax was decreased in the dark phase from the 8th day after surgery. However, this decrease was significantly reduced by the continuous administration of BEO (**Figure 4E**). The effect of BEO was significantly antagonized 2 days after co-administration with naloxone hydrochloride (12 days after surgery; PSNL-jojoba wax: 972.00 ± 543.95, PSNL-BEO: 4,070.33 ± 693.64, PSNL-BEO/Naloxone: 1,431.33 ± 287.24) (**Figure 4E**). These results indicate that the continuous administration of BEO suppressed the decrease in the running wheel activity that was observed in PSNL mice *via* opioid receptors.

Single Subcutaneous Injection of BEO Suppressed the Increased Two-Dimensional Planar Motion but Did Not Affect the Running Wheel Activity in PSNL Mice

Next, we examined whether the behavioral and pharmacological changes induced by the continuous administration of BEO in PSNL mice could be reproduced by a single injection. To examine the effect of a single subcutaneous administration of BEO on a two-dimensional planar movement, the administration was performed at 10 am on the 5th day after surgery. Representative data showed that the increase in the two-dimensional planar motor counts in PSNL mice during the light phase was temporarily suppressed by a single subcutaneous injection of BEO (**Figure 5A**). In the latter half of the light period, the medicinal properties of BEO disappeared and the two-dimensional planar behavior increased again (**Figure 5A**). The total number of the two-dimensional planar counts during the light phase on the 5th day after surgery in PSNL mice was significantly reduced with a single subcutaneous injection of BEO (PSNL: 1,521.44 ± 214.61, PSNL-BEO: 888.77 ± 234.21) (**Figure 5B**).

On the other hand, to examine the effect of a single subcutaneous injection of BEO on the running wheel activity, the injection was performed at 18 o'clock on the 11th day after

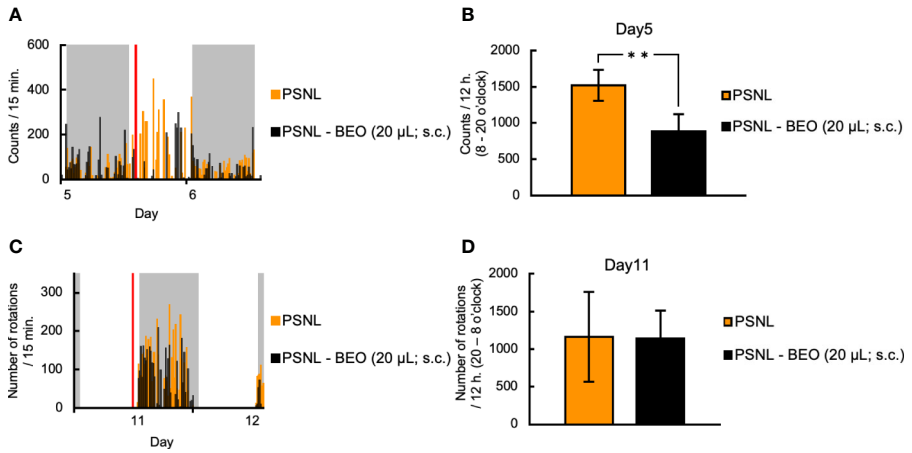

FIGURE 5 | Assessment of behavioral activities in PSNL mice with a single subcutaneous injection of BEO. **(A, B)** Single subcutaneous injection of BEO in PSNL mice was performed at 10 o'clock on the 5th postoperative day. **(A)** Representative data of two-dimensional planar motion every 15 min from 5 to 6 days in PSNL mice with a single subcutaneous administration of BEO. **(B)** Assessment of two-dimensional planar motion during the light period (8 o'clock–20 o'clock) in PSNL mice with a single subcutaneous administration of BEO on the 5th postoperative day. **(C, D)** Single subcutaneous injection of BEO in PSNL mice was performed at 18 o'clock on the 11th postoperative day. **(C)** Representative data of running wheel activity every 15 min from 11 to 12 days in PSNL mice with a single subcutaneous administration of BEO. **(D)** Assessment of running wheel activity during the dark period (20 o'clock–8 o'clock) in PSNL mice with a single subcutaneous administration of BEO on the 11th postoperative day. **(B, D)** Values are the means ± S.E.M. (PSNL: n = 9, PSNL-BEO [20 µl; s.c.]: n = 13). **(B)** $^{**}P < 0.01$ (Student's t-test).

surgery. Representative data showed that the running wheel activity in the dark phase of PSNL mice was not affected by a single subcutaneous injection of BEO (**Figure 5C**). The total number of rotations during the dark phase of PSNL mice was also unaffected by a single subcutaneous injection of BEO (PSNL: 1,160.50 ± 597.17, PSNL-BEO: 1,145.11 ± 367.81) (**Figure 5D**).

Inhalation of BEO Did Not Affect the Two-Dimensional Planar Motion and Running Wheel Activity in PSNL Mice

To clarify the behavioral and pharmacological effects of BEO inhalation, we investigated using a double activity monitoring system®. To examine the effect of a single inhalation of BEO on two-dimensional planar movement, the mice underwent inhalation for 1 h from 10 am on the 5th day after surgery. To confirm activation of the olfactory signal by inhalation of BEO, olfactory bulbs were sampled from PSNL mice immediately after inhalation of BEO, and fluorescent immunostaining for c-fos, a nerve activity marker, was performed. Representative images of fluorescent immunostaining confirmed that c-fos expression had increased and the olfactory signal was enhanced in this group compared to the group without inhalation (**Figure 6A**). When comparing the two-dimensional planar movement under these conditions, representative data showed that the two-dimensional planar movement in the light period of PSNL mice was not affected by the inhalation of BEO (**Figure 6B**). The total number of two-dimensional planar counts during the light phase on the 5th day after surgery in PSNL mice was not affected by the single inhalation of BEO (PSNL: 1,521.44 ± 214.61, PSNL-BEO inhalation: 1,245.63 ± 129.74) (**Figure 6C**).

On the other hand, to examine the effect of a single inhalation of BEO on the running wheel activity, inhalation was performed at 18 o'clock on the 11th day after surgery. Representative data showed that the running wheel activity in the dark phase of PSNL mice was not affected by a single inhalation of BEO (**Figure 6D**). The total number of rotations during the dark phase of PSNL mice was also unaffected by a single inhalation of BEO (PSNL: 1,160.50 ± 597.17, PSNL-BEO inhalation: 1,103.50 ± 301.09) (**Figure 6E**).

Inhalation of BEO Did Not Affect Mechanical Allodynia in PSNL Mice

Finally, we examined the effect of inhalation of BEO on mechanical allodynia in PSNL mice. The von Frey test showed that inhalation of BEO at 10 o'clock on days 7, 14, and 21 at a dose of 0.004, 0.04, 0.4, 4, 40, 400 µl did not affect mechanical allodynia (**Figures 7A–C**).

DISCUSSIONS

This study aimed to elucidate whether the analgesic action of BEO in PSNL mice is mediated by olfactory stimulation by its volatile components. We conducted behavioral pharmacology studies using the double activity monitoring system®, which can simultaneously detect the two-dimensional planar motion and running wheel activity at the same time. Besides, BEO was continuously administered subcutaneously with an osmotic pump to eliminate the effects of scent as much as possible.

Few papers regarding behavioral experiments in mice have measured two-dimensional planar motion and running wheel activity at the same time, except for those using the double activity monitoring system®. However, many papers have measured each of these independently.

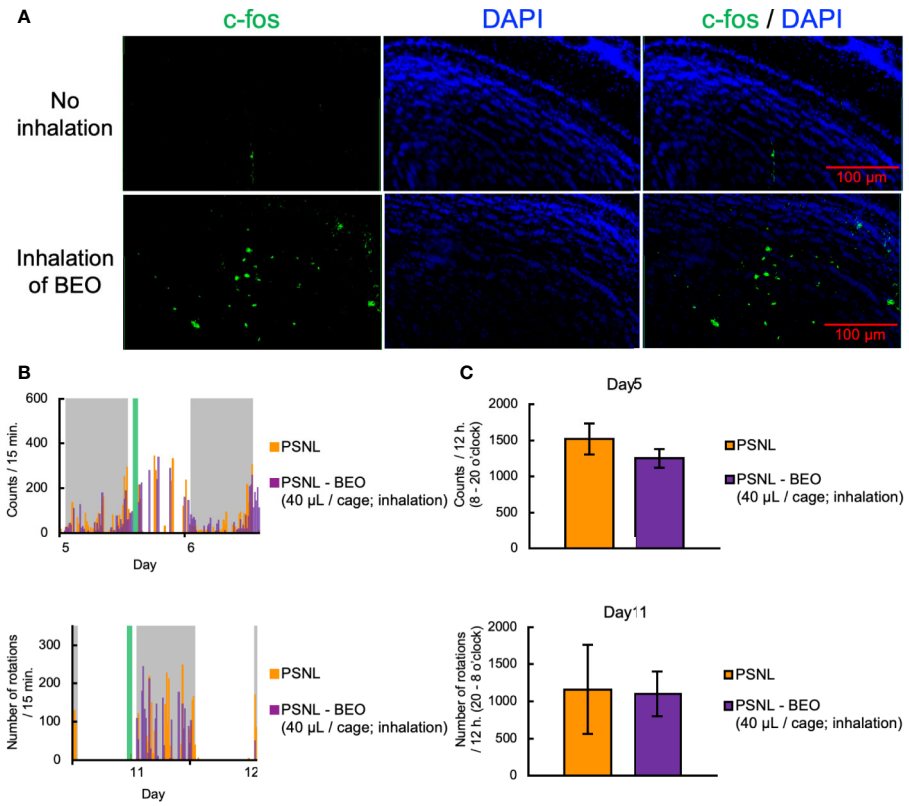

FIGURE 6 | Assessment of behavioral activities in PSNL mice with inhalation of BEO. **(A–C)** Inhalation of BEO by PSNL mice was performed from 10 o'clock to 11 o'clock on the 5th postoperative day. **(A)** Fluorescent immunostaining images of c-fos protein in the olfactory bulb in PSNL mice with inhalation of BEO or no inhalation. **(B)** Representative data of two-dimensional planar motion every 15 min from 5 to 6 days in PSNL mice with inhalation of BEO. **(C)** Assessment of two-dimensional planar motion at the light period (8 o'clock–20 o'clock) in PSNL mice with inhalation of BEO on the 5th postoperative day. **(D, E)** Inhalation of BEO in PSNL mice was performed from 18 o'clock to 19 o'clock on the 11th postoperative day. **(D)** Representative data of running wheel activity every 15 min from 11 to 12 days in PSNL mice with inhalation of BEO. **(E)** Assessment of running wheel activity at the dark period (20 o'clock–8 o'clock) in PSNL mice with inhalation of BEO on the 11th postoperative day. **(C, E)** Values are the means ± S.E.M. (PSNL: n = 9, PSNL-BEO [40 μl/cage; inhalation]: n = 8).

Two-dimensional planar motion is commonly used in "open field tests" and "home cage activity tests" to assess locomotor activity. An open field test is a test that measures spontaneous activity in a new environment (Streng, 1971). On the other hand, a home cage activity test is a test that measures spontaneous activity in the home cage over a medium to long term period (Miyakawa et al., 2003). The ability to capture mouse activity patterns over time can be used to analyze circadian rhythms. Regarding the circadian rhythms of PSNL mice, we reported that the paw withdrawal threshold in PSNL mice fluctuated over about 24 h, with the peak of allodynia persisting from the late light phase to the early dark phase (Kusunose et al., 2010). During the evaluation of the two-dimensional planar motion in this study, using the double activity monitoring system®, a peak of abnormal behavior count was observed from 19 o'clock to 20 o'clock, just before the dark period in PSNL mice (**Figure 2B**), which indicates that allodynia symptoms were reflected. On the other hand, the theoretical sustained elimination period of BEO initiated by an osmotic pump at the same time of PSNL surgery is 7 days, and the increased counts of PSNL of the light phase was abolished over the 14-day measurement period (**Figures 2A, D, F**). On comparing mechanical allodynia with the von Frey test, the effect of BEO disappeared at 7 days (data not shown in figures). The PSNL mice showed transient mechanical allodynia with the von Frey test, returning to baseline in the sham-operated group at 42 days post-operative, while two-dimensional planar activity did not return to baseline (data not shown in figures). These results suggest that the changes in the two-dimensional planar activity may reflect factors other than allodynia in PSNL mice. Concerning this phenotype, we considered whether sleep is involved. It has been reported that PSNL mice showed decreased non-rapid eye movement sleep (NREM) and sleep duration in the light phase compared to sham-operated mice (Takemura et al., 2011; Wang et al., 2015). Also, certain essential oils, such as bergamot, have been shown to have a hypnotic effect in humans (Lillehei and Halcon, 2014). Therefore, it was considered that the evaluation of the two-dimensional planar motion using the double activity monitoring system® can obtain a phenotype that reflects on sleep and allodynia symptoms.

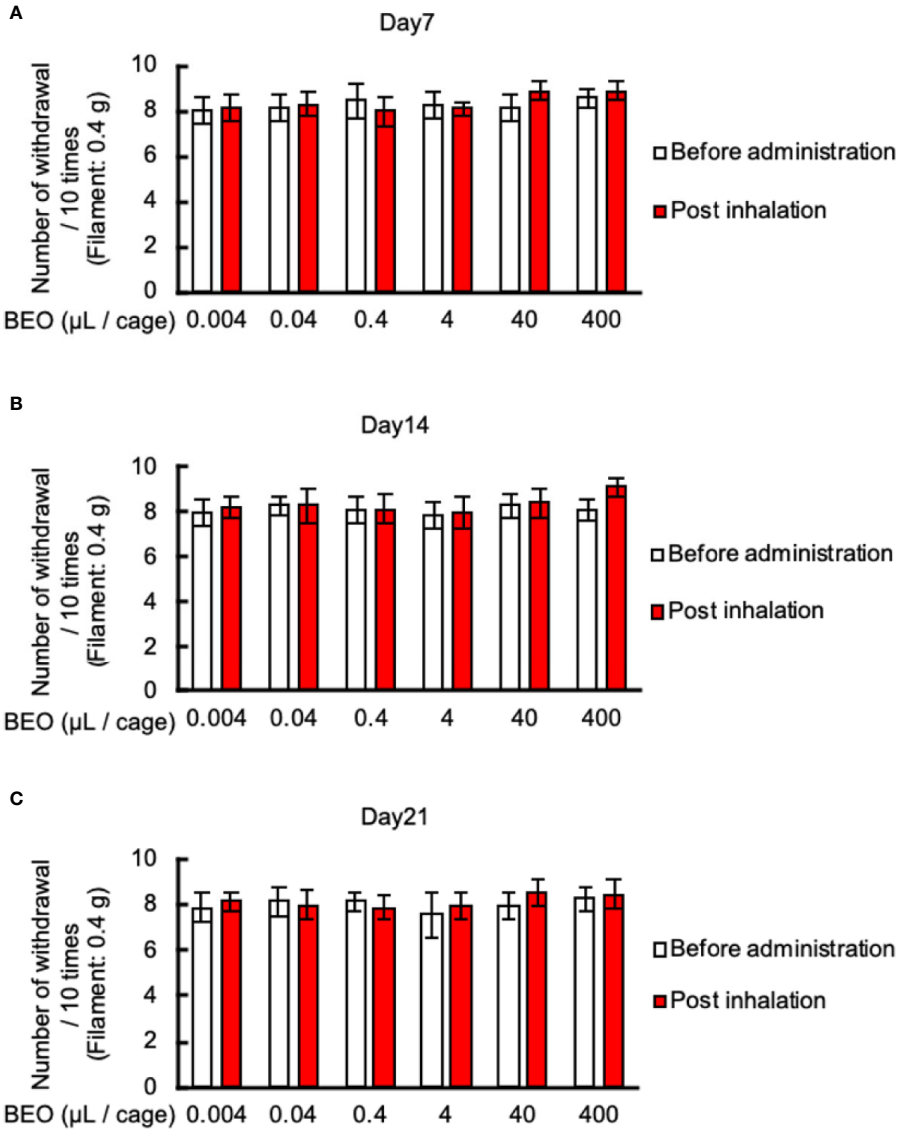

FIGURE 7 | Effects of BEO inhalation on mechanical allodynia in PSNL mice. **(A–C)** Inhalation of BEO by PSNL mice was performed from 10 o'clock to 11 o'clock. After that, mice were removed from the glass cages, and the von Frey test was performed. Effect of BEO inhalation on mechanical allodynia in PSNL mice on the 7th **(A)**, 14th **(B)**, and 21st **(C)** day after PSNL mouse surgery (non-ligation side of Day 7: 1.50 ± 0.23, Day 14: 1.35 ± 0.27, Day 21: 1.61 ± 0.20). Values are the means ± S.E.M. for all experiences (BEO 0.04 μl/cage: n = 8, other groups: n = 7).

Regarding the results of two-dimensional planar motion in PSNL mice, the number of counts in the light phase increased (**Figure 2D**) but not in the dark phase (**Figure 2C**). This decrease during the dark period may be due to the rebound effect of increased activity during the light period (sleep period).

The effect of BEO on the two-dimensional planar counts in mice with PSNL was antagonized by naloxone hydrochloride (**Figure 3B**). We performed a continuous administration experiment of the selective μ-opioid receptor antagonist β-FNA and the selective κ-opioid receptor antagonist norbinaltorphimine, instead of the nonspecific opioid receptor antagonist, naloxone hydrochloride. However, at present, it could not be measured well due to the decrease in the number of two-dimensional planar activities during the day and night (sedative effect) (data not shown in figure). In any case, we had previously reported that PSNL mouse-induced allodynia is suppressed by an intra-plantar injection of BEO (Komatsu et al., 2018). This inhibitory effect of BEO on PSNL-induced allodynia is also antagonized by β-FNA but not by naltrindole and norbinaltorphimine (Komatsu et al., 2018). These results suggest that the increased counts produced by PSNL, that were abolished by the continuous administration of BEO in the light phase, may be triggered by activation of peripheral μ-opioid receptors.

The voluntary wheel-running activity is commonly used to assess circadian rhythm or motor function and has been

proposed as an observer-independent measure for ongoing pain in inflammatory models (Cobos et al., 2012; Kandasamy et al., 2016). In the neuropathic pain model, it has been reported that the running wheel mileage decreases in spared nerve injury (SNI) model mice (Pitzer et al., 2016), which is almost in agreement with our results in PSNL mice (**Figure 4B**).

We consider that the decrease in the wheel-running activity in PSNL mice was not due to changes in circadian rhythm phases, but due to reduced motivation from chronic pain. Concerning changes in circadian rhythm, the typical wheel-running activity occurred only in the dark phase for both PSNL and sham-operated mice (**Figure 4A**). In the previous report, the expression phase of various clock genes on day 7 after surgery in PSNL mice was unchanged (Koyanagi et al., 2016). This finding supports that reduced running wheel activity in PSNL mice does not reflect changes in the rhythmic phase.

It is widely known that anxiety and depression are secondary to chronic allodynia. A previous report demonstrated that in the 4th week after surgery, PSNL caused significant depression-like behavior in mice, evaluated using the forced swimming test (FST) and the tail suspension test (TST), which was accompanied by increased pain sensitivity (Gai et al., 2014). The delayed appearance of decreased motivation associated with pain may contribute to the explanation that a decrease in running wheel activity in PSNL mice has a time lag from the 8th day after surgery. Our study showed that a single dorsal subcutaneous injection of BEO did not affect the running wheel activity in PSNL mice (**Figures 5C, D**) and that continuous administration of BEO by osmotic pump corrected the decrease in PSNL mice (**Figures 4C–E**). These results suggest that BEO's effects on the running wheel activity are mediated by something that is not an opioid receptor but is important in analgesia and two-dimensional planar behavior. It was reported that altered serotonergic (5-HT) neurotransmission is implicated in the antidepressant and anxiolytic properties of physical activity, assuming that the running wheel activity in the double activity monitoring system reflects the pathology of anxiety and depression (Greenwood et al., 2005). We had recently reported that BEO exerts anxiolytic effects by correcting the neuronal activity of the serotonin nervous system *via* the serotonin 5-HT_{1A} receptor (Rombola et al., 2020). In light of the previous reports, continuous administration of BEO did not reduce motivation due to the early analgesic action mediated by opioid receptors, and the anxiolytic action mediated by serotonin 5-HT_{1A} receptors. These effects are considered to be why the reduction in running wheel activity by PSNL mice did not occur after the 8^{th} postoperative day when the release of BEO by the osmotic pump was theoretically completed. On the other hand, a single subcutaneous injection of BEO is expected to increase running wheel activity due to the anxiolytic effect mediated by the serotonin 5-HT_{1A} receptor. However, even in the previous paper, a single injection of BEO did not obtain sufficient anxiolytic effect. Therefore, these findings are consistent with those of the present study in which a single-dose was not efficacious. Based on the above, the running wheel activity in the double activity monitoring system may be decreased in PSNL mice due to anxiety and depression secondary to chronic allodynia pathology. It is considered that recovery by BEO is due to the regulation of the 5-HT_{1A} receptor function.

We have reported that among the constituents of BEO, linalool is important in analgesia (Kuwahata et al., 2009; Sakurada et al., 2009; Sakurada et al., 2011; Kuwahata et al., 2013; Katsuyama et al., 2015). Linalool is one of the major volatile aromatic components contained in BEO. The pharmacokinetics of linalool have been reported to be excreted from urine (approximately 60%), exhaled breath (approximately 23%), and feces (approximately 15%) within 72 h after oral administration of radiolabeled linalool to rats (Parke et al., 1974). The primary metabolite of linalool is in the form of a glucuronide conjugate and is known to be excreted in the feces (Antoine et al., 1993). It is assumed that the metabolites excreted in the feces also have a scent. Therefore, at the beginning of the experiment, it was thought that the scent could be removed by an osmotic pump, but as it is excreted in feces and urine, it is challenging to remove the scent of BEO altogether. However, there are no reports that the metabolites of linalool have an analgesic effect, and it is unlikely that the scents of linalool and linalool metabolites found in manure act in all other complex scents.

In the inhalation experiment of BEO to PSNL mice, we compared the expression of c-fos, a kind of neural activity marker, by fluorescent immunostaining to confirm that the neural activity of the olfactory bulb was activated. The activation occurred only in a part of the olfactory bulb (**Figure 6A**), which was consistent with previous reports (Loch et al., 2013). Generally, when inhaling a scented substance, there are three main routes to be absorbed into the body (Lv et al., 2013). First, it enters the lungs through the respiratory tract and enters the bloodstream through the capillaries of the alveoli. Second, it is absorbed from the capillaries of the nasal mucosa and enters the bloodstream. Third, through a pathway in which it binds to an olfactory receptor of the olfactory cells of the nasal olfactory epithelium, and acts on the central nervous system as an olfactory neurotransmission signal. Of these three routes, the action of the third is produced at the lowest dose. Therefore, in this study, based on the dose (400 µl/cage) used for the reduction of formalin-induced nociceptive behavior by the inhalation of BEO, we investigated every 1/10 up to the dose of 1/100,000 on the low dose side. As a result, no effect of inhalation was found (**Figures 7A–C**) (Scuteri et al., 2018). We consider that by increasing the inhalation dose and exposure time of BEO and increasing its blood transfer, it is possible to confirm the antiallodynic effect, even in inhalation.

CONCLUSION

This study suggests that BEO exhibits an analgesic action, which is mediated by opioid receptors but not by the olfactory system. There have been several reports that aromatherapy massage relieves pain in humans. However, our results indicate that at

least in mice, olfactory receptors are not involved in the analgesia of severe pain, such as neuropathic pain. Our data also indicates that the running wheel activity in the double activity monitoring system® is useful as a model of anxiety and depression secondary to chronic allodynia.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, T.S., upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by: All experiments were performed following the approval of the Ethics Committee for Animal Experiments at Daiichi University of Pharmacy (Examination number: H30-006, approval number: 29004) and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. KH, SK, TK, KA, DS, GB, and TS designed the research. KH, SK, and TK acquired the data. KA, DS, GB, and TS supplied the experimental materials. KH and KA drafted the manuscript.

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Intrathecally Administered Apelin-13 Alleviated Complete Freund's Adjuvant-Induced Inflammatory Pain in Mice

Shuangyu Lv[1], Xiaomei Zhang[1], Yuchen Zhou[1], Yu Feng[1], Yanjie Yang[1] and Xinchun Wang[2]**

[1] Institute of Molecular Medicine, School of Basic Medical Sciences, Henan University, Kaifeng, China, [2] Key Laboratory of Clinical Resources Translation, The First Affiliated Hospital of Henan University, Kaifeng, China

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***Correspondence:**
Yanjie Yang
yangyj@henu.edu.cn
Xinchun Wang
wxc619533@sina.com

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Apelin is the endogenous ligand for APJ, a G-protein-coupled receptor. Apelin gene and protein are widely distributed in the central nervous system and peripheral tissues. The role of apelin in chronic inflammatory pain is still unclear. In the present study, a mouse model of complete Freund's adjuvant (CFA)-induced inflammatory pain was utilized, and the paw withdrawal latency/threshold in response to thermal stimulation and Von Frey filament stimulation were recorded after intrathecal (i.t.) injection of apelin-13 (0.1, 1, and 10 nmol/mouse). The mRNA and protein expression, concentration of glutamic acid (Glu), and number of c-Fos immunol staining in lumbar spinal cord (L4/5) were determined. The results demonstrated that *Apln* gene expression in the lumbar spinal cord was down-regulated in the CFA pain model. Apelin-13 (10 nmol/mouse, i.t.) alleviated CFA-induced inflammatory pain, and it exhibited a more potent antinociceptive effect than apelin-36 and (pyr)apelin-13. The antinociception of apelin-13 could be blocked by APJ antagonist apelin-13(F13A). I.T. apelin-13 attenuated the increased levels of *Aplnr*, *Grin2b, Camk2d*, and *c-Fos* genes expression, Glu concentration, and NMDA receptor 2B (GluN2B) protein expression caused by CFA. Apelin-13 significantly reduced the number of Fos-positive cells in laminae III and IV/V of the dorsal horn. This study indicated that i.t. apelin-13 exerted an analgesic effect against inflammatory pain, which was mediated by activation of APJ, and inhibition of Glu/GluN2B function and neural activity of the spinal dorsal horn.

Keywords: apelin, inflammatory pain, NMDA receptor, spinal cord, Fos

INTRODUCTION

Apelin, an endogenous peptide, was identified as the natural ligand of the orphan receptor APJ (Tatemoto et al., 1998). The APJ (putative receptor protein related to the type 1 angiotensin receptor) is a G-protein-coupled receptor (GPCR) and was cloned from a human genomic library cDNA in 1993 (O'Dowd et al., 1993). Apelin was initially isolated from bovine stomach extracts and was given the name apelin (Tatemoto et al., 1998). The human *Apln* gene is located on chromosome Xq25-26 and has 3 exons and 2 introns (Lee et al., 2000). Apelin is generated from a 77-amino-acid precursor, named preproapelin, which can be hydrolyzed by endopeptidases into several active biological fragments, including apelin-36 (apelin42–77), apelin-17 (apelin61–77), apelin-16 (apelin62–77), apelin-13 (apelin65–77), and apelin-12 (apelin66–77) and pyroglutaminated apelin-13 [(pyr)apelin-13] (Kawamata et al., 2001). Preproapelin contains N and C termini with potential proteolytic cleavage sites, and the sequence of 23 amino acids between tryptophan 55 and phenylalanine 77 is fully conserved among different species (Medhurst et al., 2003). Among these, apelin-13 is the most potent activator for APJ expressed in cell lines, and apelin-13 and apelin-36 are the most widely studied (Tatemoto et al., 1998; Habata et al., 1999; Kawamata et al., 2001).

The apelin/APJ system is involved in a broad range of physiological functions and pathological processes, including cardiovascular function (Wysocka et al., 2018; Esmaeili et al., 2019), cardiac contractility (Zhong et al., 2017; Esmaeili et al., 2019), angiogenesis (Wu et al., 2017; Cheng et al., 2019), energy metabolism (Bertrand et al., 2015; Castan-Laurell et al., 2019), liver diseases (Principe et al., 2008; Lv et al., 2017), ischemia/reperfusion injury (Yang et al., 2015; Chen et al., 2016) and cancer (Yang et al., 2016a; Masoumi et al., 2020). Recent studies were focused on the role of apelin in psychosis and neuropathy (Lv et al., 2020). Xiao et al. (2018) demonstrated that intrahippocampal infusion of apelin-13 (1-4 μg/rat) exhibited an anti-depressive effect in forced swim test. Chronic intracerebroventricular (i.c.v.) administration of apelin-13 (2 μg/d) alleviated chronic stress-induced depression-like phenotypes by ameliorating hypothalamic–pituitary–adrenal (HPA) axis and hippocampal glucocorticoid receptor dysfunction in rats (Dai et al., 2018). Peripheral injection of apelin-13 induced anxiolytic activity in a mouse model of chronic normobaric hypoxia by suppressing the nuclear factor κB (NF-κB) pathway, and silent mating type information regulation 2 homolog 1 (SIRT1) was involved in this process (Fan et al., 2017; Fan et al., 2018). In addition, the apelin/APJ system had a protective effect on memory impairment (Li et al., 2016; Haghparast et al., 2018), ischemic stroke (Yang et al., 2016b; Wu et al., 2018), and brain damage (Bao et al., 2015; Chu et al., 2016). However, the role of apelin in inflammatory pain is still unclear.

Apelin/APJ gene and protein are abundantly distributed in the central nervous system (CNS) and peripheral tissues in humans and rodents. In the periphery, apelin and APJ were expressed in lungs, heart, kidneys, stomach and intestine (Hosoya et al., 2000; Lee et al., 2000; O'Carroll et al., 2000). In the CNS, they were found in the spinal cord, dorsal raphe nucleus, amygdala and hypothalamus (Hosoya et al., 2000; O'Carroll et al., 2000; Reaux et al., 2001), which are the major regions closely related to pain intensity. The distribution pattern suggests that the apelin/APJ system plays a potential role in regulating pain. Our previous studies showed that i.c.v. administration of apelin-13 (0.5, 1 and 3 μg/mouse) induced an antinociceptive effect in a visceral pain model using acetic acid-induced writhing test (Lv et al., 2012), and apelin-13 infused intrathecally (i.t.) induced an antinociceptive effect in an acute pain model using tail immersion test (Lv et al., 2013). Chronic inflammatory pain is a common clinical disease characterized by persistent spontaneous pain and hyperalgesia. It is necessary to explore the regulatory effect of apelin/APJ on chronic inflammatory pain.

Infusion of complete Freund's adjuvant (CFA) into rodents' hind paw is considered to be a valid model to investigate the mechanism of chronic inflammatory pain, as well as to screen for anti-inflammatory hyperalgesic drugs (Beyer et al., 1997; Sun et al., 2012). The present study was designed to study the effect and mechanism of i.t. treatment with apelin on chronic inflammatory pain using a model induced by intraplantar injection of CFA, real-time quantitative polymerase chain reaction (qPCR), western blotting, high performance liquid chromatography (HPLC), and immunohistochemistry.

MATERIALS AND METHODS

Animals

Male Kunming mice (aged 6–8 weeks) were supplied by the Animal Center of Henan Province (Zhengzhou, China). The animals were housed under controlled conditions with a 12:12 h light/dark cycle with food and water available *ad libitum*. Behavioral testing was performed from 09:00 to 15:00 h in a quiet room after animals had been acclimatized to the environment for at least 30 min. The animal experimental protocol was approved by the Committee of Medical Ethics and Welfare for Experimental Animals, Henan University School of Medicine. All efforts were made to minimize animal suffering and the number of the animals used in the following experiments.

Chemicals and I.T. Injection

Apelin-13, (pyr)apelin-13, apelin-36, and apelin-13(F13A) were purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China).

Abbreviations: CFA, complete Freund's adjuvant; CNS, central nervous system; Creb1, cAMP-response element binding protein 1; Dr1, down-regulator of transcription 1; Egr1, Early growth response 1; FLI, c-Fos-like immunoreactive; Fos, FBJ osteosarcoma oncogene; Glu, glutamic acid; GPCR, G protein-coupled receptor; HPA, hypothalamic–pituitary–adrenal; HPLC, high performance liquid chromatography; i.c.v., intracerebroventricular; i.t., intrathecal; NR2B, NMDA Receptor 2B; NF-κB, nuclear factor κB; NMDARs, N-methyl-D-aspartate receptors; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; RT-qPCR, real-time quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate- poly acrylamide gel electrophoresis; SIRT1, silent mating type information regulation 2 homolog 1.

The peptides were dissolved and diluted in physiological saline before treatment. The CFA was supplied by Sigma–Aldrich (St. Louis, MO, USA).

To evaluate the roles of spinal apelin in regulating inflammatory pain, peptide or antagonist was administered by i.t. delivery, as described by Hylden and Wilcox (Hylden and Wilcox, 1980). A stainless needle connected to a Hamilton microsyringe (25 μl) was inserted into the L5/6 intervertebral space in conscious mice. The reflexive lateral flick of the tail or formation of an S shape by the tail indicated successful treatment. The drug solution or vehicle was delivered in a total volume of 5 μL for 10 s. The correctness of the i.t. injection site was confirmed by injecting an appropriate volume of methylene blue solution (1.0%).

Behavioral Analysis

To induce a model of inflammatory pain, CFA was injected into the plantar surfaces of hind paws of the mice in a volume of 10 μL. The vehicle control group was injected with an equivalent volume of saline. The pain sensitivity was determined blindly as previous report (Wang et al., 2015). Heat hyperalgesia was assessed by evaluating the paw withdrawal latency (PWL) in response to a radiant heat source. The mice were placed in plastic boxes and allowed to be acclimatized for 30 min before the test. PWL was tested using the Thermal Stimulator System (PL-200; TaiMeng Technology Corporation, Chengdu, China). The animals were placed on a clear glass plate, and a radiant heat stimulus using a projector lamp bulb (8 V, 50 W) was focused on the plantar surface of each hind paw, with a cutoff of 10 s. The duration between the start of heat application and paw withdrawal was calculated as the PWL.

Mechanical allodynia was assessed by analyzing 50% paw withdrawal threshold (PWT) in response to Von Frey filament (Stoelting Co., Wood Dale, IL, USA) stimulation. Mice were put in boxes on an elevated metal mesh floor and allowed to habituate for 30 min before the test. The Von Frey filaments were applied perpendicularly to the central region of the plantar surface of one hind paw until the filaments were bent. The 50% PWT was measured using Dixon's up–down method.

Real-Time qPCR

Total RNA was extracted from lumbar spinal cord (L4/5) using TRIzol reagent. RNA was quantified by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (0.5 μg) from each mouse sample was reverse transcribed to synthesize cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The mRNAs level of apelin (*Apln*), apelin receptor (*Aplnr*), NMADR2A (*Grin2a*), NMDAR2B (*Grin2b*), CamK II (*Camk2d*), FBJ osteosarcoma oncogene (*Fos*), cAMP-response element binding protein 1 (*Creb1*), down-regulator of transcription 1 (*Dr1*), and Early growth response 1 (*Egr1*) were measured by real-time qPCR. Real-time PCR was carried out using the 7500HT Thermal Cycler and SYBR Green Master Mix (Applied Biosystems). Primers used were supplied by Wuhan Protein Interaction Bio Co. Ltd. (Wuhan, China), and they were designed as described previously (Kasai et al., 2010; Ze et al., 2014). Dissociation curve analysis was completed after each real-time qPCR. Ct values of mRNA expression of targeted genes were normalized to *36B4*. The relative fold change of target genes was analyzed by the equation $2^{-\Delta\Delta Ct}$. Sequences of the primers used for real-time qPCR was listed in **Table 1**.

Western Blotting

The mouse L4/5 spinal segments were dissected out and homogenized with RIPA lysis buffer in the presence of protease inhibitor (Beyotime, Shanghai, China). After centrifugation of the lysates (14,000 *g*, 10 min at 4°C), the protein concentration was determined by Bicinchoninic Acid Protein Assay Kit (Beyotime). Protein samples were loaded into each well, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 40 min at 120 V, and then blotted onto polyvinylidene fluoride membranes for 70 min at 120 V. The blots were blocked with 5% milk at room temperature for 1 h, and then membranes were incubated with the rabbit antibodies against N-methyl-D-aspartate receptor (NMDAR)2A and NMDAR2B (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. Thereafter, they were incubated with horseradish-peroxidase-conjugated secondary antibody (Proteintech, Wuhan, China) for 1 h. The relative intensities of target proteins were normalized using β-actin (1:1000, Beyotime) as an internal control. The membranes were incubated with enhanced chemiluminescent substrates (Thermo Scientific, Wilmington, DE, USA) and detected using automatic multifunction chemiluminescent detection system (Tanon, Shanghai, China). The protein levels were assessed by densitometry using Image-J Software.

HPLC

The lumbar spinal cord (L4/5) was isolated and homogenized with cold PBS. The protein was precipitated by methanol, and the supernatants were obtained after centrifugation (10,000 *g*,

TABLE 1 | Primer sequence used for RT-qPCR.

Primers name	Primer sequence	Size (bp)
Apln-F	5'- GTTGCAGCATGAATCTGAGG-3'	247
Apln-R	5'- CTGCTTTAGAAAGGCATGGG-3'	
Aplnr-F	5'-CCACCTGGTGAAGACTCTCTACA-3'	110
Aplnr-R	5'- TGACATAACTGATGCAGGTGC-3'	
Grin2a-F	5'-ATGAACCGCACTGACCCTAAG-3'	246
Grin2a-R	5'-GGCTTGCTGCTGGATGGA-3'	
Grin2b-F	5'-AATGTGGATTGGGAGGATAGG-3'	255
Grin2b-R	5'-ATTAGTCGGGCTTTGAGGATACT-3'	
Camk2d-F	5'- AGAAGTTCAAGGCGACCAGCA -3'	150
Camk2d-R	5'- GGGTATCCCACCAGCAAGATGTAG -3'	
c-Fos-F	5'-GGTGAAGACCGTGTCAGGAGGCAG-3'	117
c-Fos-R	5'-GCCATCTTATTCCGTTCCCTTCGG-3'	
Creb1-F	5'-TACGGATGGGGTACAGGGC -3'	197
Creb1-R	5'-CAATGGTGCTCGTGGGTG -3'	
Dr1-F	5'-CTGGGAGTGGTGTCCCTAGA-3'	479
Dr1-R	5'-GCCCAAACTTTCCAGTGCTTG-3'	
Egr1-F	5'-GAGCACCTGACCACAGAGTC-3'	172
Egr1-R	5'-AAAGGGGTTCAGGCCACAAA-3'	
36B4-F	5'-CGACCTGGAAGTCCAACTAC-3'	109
36B4-R	5'-ATCTGCTGCATCTGCTTG-3'	

F, forward; R, reverse.

15 min). The content of Glu was detected by Qiangdao Sci-tech Innovation Testing Limited Company (Qingdao, China). The samples were analyzed using LC-10A HPLC system (Shimadzu, Japan), with L-homoserine as an internal standard. Reversed phase column (ODS-C18, 250×4 mm) was used at 38°C. The excitation wavelength and emission wavelength of the fluorescent detector were set at 340 nm and 450 nm, respectively. The mobile phase was methanol: water (50: 50) at a flow rate of 1.0 mL/min. The samples were mixed with L-homoserine and o-phthalaldehyde for 2 min, and the injection volume was 10 μL.

Immunohistochemistry

After the behavior test, mice were immediately anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneally). Animals were perfused transcardially and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Each spinal cord was instantly removed, fixed with the above fixative overnight, and then embedded in paraffin. Transverse sections (5 μm thick) of the L4/5 spinal cord were cut using a vibratome (Leica Biosystems, Nussloch, Germany), and stained as previously described (Bao et al., 2017). Serial sections were blocked in 10% normal goat serum at room temperature for 1 h. A rabbit anti-c-Fos antibody (1:100, Abcam Inc., Burlingame, CA, USA) was applied and sections were incubated overnight at room temperature. Following incubation with a biotinylated secondary antibody (Proteintech, Wuhan, China) for 2 h, all sections were processed with the avidin–biotin–peroxidase complex (Corning Inc., Corning, NY, USA) for 30 min. The results of the immunostaining were revealed by 3,3-diaminobenzidine kit (ZSGB-bio, Beijing, China). To calculate in detail, spinal dorsal horn was divided into three areas, including laminae I/II (superficial dorsal horn), laminae III/IV (nucleus proprius), and laminae V/VI (neck of the dorsal horn). These areas were selected because they are crucial for nociceptive transmission in the dorsal horn (Besson and Chaouch, 1987). The left (ipsilateral) side of each section was used for data analysis, and the quantity of c-Fos-like immunoreactive (FLI) cells was assessed by Image-J.

Data Analysis

All values are expressed as mean ± SEM. Data were analyzed using one-way analysis of variance followed by Dunnett's test for *post hoc* comparisons. A two-tailed un-paired Student's *t* test was performed to evaluate the difference between the two groups. The level of significance was set at $p < 0.05$.

RESULTS

Relative *Apln* mRNA Level Was Decreased in CFA-Treated Mice

To examine the possible changes of *Apln* gene between CFA-induced inflammatory pain model and vehicle control, the tissues, including L4/5 spinal cord, prefrontal cortex, hippocampus, and hypothalamus, were collected and mRNAs were determined. As shown in **Figure 1**, *Apln* mRNA was significantly decreased in L4/5 spinal cord of the CFA-induced inflammatory hyperalgesia mouse model, compared with the vehicle control ($p < 0.05$). However, *Apln* mRNA in prefrontal cortex ($p = 0.505$) or hypothalamus ($p = 0.936$) was not changed.

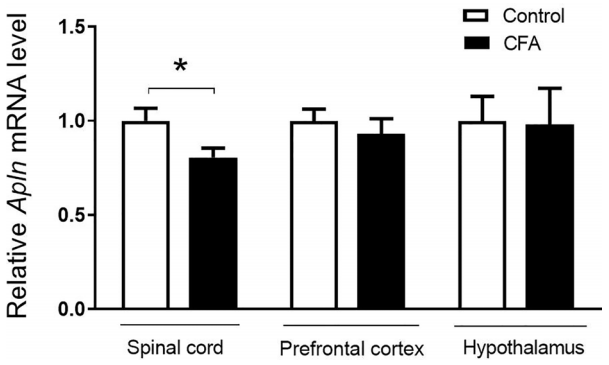

FIGURE 1 | *Apln* gene expression in the CFA-induced inflammatory pain in mice. mRNA levels in L4/5 spinal cord, prefrontal cortex and hypothalamus were detected by real-time qPCR, normalized with the housekeeping gene *36B4*. Data are expressed as means ± SEM. n = 8 or 9 per group. *$p < 0.05$, compared with vehicle control.

I.T. Application of Apelin Alleviated Inflammatory Pain

To explore the effect of apelin-13 on inflammatory pain, CFA was injected subcutaneously into the plantar surface of the left hind paws of mice, and the responses to painful stimuli were detected. As expected, CFA injection induced hypersensitivity, which presented as reduced PWL in response to thermal stimuli (**Figure 2A**) and reduced PWT of mice in response to mechanical stimuli (von Frey assay) (**Figure 2B**). Apelin or saline were i.t. injected at 24 h after CFA treatment, and dose of apelin-13 was selected according to the previous report (Lv et al., 2013). Apelin-13 at the dose of 10 nmol/mouse significantly elevated the PWL of CFA-injected mice at 15, 30 and 45 min (each $p < 0.05$), and increased PWT values at 15 and 30 min (each $p < 0.05$). In addition, 1 nmol apelin-13 increased the PWL and PWT values of CFA-injected mice at 30 min (each $p < 0.05$). These results indicate that i.t. apelin-13 ameliorated CFA-induced hypersensitivity in response to mechanical and thermal stimuli in mice.

To compare the analgesic effect of different fragments of apelin, apelin-36, apelin-13 and (pyr)apelin-13 were i.t. administered at 10 nmol/mouse, and the nociceptive response was evaluated. Our result demonstrated that apelin-36, apelin-13 and (pyr)apelin-13 produced an obvious increase in PWL values of CFA-injected mice at 15 min (each $p < 0.05$), indicating an antinociceptive effect of these three forms of apelin (**Figure 2C**). Among them, apelin-13 exhibited the most potent analgesic effect in the CFA-induced inflammatory pain mouse model.

APJ Was Involved in Antinociceptive Effect of I.T. Apelin-13

To verify whether APJ was involved in the analgesic effect of apelin-13, the specific APJ antagonist apelin-13(F13A) was

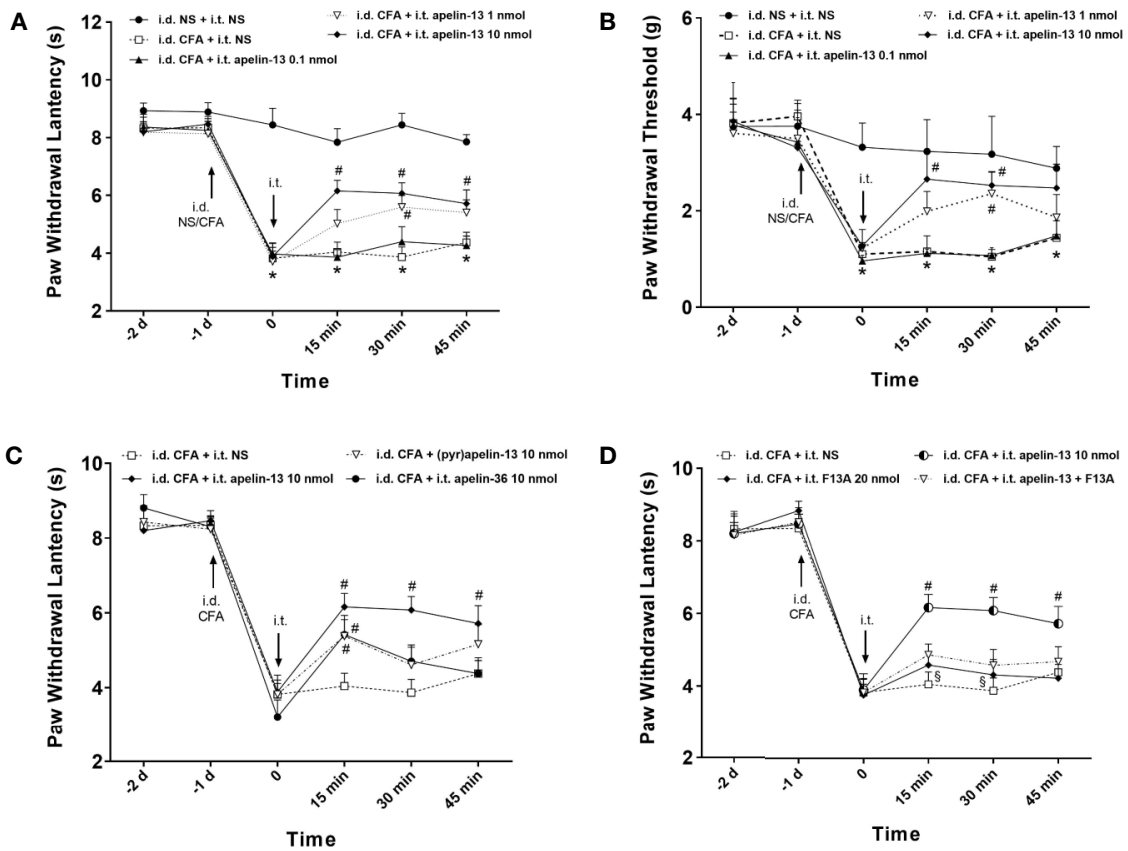

FIGURE 2 | I.T. injection of apelin alleviated the inflammatory pain induced by intradermal injection of CFA in mice. Normal saline (NS) was used as a control. The upward and downward arrows indicate the time points when i.d. and i.t. injection were administered. **(A, B)** Effect of apelin-13 (0.1, 1 and 10 nmol/mouse, i.t.) on PWL in response to thermal stimulation and PWT in response to Von Frey filament stimulation. **(C)** The effect of i.t. application of 10 nmol apelin-13, (pyr)apelin-13 and apelin-36 on PWL. **(D)** Effect of APJ receptor antagonist apelin-13(F13A) (20 nmol/mouse) on antinociception of (pyr)apelin-13 (10 nmol/mouse). Data are expressed as means ± SEM. n = 6–10 per group. $^{*}p < 0.05$, compared with vehicle control (i.d. NS + i.t. NS); $^{\#}p < 0.05$, compared with CFA group (i.d. CFA + i.t. NS); $^{\S}p < 0.05$, compared with apelin-13-treated group (i.d. CFA + i.t. apelin-13). F13A, apelin-13(F13A).

selected. Apelin-13(F13A) (i.t., 20 nmol/mouse) had no influence on the PWL values of CFA-injected mice (**Figure 2D**). However, it significantly blocked the increased PWL values induced by apelin-13 (10 nmol/mouse) in CFA-treated mice at 15 min and 30 min (each $p < 0.05$, compared with apelin-13 treated group). These results indicated that the inhibitory effect of apelin-13 on CFA-induced hyperalgesia was mediated by APJ.

To further determine whether apelin-13 influence *Aplnr* gene expression, the relative *Aplnr* mRNA level in lumbar (L4/5) spinal cord was detected. As shown in **Figure 3A**, the *Aplnr* mRNA level significantly reduced in CFA treated mice ($p < 0.05$, compared with control), which was significantly reversed by i.t. apelin-13 (compared with apelin-13 treated group), suggesting the involvement of APJ in the antinociception of apelin-13.

Apelin-13 Reduced *Grin2b* and *Camk2d* mRNAs in Mice With CFA-Induced Inflammatory Pain Model

To explore whether the key molecules, NMADR2A (NR2A), NMDAR2B (NR2B) and CamK II were involved in pain transmission for apelin-13, the relative mRNAs level of *Grin2a*, *Grin2b*, and *Camk2d* in the mouse lumbar (L4/5) spinal cord were determined. The results demonstrated that CFA or apelin-13 did not affect *Grin2a* gene expression ($p = 0.662$, compared with control; $p = 0.331$, compared with CFA treated group, **Figure 3B**). However, CFA caused an increase of *Grin2b* and *Camk2d* gene expression, compared with saline-treated group (each $p < 0.05$) (**Figures 3C, D**). The increased gene expression was significantly reduced after i.t. apelin-13 (each $p < 0.05$, compared with CFA treated group, **Figures 3C, D**).

Apelin-13 Mitigated the Elevated NR2B Expression and Glu Concentration Induced by CFA

To confirm the involvement of NR2A/B in the antinociception of apelin-13, the relative protein expression was detected using western blotting. As shown in **Figures 4A–C**, CFA induced an increase in GluN2B expression in the mouse lumbar spinal cord ($p < 0.05$, compared with control), but not GluN2A ($p = 0.80$, compared with control). The elevated GluN2B expression was

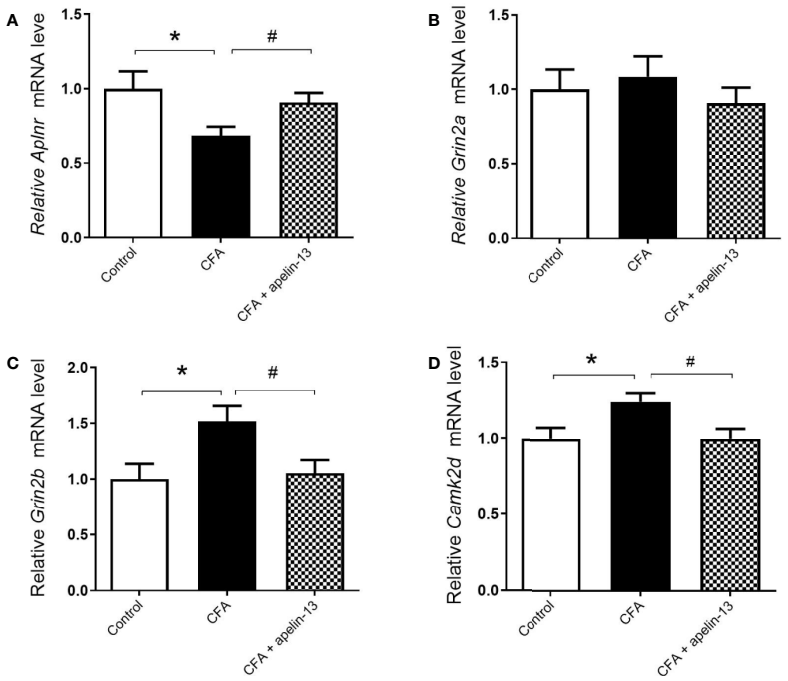

FIGURE 3 | Effect of i.t. apelin-13 on gene expression in the L4/5 spinal cord of mice. mRNA expression levels of *Aplnr* **(A)**, *Grin2a* **(B)**, *Grin2b* **(C)** and *CamK2d* **(D)** were normalized with the housekeeping gene *36B4* mRNA expression using real-time qPCR. Data are expressed as means ± SEM. n = 6–9 per group. $^{*}p < 0.05$, compared with vehicle control (i.d. NS + i.t. NS); $^{\#}p < 0.05$, compared with CFA group (i.d. CFA + i.t. NS).

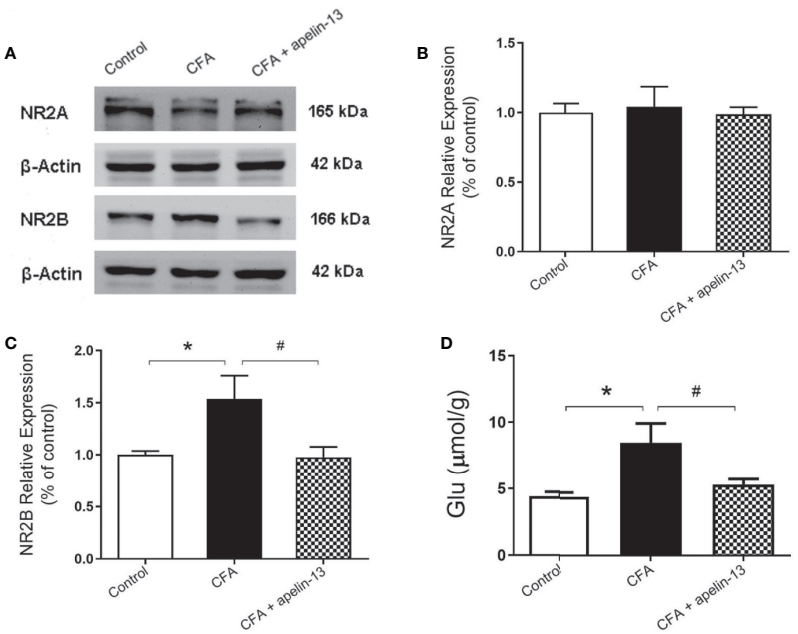

FIGURE 4 | Effect of i.t. apelin-13 on NR2A and NR2B expression, and Glu concentration in mice. **(A)** Expression of NR2A and NR2B in the mouse L4/5 spinal cord determined by western blotting. β-Actin was used as a loading control. **(B, C)** Quantitative analysis of normalized optical density (NR2A/β-actin, NR2B/β-actin) in the three groups. **(D)** Concentration of Glu in the mouse L4/5 spinal cord detected by HPLC. Data are expressed as means ± SEM. n = 4–6 per group. $^{*}p < 0.05$, compared with vehicle control (i.d. NS + i.t. NS); $^{\#}p < 0.05$, compared with CFA group (i.d. CFA + i.t. NS).

significantly reduced by i.t. aplein-13 ($p < 0.05$, compared with CFA treated group). In addition, to measure the change of Glu, a neuroexcitatory neurotransmitter, in the lumbar spinal cord, the free Glu was detected by HPLC. The concentration of Glu was significantly induced by CFA compared with the control group ($p < 0.05$), which significantly decreased after apelin-13 treatment, compared with the CFA group ($p < 0.05$, **Figure 4D**).

Apelin-13 Reduced *Fos* Gene Expression and the Number of Fos-Positive Cells in Laminae III and IV/V of the Dorsal Horn

To verify whether the transcription factors were involved in the antinociception of apelin-13, the mRNAs level of *c-Fos*, *Creb1*, *Dr1*, and *Egr1* were measured. Compared with the saline-treated control group, CFA obviously up-regulated *c-Fos* gene expression in the mouse lumbar spinal cord ($p < 0.05$, **Figure 5A**). However, it had no influence on *Creb1* ($p = 0.89$, **Figure 5B**), *Dr1* ($p = 0.51$, **Figure 5C**) and *Egr1* ($p = 0.54$, **Figure 5D**) gene expression. The increased *c-Fos* mRNA was significantly down-regulated by apelin-13 ($p < 0.05$).

To study the role of apelin in regulating chronic inflammatory pain, CFA-induced Fos expression was used as a functional marker to identify the activation of spinal neurons. Intradermal (i.d.) injection of CFA into the hind paw of the mice stimulated Fos expression within the L4/5 segments of the ipsilateral side of the spinal cord (**Figures 6A–E**). Compared with the control group, the number of FLI neurons in the CFA-treated group reached statistical significance in laminae III ($p < 0.05$) and laminae IV/V ($p < 0.05$), but not in laminae I/II ($p = 0.10$, **Figure 6E**). The elevated number of Fos-labeled neurons induced by CFA was reduced by i.t. apelin-13 in laminae IV/V ($p < 0.05$), but not in laminae III ($p = 0.19$).

DISCUSSION

Our present study indicated that *Apln* mRNA was down-regulated in the lumbar spinal cord of mice with inflammatory pain induced by CFA, compared with the vehicle control group. Apelin-13 (1 and 10 nmol/mouse, i.t.) alleviated CFA-induced hypersensitivity to both mechanical stimuli and thermal stimuli. Apelin-13 exerted more potent analgesic activity than apelin-36 and (pyr)apelin-13 in the CFA-induced inflammatory pain model. Wang et al. found that electroacupuncture stimulation alleviated CFA-induced inflammatory pain by restoring apelin and APJ mRNA and protein expression (Wang et al., 2016),

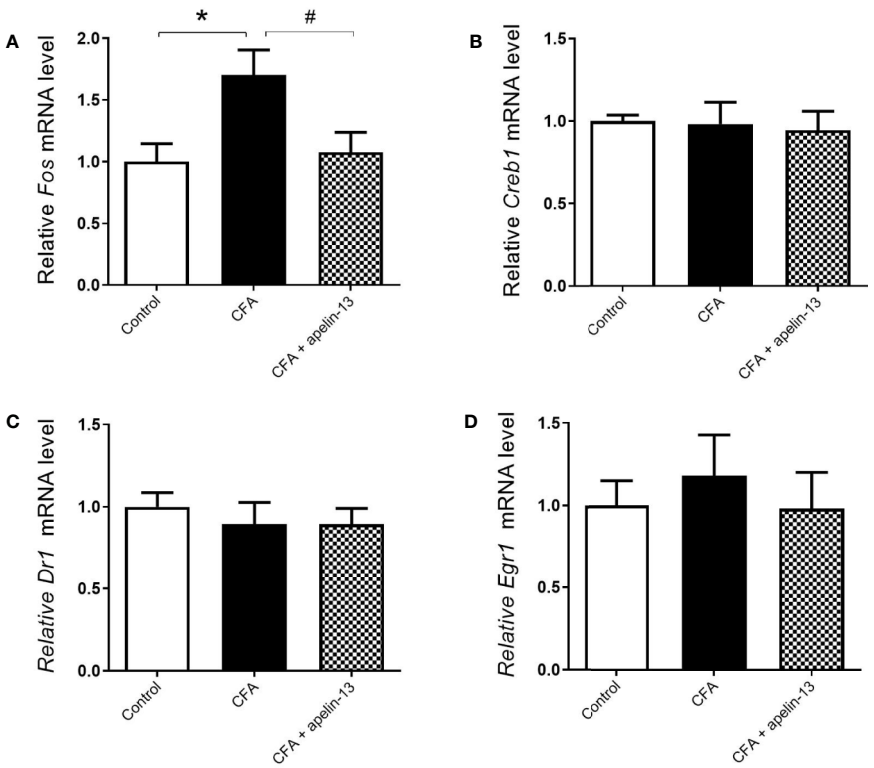

FIGURE 5 | Effect i.t. apelin-13 on the *c-Fos*, *Creb1*, *Dr1* and *Egr1* gene expression in mouse L4/5 spinal cord. mRNA expression levels of *Fos* **(A)**, *Creb1* **(B)**, *Dr1* **(C)** and *Egr1* **(D)** were determined by real-time qPCR and normalized with the housekeeping gene *36B4* mRNA expression. Data are expressed as means ± SEM. $^{*}p < 0.05$, compared with vehicle control (i.d. NS + i.t. NS); $^{\#}p < 0.05$, compared with CFA group (i.d. CFA + i.t. NS). n = 8 or 9 per group.

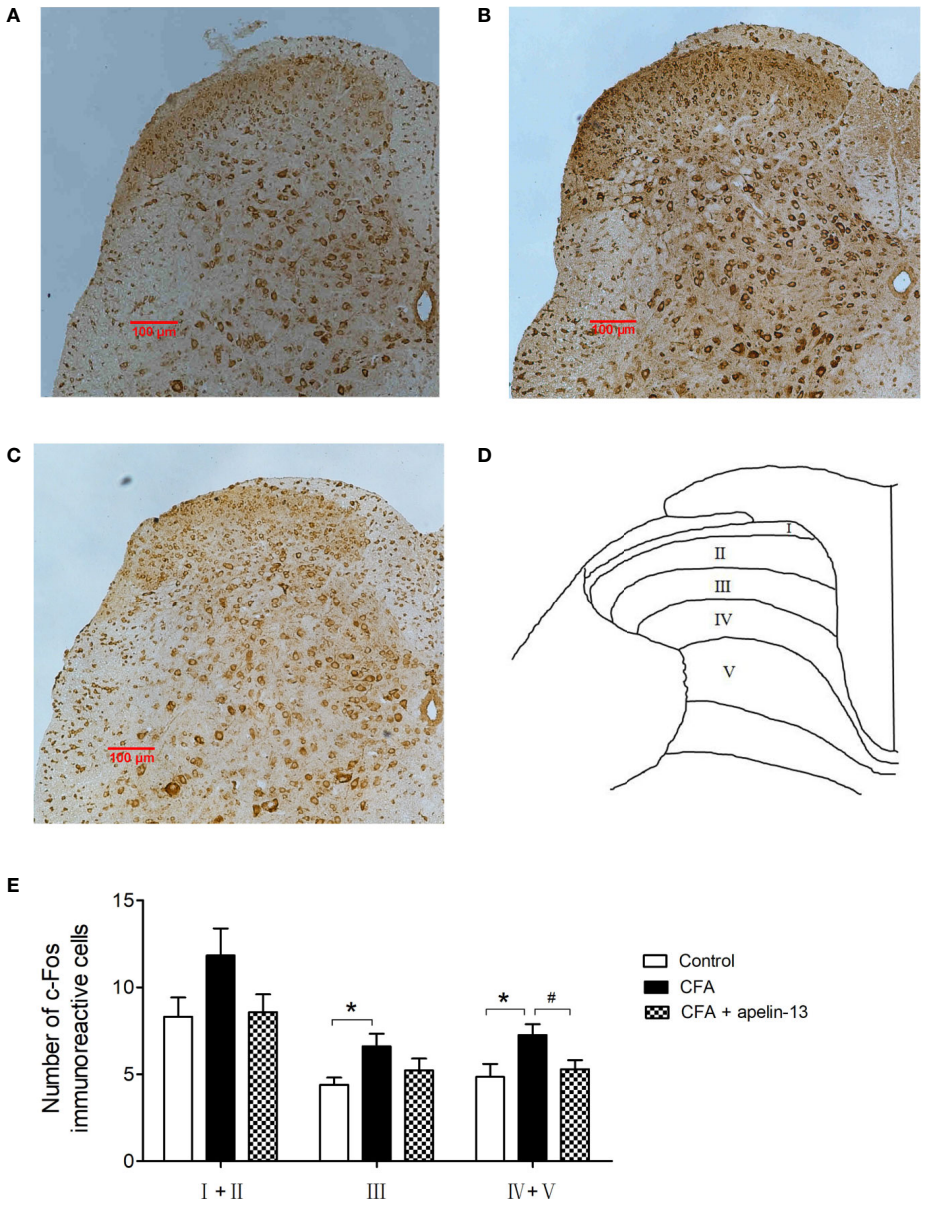

FIGURE 6 | Effect of apelin-13 on Fos protein expression in L4/5 spinal cord in mice. Representative sections of the lumbar spinal cord showing Fos-positive cells in the vehicle control (i.d. NS + i.t. NS, **A**), CFA group (i.d. CFA + i.t. NS, **B**) and apelin-13-treated group (i.d. CFA + i.t. NS, **C**). **(D)** Objective to measure and evaluate the distribution of Fos-positive cells of the lumbar spinal cord in mice. **(E)** Quantification of the number of Fos-positive cells in the spinal cord L4/5 segments from mice. Data are expressed as means ± SEM. $^{*}p < 0.05$, compared with vehicle control (i.d. NS + i.t. NS); $^{\#}p < 0.05$, compared with CFA group (i.d. NS + i.t. CFA). n = 6 per group. Scale bars = 100 μm.

suggesting that the apelin/APJ system has a close relationship with inflammatory pain. The present result confirmed that apelin induced an inhibitory effect on chronic inflammatory pain induced by CFA at the spinal level. Our previous studies showed that i.c.v. or i.t. apelin-13 inhibited acute pain in acid-induced writhing and tail immersion tests (Lv et al., 2012; Lv et al., 2013). In addition, it was reported that chronic injection of (pyr)apelin-13 (i.t., 1 and 5 μg/rat) ameliorated neuropathic pain after spinal cord injury (Xiong et al., 2017), and APJ antagonist ML221 mitigated neuropathic pain induced by chronic constriction injury (Hajimashhadi et al., 2017). It was reported that chronic apelin (3 μg/rat, i.t.) produced thermal antinociception and down-regulated spinal APJ. However, apelin could induce tolerance to its antinociceptive effect (Abbasloo et al., 2016). These results demonstrate that apelin induces consistent analgesic effects in different types of pain models.

The apelin receptor, APJ, shares 40–50% sequence homology with angiotensin II type 1 receptor, but it does not bind to angiotensin II (O'Dowd et al., 1993). Given the wide range of tissue distribution and physiological functions of APJ, it is considered as an interesting target. The specific APJ receptor antagonist was designed by mutation of the carboxyl-terminal phenylalanine, named apelin-13(F13A), and it blocked the hypotensive effects of apelin-13 (Lee et al., 2005). Our results indicated that apelin-13(F13A) significantly antagonized the inhibitory effect of apelin-13 on hyperalgesia response induced by CFA, whereas, apelin-13(F13A) alone did not influence CFA-induced hyperalgesia. Additionally, the down-regulated *Aplnr* mRNA in the CFA-treated group was restored by apelin-13 infusion. These results demonstrated that the antinociception of apelin-13 was mediated by APJ, which was supported by the anatomical site of APJ, such as the spinal cord (Hosoya et al., 2000; O'Carroll et al., 2000).

NMDARs, as glutamate-gated ion channels, play a key role in regulating synaptic plasticity. NMDARs consisted of three homologous subunits, including GluN1/NR1, GluN2/NR2 (GluN2A–GluN2D), and GluN3/NR3 (GluN3A–GluN3B). The major NMDAR subtypes in spinal dorsal horn are GluN1 (NR1A), GluN2A (NR2A), and GluN2B (NR2B) subunits. Our result showed that the gene and protein levels of GluN2B in lumbar spinal cord were significantly increased in the mouse model of CFA-induced inflammatory pain, compared with the control group. This is supported by the established theory that peripheral inflammation causes specific accumulation of NR2B receptors at spinal cord synapses (Tan et al., 2005; Yang et al., 2009; Zhuo, 2009). In the present study, i.t. apelin-13 attenuated the elevated NR2B gene and protein expression in the lumbar spinal cord of mice with CFA-induced chronic inflammatory pain, indicating that NR2B was involved in the antinociception of apelin-13. It has been proved that glutamate is a crucial transmitter of excitatory pathways to the spinal cord (Gougis et al., 2002). HPLC analysis showed that apelin-13 diminished the up-regulated glutamate level induced by CFA in mouse lumbar spinal cord. We suppose that the antinociception of apelin-13 may be caused by inhibiting release of the excitatory neurotransmitter in spinal cord.

Spinal dorsal horn neurons are reactive to nociceptive stimuli and participate in the transmission of painful information to the brain. *Fos* was a proto-oncogene expressed in neurons, and its rapid and transient expression had been identified as an indicator of neuronal excitation (Morgan et al., 1987). In the spinal cord, Fos expression was one of the long-term intracellular events, which was described as an indirect marker of nociceptive processes (Chapman and Besson, 1997). Our result demonstrated CFA injection up-regulated *Fos* mRNA expression in lumbar spinal cord and increased Fos-positive staining of laminae III and IV/V of the dorsal horn compared with the vehicle control, which was consistent with previous studies (Bao et al., 2017; Choi et al., 2018). In this study, we found that i.t. apelin-13 reduced the increased *Fos* mRNA level in lumbar spinal cord and the number of FLI cells located in laminae III and IV/V, suggesting that the inhibitory effect of apelin-13 on inflammatory pain was related to the reduction of neuronal activity in spinal dorsal horn.

CONCLUSION

We found that i.t. apelin-13 (1, 10 nmol/mouse) alleviated hyperalgesia in response to thermal and mechanical stimulation in a model of chronic inflammatory pain induced by injecting CFA into one hind paw of each mouse. Apelin-13 exhibited more potent antinociceptive activity than apelin-36 and (pyr)apelin-13. The antinociception was blocked by APJ antagonist apelin-13(F13A). Additionally, i.t. apelin-13 restored the decreased *Aplnr* mRNA, and reduced the up-regulated Glu concentration, and NR2B and Fos gene and protein level induced by CFA. We suppose that the antinociception of apelin-13 potentially was mediated by APJ activity, and inhibiting the Glu/NR2B function and neural activity of the spinal dorsal horn. Apelin-13 is potentially a preclinical drug for the treatment of inflammatory pain.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee of Medical Ethics and Welfare for Experimental Animals, Henan University School of Medicine (Approval No. HUSOM2016-042).

AUTHOR CONTRIBUTIONS

YY and XW developed the idea and designed the research. SL, XZ, YZ, and YF performed the experiment and analyzed the data. SL wrote the draft of the manuscript. YY and XW contributed to revise the writing. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Resting-State Functional Connectivity Patterns Predict Acupuncture Treatment Response in Primary Dysmenorrhea

Siyi Yu[1†], Mingguo Xie[2†], Shuqin Liu[2], Xiaoli Guo[1], Jin Tian[1], Wei Wei[1], Qi Zhang[1], Fang Zeng[1], Fanrong Liang[1] and Jie Yang[1*]*

[1] Brain Research Center, Department of Acupuncture & Tuina, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2] Department of Radiology, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, China

Primary dysmenorrhea (PDM) is a common complaint in women throughout the menstrual years. Acupuncture has been shown to be effective in dysmenorrhea; however, there are large interindividual differences in patients' responses to acupuncture treatment. Fifty-four patients with PDM were recruited and randomized into real or sham acupuncture treatment groups (over the course of three menstrual cycles). Pain-related functional connectivity (FC) matrices were constructed at baseline and post-treatment period. The different neural mechanisms altered by real and sham acupuncture were detected with multivariate analysis of variance. Multivariate pattern analysis (MVPA) based on a machine learning approach was used to explore whether the different FC patterns predicted the acupuncture treatment response in the PDM patients. The results showed that real but not sham acupuncture significantly relieved pain severity in PDM patients. Real and sham acupuncture displayed differences in FC alterations between the descending pain modulatory system (DPMS) and sensorimotor network (SMN), the salience network (SN) and SMN, and the SN and default mode network (DMN). Furthermore, MVPA found that these FC patterns at baseline could predict the acupuncture treatment response in PDM patients. The present study verified differentially altered brain mechanisms underlying real and sham acupuncture in PDM patients and supported the use of neuroimaging biomarkers for individual-based precise acupuncture treatment in patients with PDM.

Keywords: functional connectivity, primary dysmenorrhea, machine learning, multivariate pattern analyses, acupuncture

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***Correspondence:**
Fanrong Liang
acureaserch@126.com
Jie Yang
jenny_yang_jie@126.com

† These authors have contributed equally to this work

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INTRODUCTION

Primary dysmenorrhea (PDM), cyclic menstrual pain in the absence of pelvic anomalies, is a common, and often debilitating, gynecological condition that affects between 45 and 95% of menstruating women (Coco, 1999). Despite the high incidence rate of PDM, it is unfortunately often underdiagnosed and poorly treated (O'Connell et al., 2006). Acupuncture, a traditional Chinese medicine procedure, has been widely used to alleviate diverse types of pain for over 2000 years (Zhao, 2008). The National Institutes of Health has also recommended acupuncture

as an effective tool for certain health problems, including menstrual pain (Campbell and McGrath, 1999). Subsequently, the efficacy and safety of acupuncture for PDM have been reported in our systematic review (Yu et al., 2017) and several randomized controlled trials (RCTs) (Witt et al., 2008; Ma et al., 2013; Armour et al., 2017).

Although acupuncture has been shown to be effective in PDM, patients' responses to acupuncture treatment vary widely between individuals (Liu et al., 2017; Liu et al., 2019; Tu et al., 2019b). In addition, responses to other analgesic therapies have also been characterized by robust individual differences (Coghill and Eisenach, 2003; Wager et al., 2011; Angst et al., 2012). If we can identify the interindividual differences in pain processing, this would greatly help to achieve improved and personalized treatment. By definition, pain is a subjective and highly personal experience, and treatment outcomes are likely to be affected by an individual's baseline characteristics, such as demographic characteristics (e.g., sex, race, and age), levels of clinical pain and some objective biological markers (Wandner et al., 2012; Fillingim, 2017). Thus, baseline characteristics of individuals would be useful to predict the differential response to intervention strategies. Previous research has found that baseline clinical and demographic factors influence treatment response, but these characteristics have not achieved the accuracy required for prediction (Underwood et al., 2007; Azevedo et al., 2019; Witt et al., 2019). Some studies have focused on quantitative sensory testing (QST) in the prediction of analgesic effects, but with contradictory results (Grosen et al., 2013). In light of these studies showing limited individual predictive value for clinical measures, brain-based biomarkers have recently shown promise at predicting response to treatment (Chen et al., 2018; Reggente et al., 2018).

PDM has been proposed to be part of the central sensitization syndromes together with several chronic pain conditions, including fibromyalgia, irritable bowel syndrome, idiopathic low back pain, headache and migraine (Yunus, 2012; Iacovides et al., 2015). In recent years, using neuroimaging techniques, our group (Shen et al., 2019; Zhang et al., 2019) and other groups (Tu et al., 2009; Liu et al., 2018; Chen et al., 2019) have confirmed that PDM is associated with significant changes in the central nervous system's anatomy, metabolism, and resting-state function. Although the exact mechanisms of the analgesic effects of acupuncture are not known, studies have postulated that acupuncture can alleviate pain by modulating brain regions and networks associated with pain processing (Chen et al., 2015; Maeda et al., 2017). Neuroimaging research has demonstrated that chronic pain may be associated with alterations in multiple brain networks, such as the default mode network (DMN), sensorimotor network (SMN), salience network (SN), and descending modulation pathways (DPMS) (Kucyi and Davis, 2015; Kucyi and Davis, 2017). These particular networks, involved in the cognitive, sensorimotor, and affective aspects of pain, have been implicated in the core symptomatology of chronic pain and treatment response (Shi et al., 2015; Wu et al., 2016; Low et al., 2017; Lee et al., 2019; Zhang et al., 2019). However, it remains largely unknown where and how specific changes in these pain-related networks give rise to symptom improvement in patients with PDM after acupuncture treatment and whether network-level markers can predict the clinical response before intervention.

In this study, we used a longitudinal study design to investigate brain plasticity following acupuncture treatment. Our main aim was to explore whether particular pretreatment functional connectivity (FC) patterns (including those of the DMN, SMN, SN and DPMS) would predict the real and sham acupuncture response in PDM patients. First, we assessed FC alterations after acupuncture over the course of three menstrual cycles. Second, the different FC alterations between real and sham acupuncture treatment were explored. Third, we used multivariate pattern analyses (MVPA) based on a machine learning approach (support vector regression, SVR) to explore whether the different FC patterns predicted the acupuncture treatment response in PDM patients. We hypothesized that there were different neural mechanisms underlying real and sham acupuncture treatment that could predict the treatment response in PDM patients.

MATERIALS AND METHODS

Participants

Fifty-four patients with PDM were recruited from advertisements and word of mouth to participate in a dysmenorrhea study, and all participants were screened using telephone and in-person structured interviews. The Research Ethics Committee of Chengdu university of Traditional Chinses Medicine (CDUTCM) approved this study, and all participants gave written informed consent. The inclusion criteria for patients with PDM were (1) a regular menstrual cycle (27–32 days); (2) a history of PDM longer than 1 year; (3) no exogenous hormones or centrally acting medication in the last 6 months; (4) lower quadrant abdominal pain (including cramping, swelling, tingling, etc.) during menstruation in the last 6 months rated higher than 4 on a visual analog scale (VAS) (0 = not at all, 10 = the worst pain sensation); and (5) right-handedness, as confirmed by the Edinburgh Handedness Inventory (Oldfield, 1971). The exclusion criteria for patients with PDM were as follows: (1) other chronic pain conditions, such as low back pain; (2) organic pelvic disease or abnormalities found in gynecological ultrasonography; (3) visceral pain and other neurology that may cause hyperalgesia; (4) a positive pregnancy test or plan for pregnancy; (5) a neurologic or psychiatric disorder history; and (6) any contraindication for MRI scanning. Ten patients dropped out before the baseline clinical assessment and MRI scan, and nine patients dropped out during the treatment period. All the participants did not have any acupuncture experience. Finally, thirty-five patients (20 in the real and 15 in the sham acupuncture groups) completed all the clinical assessments and image scans and received real or sham acupuncture treatment during 3 menstrual cycles (**Table 1**). The details of the study design can be found in **Figure 1**.

Clinical Assessment

The primary outcome assessed in this trial was abdominal pain severity, as measured by the 0–10 VAS rom "no pain at

TABLE 1 | The demographic and clinical information of each group.

Items	Real acupuncture (*n* = 20)	Sham acupuncture (*n* = 15)	*T*	*P*
Age	24.70(2.10)	24.33(1.84)	0.54	0.59
BMI	19.55(1.38)	19.30(1.69)	0.49	0.63
Duration (months)	91.05(34.88)	91.00(33.72)	0.01	0.99
SAS	41.87(4.77)	40.46(7.82)	0.66	0.51
SDS	40.36(6.61)	43.33(10.17)	0.17	0.30
Baseline VAS	6.17(0.99)	5.87(1.18)	0.94	0.41
Post-treatment VAS	3.35(1.49)	5.39(1.38)	4.03	<0.001
VAS change	3.40(1.53)	1.03(1.84)	4.07	<0.001
VAS change rate (%)	50.80(22.57)	19.42(36.59)	3.49	<0.001

BMI, body mass index; SAS, self-report anxiety scale; SDS, self-report depression scale; VAS: visual analog scale.

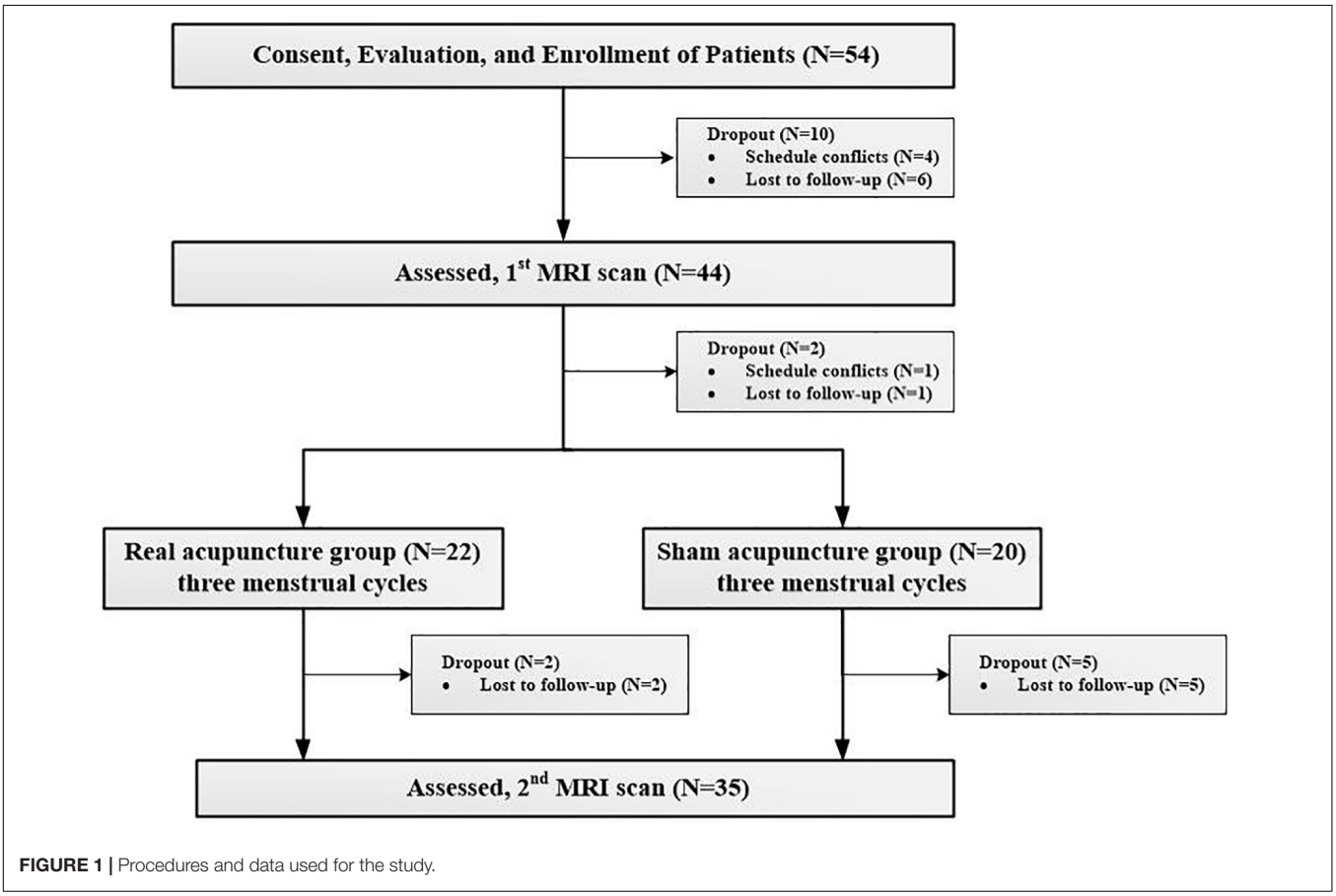

FIGURE 1 | Procedures and data used for the study.

all" to "unbearable pain (Larroy, 2002). In addition, the self-rating anxiety scale (SAS) and self-rating depression scale (SDS) were applied as secondary outcomes to evaluate the anxiety and depression levels of the PDM patients (Zung et al., 1965; Zung, 1971). All clinical outcomes were measured at baseline and after completion of three sessions of treatment during the periovulatory phase (days 12–16 of the menstrual cycle).

Acupuncture Treatment

Patients were randomized using a computer-generated, random-allocation sequence and then assigned to either the real acupuncture group or the sham acupuncture group. All patients and study staff were blinded to the treatment groups. Only the acupuncturist, who had to know whether to deliver real or sham treatment, was not blinded.

Real Acupuncture Treatment

For the real acupuncture treatment group, *sanyinjiao* (SP6) was selected based on data mining from our previous review and expert opinions. SP6 is located on the tibial aspect of the leg, posterior to the medial border of the tibia and 3 *cun* (proportional bone cun), above the medial malleolus (World Health Organization, 2008). The acupuncture procedures were as follows: after the skin was cleaned with tincture of iodine

and alcohol, 0.25 × 40 mm stainless needles (Hwatuo, Suzhou, China) were inserted 1.0–1.2 *cun* and gently twisted, lifted and thrust with even amplitude, force and speed four to six times until *deqi* was obtained (soreness, numbness, distension and heaviness). Needles were retained at the acupoints for 30 min, and the above manipulation was repeated twice every 10 min for 30 s each time.

Sham Acupuncture Treatment

An adjacent sham acupoint located at the midpoint between the stomach and gall bladder meridians in the same level of SP6 and *xuanzhong* (GB39) was selected in the sham group (Ma et al., 2010). The patients in this group underwent an acupuncture procedure similar to the patients in the real acupuncture group, but no needle manipulation was performed after needle insertion, and *deqi* sensation was not obtained. The real and sham acupuncture targets are displayed in **Supplementary Figure S1**.

The acupuncture interventions for both groups were performed by two licensed acupuncturists with over 3 years of experience. Acupuncture treatments started 7 days before the beginning of menses and did not stop until the onset of the next menstruation. All participants received acupuncture treatment once a day, there were 7 days in a session, and there were 3 sessions over 3 menstrual cycles.

Imaging Acquisition

All participants underwent two MRI scans on the same 3.0-Tesla magnetic resonance scanner (Discovery MR750, General Electric, Milwaukee, WI, United States) in the Department of Radiology at the Affiliated Hospital of CDUTCM at baseline and the forth periovulatory phase after each clinical assessment. Tight, but comfortable, foam padding was used to minimize head motion, and earplugs were used to reduce scanner noise. Sagittal 3D T1-weighted images were acquired using a brain volume sequence with the following parameters: repetition time (TR) = 8.16 ms; echo time (TE) = 3.18 ms; flip angle (FA) = 7°; field of view (FOV) = 256 × 256 mm; matrix = 256 × 256; slice thickness = 1 mm, no gap; and 188 sagittal slices. The resting-state functional fMRI (rs-fMRI) datasets were obtained in 7 min with a gradient-recalled echo-planar imaging pulse sequence. The rs-fMRI imaging parameters were TR = 2000 ms, TE = 30 ms, FA = 90°, acquisition matrix = 64 × 64, FOV = 240 × 240 mm, thickness = 4.0 mm, voxel size = 3.5 × 3.5 × 4.02 mm^3, gap = 0.5 mm, NEX = 1.0, and number of slices = 33. A total of 210 volumes were acquired. All subjects were scanned during the first three days of the menstrual phase. During the data scans, all subjects were instructed to relax and maintain closed eyes, and all participants reported that they did not fall asleep during the scanning.

fMRI Data Preprocessing

MRI data were preprocessed and analyzed using the SPM8 toolbox[1] implemented in MATLAB 8.0 (Mathworks Inc., Sherborn, MA, United States). Structural images were coregistered with resting-state functional images. Conventional preprocessing steps were performed, which included (1) removing the first 5 time points; (2) slice timing correction; (3) realignment (participants with head motion greater than 1.5mm maximum displacement in any direction (x, y, z) or 1.5° of angular motion were excluded); (4) normalization of images with a T1 template in the Montreal Neurological Institute (MNI) atlas space and resampling to 3? × 3 × 3mm^3 cubic voxels; (5) linear detrending; (6) nuisance covariate regression including six motion parameters, average signals of cerebrospinal fluid and white matter, and time points having spike motion of framewise displacement (FD) > 0.5; (7) temporal filtering (bandpass 0.01–0.1 Hz); and (8) spatial smoothing using a Gaussian kernel of 6-mm full-width at half-maximum (FWHM). The images with all preprocessing steps were used for region of interest (ROI) to ROI functional connectivity analysis, while the images after the first four preprocessing steps were used for group independent component analysis (GICA). There were no differences in head motion parameters (FD) within or between groups.

ROI Selection and Functional Connectivity Analyses

First, GICA analysis was conducted using group ICA of the fMRI toolbox (GIFT 3.0b, Medical Image Analysis Lab, University of New Mexico, Albuquerque, NM, United States) implemented in MATLAB 8.0 and SPM8. A set of independent components (ICs) were identified as intrinsic resting-state networks in all subjects at both the baseline and post-treatment periods (Calhoun et al., 2001). The optimal number of ICs was automatically estimated by minimum description length (MDL) criteria in GIFT, and the median of the MDL over all subjects was 66 (Li et al., 2006). The ICA components were calculated by infomax algorithms, and spatial maps were transformed to z-scores.

Second, the default mode network (DMN), sensorimotor network (SMN), and salience network (SN) were chosen as components of interest to be evaluated from the resting-state data. ten ROIs in these resting-state networks (RSNs) were manually chosen from the 66 extracted ICs, which were identified as anatomically and functionally classical RSNs by two experienced neuroimaging researchers (YJ and YSY) (Smith et al., 2009). Four ROIs in the DMN, including the medial prefrontal cortex (mPFC), posterior cingulate cortex (PCC) and bilateral inferior parietal cortices (IPC), are involved in pain rumination (Kumbhare et al., 2017). Four ROIs in the SN, including the bilateral dorsolateral prefrontal cortices (dlPFC) and bilateral anterior insula (aINS) represent the sustained activation during attention to pain (Kucyi and Davis, 2015, 2017), and descending pathways that modulate the transmission of ascending nociceptive signals (Hemington et al., 2016; Davis et al., 2017). Bilateral primary somatosensory cortices (S1) and bilateral thalami in the SMN represent the major ascending pathways of pain (Tracey and Mantyh, 2007; Davis et al., 2017). In addition, two key regions in the descending pain modulatory system, the periaqueductal gray (PAG) and rostroventral medulla (RVM) (Goksan et al., 2018), were also selected as ROIs for the pain-related FC matrix in PDM. The ten cortical ROIs were saved as masks by one sample *t*-test by using SPM8. The subcortical

[1]http://www.fil.ion.ucl.ac.uk/spm

ROI of the thalamus in the SMN was acquired from automated anatomical labeling, and the PAG and RVM masks were acquired from the DPMS network mask in standard space (Goksan et al., 2018; **Figure 2**).

Third, an ROI-wise FC matrix was constructed using the REST toolbox[2]. The averaged time series in each ROI were extracted, and the Pearson correlations with time series in other ROIs were calculated. Then, the FCs were transformed to Z values (Lowe et al., 1998). Thus, a 14 × 14 FC pattern matrix for each individual was separately obtained for further analysis.

Statistical Analysis

Demographic and Clinical Features

Two independent t-tests were performed to compare the demographic and clinical traits between the two groups, and paired t-tests were employed to determine whether the alterations in VAS scores were significant after treatment in each group (SPSS 20.0; SPSS Inc., Chicago, IL, United States). Pearson correlation analyses were employed to explore associations between duration of disease, baseline VAS, SDS, and SAS scores, and changes of the VAS scores. The significance level was set at $P < 0.05$.

Functional Connectivity Difference Analysis

The paired t-tests were performed to detect the FC matrix alterations after treatment in each treatment group. In addition, we employed a 2 × 2 (group × time) multivariate analysis of covariance (MANCOVA) on the FC matrix to detect whether there were different neural mechanisms underlying the different treatments, with age and duration of disease as covariates (Hand and Taylor, 1987; Davis, 2002). Traditional Pearson correlation analyses were used to explore the associations between the FCs of MANCOVA and VAS changes after three sessions of acupuncture. The significance level was set at $P < 0.05$ and corrected for multiple comparisons by the false discovery rate (FDR) approach. The in-house script was write in MATLAB to conduct the MANCOVA in the study. The codes for MANCOVA of ROI-wise functional connectivity can be downloaded from https://github.com/cdutcmysy/ROIwise_FC_mancova.

MVPA-Based Clinical Symptom Prediction Analysis

MANCOVA found differences in FC between the real and sham treatments for PDM. We proposed that these different FC patterns could reveal the neural mechanisms underlying the effects of acupuncture treatment for PDM and that these FC patterns at baseline would be used for predicting the treatment response to acupuncture in patients with PDM. Here, MVPA based on linear support vector regression (SVR, implemented by LIBSVM[3]) (Chang and Lin, 2011) was employed to verify our hypothesis. We set the change in pain severity (VAS change and VAS change rate) as the dependent variable and FCs from the MANCOVA results at baseline as independent variables (predictors) in all participants and regressed out the effects of age, treatment method and duration of disease. A leave-one-out cross-validation (LOOCV) method was used for prediction to ensure separation between training and testing samples (Plitt et al., 2015). We calculated the squared prediction-outcome correlation (R^2) as well as the mean absolute error (MAE) to evaluate the SVR predictive ability (Wager et al., 2013; Lindquist et al., 2017). Furthermore, we employed the permutation test to verify that the predictor was not from a random chance (repeated 5000 times).

RESULTS

Demographic and Clinical Features

There were no significant differences in age, BMI, duration of disease, and baseline SAS, SDS or VAS scores between the real and sham acupuncture treatment groups (all $P > 0.05$; see **Table 1**). The VAS at the post-treatment period was lower in the real group than in the sham group, and the VAS change score and VAS

[2]http://www.restfmri.net

[3]https://www.csie.ntu.edu.tw/$\sim$cjlin/libsvm/

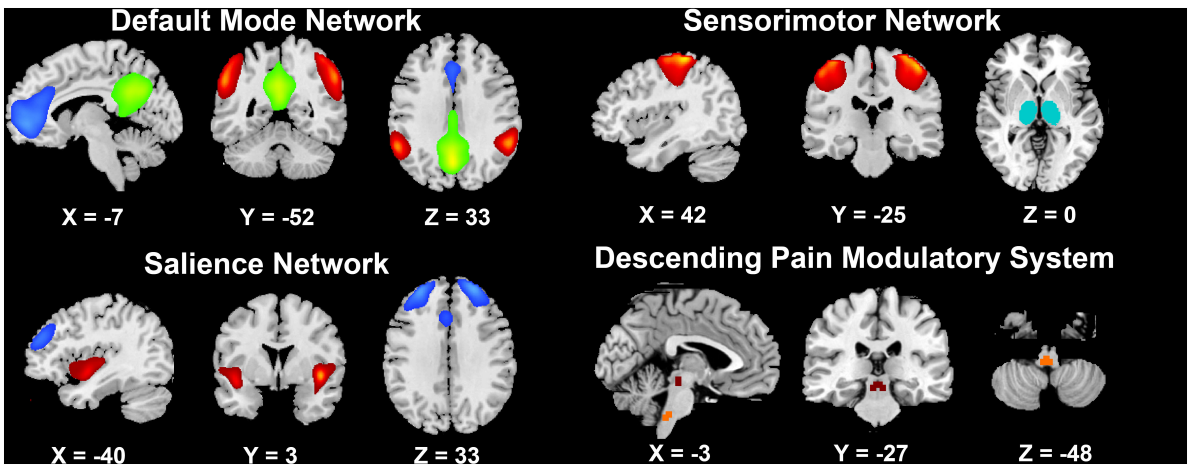

FIGURE 2 | The brain networks selected in the present study.

change rate were significantly higher in the real acupuncture treatment group than in the sham acupuncture treatment group. Paired *t*-tests showed that the VAS change in the real acupuncture treatment group was significant ($t = 9.90$, $P < 0.01$), while the VAS change in the sham acupuncture group was not significant ($t = 2.10$, $P = 0.06$). In addition, there were no significant associations between duration of disease and baseline VAS, SDS, and SAS scores and post-treatment VAS score changes in either group (all $P > 0.05$; see **Table 2**).

FC Matrix Alterations After Real and Sham Acupuncture Treatment

As illustrated in **Figures 3A–C**, after real acupuncture treatment, both increased and decreased FCs were found; specifically, FCs between the mPFC-right S1, PAG-bilateral thalami, PAG-RVM, RVM-bilateral thalami and RVM-DMN (all four ROIs in the DMN) were increased, while FC between the left S1-right aINS was decreased after real acupuncture treatment. For the sham acupuncture treatment group, increased FCs were found in the right S1-right aINS, left IPC-bilateral aINS, right dlPFC-left S1, right dlPFC-PCC and right dlPFC-right IPC, while FC between the PAG-RVM was decreased after sham treatment (**Figures 3D–F**). Interestingly, we did not find the same patterns of altered FC in the real and sham acupuncture treatment groups.

Group Differences in the FC Matrix

The MANCOVA analysis results are displayed in **Figure 4** and **Supplementary Figure S2**. Eight paired FCs showed significant differences between the two groups, including the right aINS-bilateral S1, right dlPFC-bilateral S1, right dlPFC-left IPC, RVM-bilateral thalami and RVM-PAG. Specifically, FCs that were decreased in the real group but increased in the sham group were located in the SMN-SN and DMN-SN, while FCs that were increased in the real group but decreased in the sham group were found in the DPMS-SMN and within the DPMS pathway.

TABLE 2 | The correlations between baseline clinical features, FCs and VAS changes in PDM patients.

Items	VAS change		VAS change rate	
	R	*P*	*R*	*P*
Duration of disease	0.05	0.80	0.11	0.54
Baseline VAS score	−0.15	0.39	0.05	0.78
Baseline SDS score	−0.19	0.29	−0.23	0.19
Baseline SAS score	−0.00	0.98	−0.11	0.52
L_S1-R_aINs	0.05	0.81	0.00	0.99
R_S1-R_aINs	−0.10	0.60	−0.10	0.59
L_S1-R_dlPFC	0.09	0.64	0.05	0.79
R_S1-R_dlPFC	−0.01	0.95	−0.15	0.42
RVM-L_Tha	0.15	0.42	0.33	0.07
RVM-R_Tha	0.23	0.21	0.21	0.25
RVM-PAG	−0.17	0.37	−0.24	0.20
L_IPC-R_dlPFC	0.02	0.93	0.17	0.37

FC, functional connectivity; VAS: visual analog scale; PDM, primary dysmenorrhea; SAS, self-report anxiety scale; SDS, self-report depression scale; L, left side; R, right side; S1, primary somatosensory cortex; aINS, anterior insula; dlPFC, dorsolateral prefrontal cortex; RVM, rostroventral medulla; Tha, thalamus; PAG, periaqueductal gray; IPC, inferior parietal cortex.

Clinical Prediction Results

As we found significant group differences in FC alterations between the real and sham groups, we used the baseline FCs as predictors of the treatment response and controlled for the effect of age, duration and treatment method. The SVR analyses revealed that the eight baseline FC patterns predicted the VAS change scores ($R^2 = 0.27$, $P = 0.002$, MAE = 0.36; **Figure 5A**) and VAS change rate after treatment ($R^2 = 0.30$, $P = 0.0009$, MAE = 2.26; **Figure 5B**). The permutation tests confirmed that the results could not be obtained by chance ($P < 0.001$). Conversely, the traditional bivariate correlation analyses did not find any significant associations between these differences in FC and VAS changes after acupuncture treatment in the patients with PDM (all $P > 0.05$; see **Table 2**).

DISCUSSION

This study in patients with PDM used MVPA recognition to identify neurobiological predictors of acupuncture treatment response. We first verified the efficacy of real but not sham acupuncture treatment in the patients with PDM. Then, we identified FCs involving the SMN-SN, DMN-SN, DPMS-SMN and DPMS pathway that showed different alterations after real and sham acupuncture treatment in PDM patients. Furthermore, MVPA revealed that these pretreatment multivariate FC patterns in the SMN, SN, DMN and DPMS significantly predicted individual patient pain symptoms after 3 menstrual cycles of intensive acupuncture treatment. Conversely, pretreatment clinical variables and traditional correlation analyses were not able to predict posttreatment pain severity in these patients with PDM. These findings have implications for identifying who will benefit most from acupuncture, as well as for understanding the pathophysiology of PDM as it relates to acupuncture effects.

The present study verified the effect of acupuncture on reducing menstrual pain intensity in PDM (Yu et al., 2017). In the present study, for the real acupuncture treatment, we selected *sanyinjiao* (SP6) as the acupuncture target based on data mining from our previous review (Yu et al., 2015) and expert opinions, while for the sham acupuncture treatment, an adjacent non-acupoint was selected. We found that real acupuncture was superior to sham acupuncture for pain relief. The results indicated that correct acupuncture point locations are important contributors to the treatment effects and that the acupuncture effect of pain relief cannot be explained solely in terms of placebo effects (Smith et al., 2011; Vickers et al., 2018). Subsequently, we detected the potential brain mechanisms underlying the real and sham acupuncture treatment effects in PDM.

To date, no study has explored the underlying mechanisms of pain reduction by acupuncture in patients with PDM. For other types of chronic pain, Lee et al. found that both real and sham (phantom) acupuncture could reduce pain ratings in patients with low back pain (LBP), but the reduced pain intensity was associated with reduced FC between the mPFC and posterior

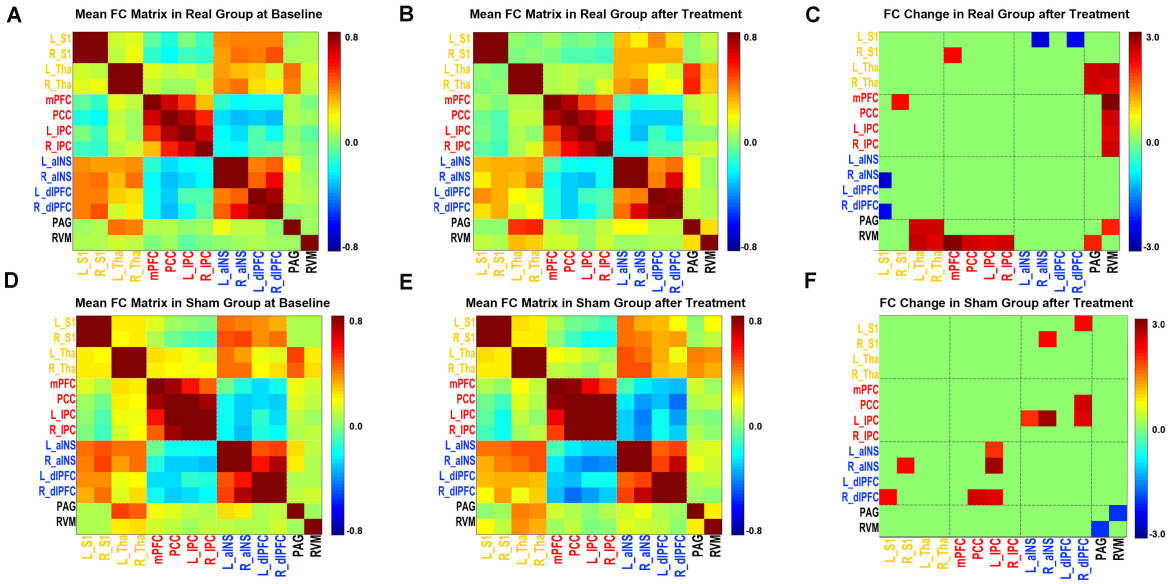

FIGURE 3 | FC matrix pattern in each group. **(A,D)** The FC matrix pattern at baseline. **(B,E)** The FC matrix pattern after treatment. The color bar indicates the correlation coefficient between two regions. **(C,F)** FC alterations after acupuncture treatment; the color bar indicates the *T* value. **(A,B,C)** Real acupuncture group. **(D,E,F)** Sham acupuncture group. Abbreviations: FC, functional connectivity; L, left side; R, right side; S1, primary somatosensory cortex; Tha, thalamus; mPFC, medial prefrontal cortex; PCC, posterior cingulate cortex; IPC, inferior parietal cortex; aINS, anterior insula; dlPFC, dorsolateral prefrontal cortex; RVM, rostroventral medulla; PAG, periaqueductal gray.

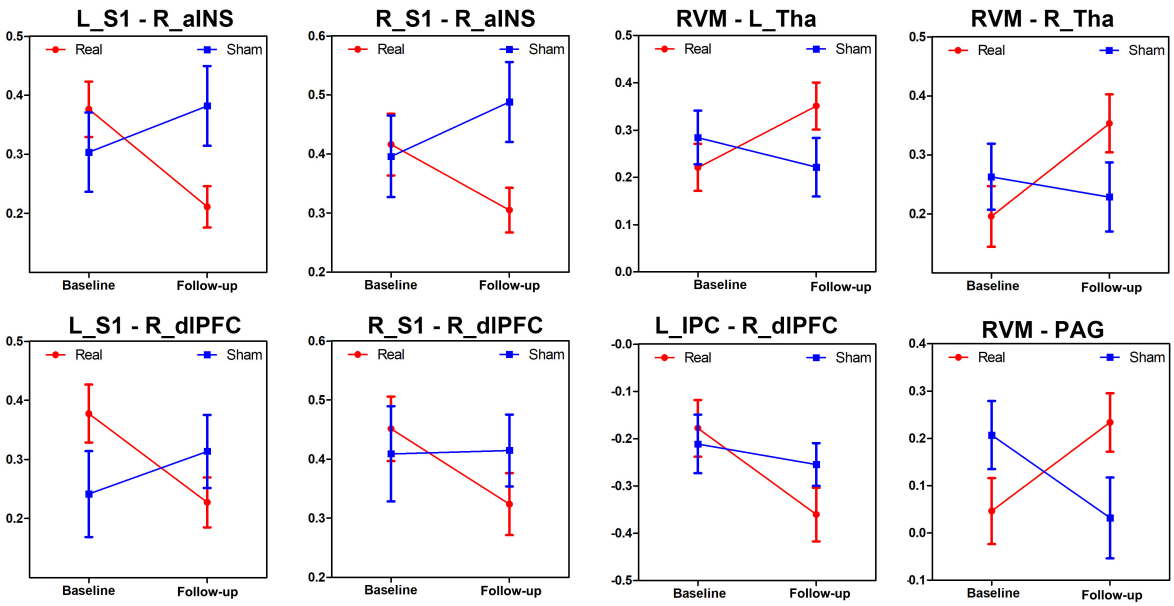

FIGURE 4 | Changes in the different brain connections following real and sham acupuncture for PDM. The line charts display the different alterations between real and sham acupuncture for PDM in these FCs after treatment. Abbreviations: FC, functional connectivity; L, left side; R, right side; S1, primary somatosensory cortex; aINS, anterior insula; dlPFC, dorsolateral prefrontal cortex; RVM, rostroventral medulla; Tha, thalamus; PAG, periaqueductal gray; IPC, inferior parietal cortex.

insula in the real acupuncture group, while the decreased pain intensity was associated with enhanced FC between the PCC and aINS (Lee et al., 2019). The results indicated differential brain influences on real and sham acupuncture in LBP. Here, we constructed the pain-related functional connectivity matrices in patients with PDM by combining the data-driven approach (GICA) and hypothesis-based ROI selection method with rs-fMRI data. The findings showed that altered pain-related FCs after real acupuncture were located in FC between the DPMS-SMN and DPMS-DMN and within the DPMS, while altered

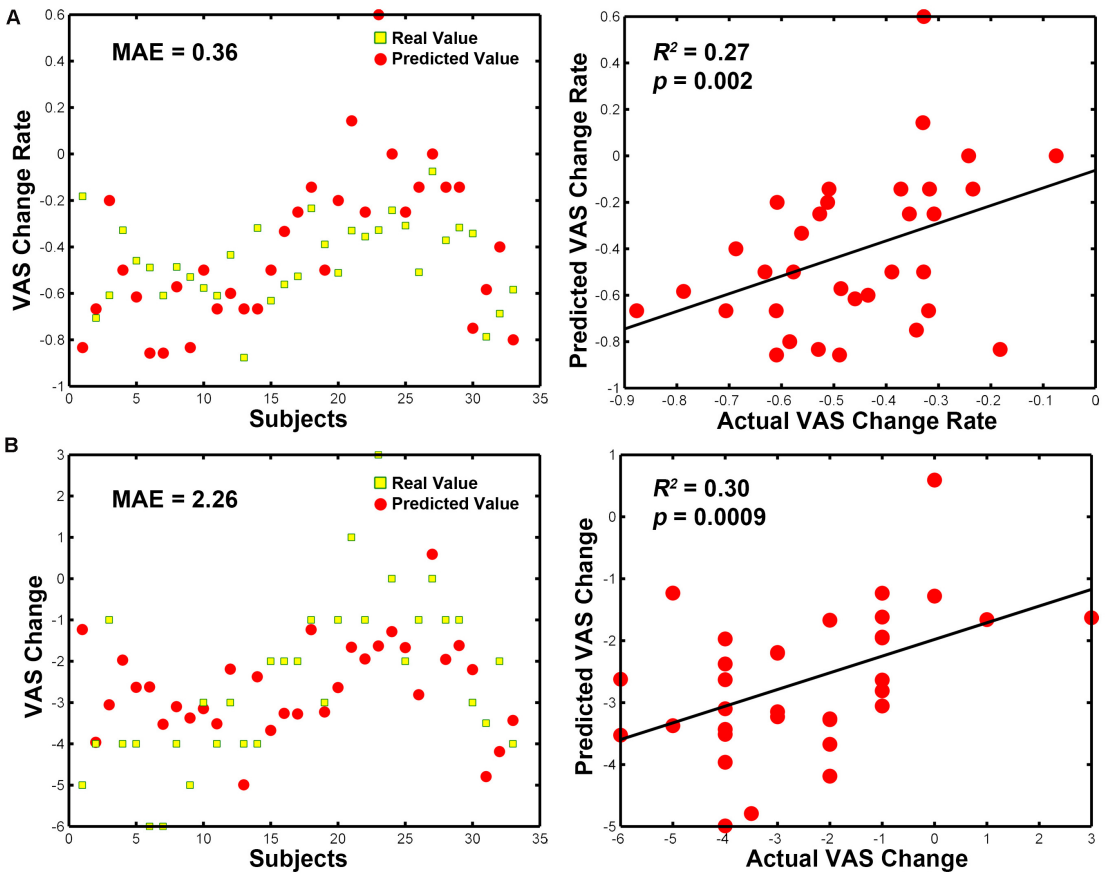

FIGURE 5 | MVPA-based clinical prediction results. **(A)** Baseline FC patterns predict VAS change scores after treatment. **(B)** Baseline FC patterns predict VAS change rates after treatment. Abbreviations: MVPA, multivariate pattern analysis; MAE, mean absolute error; VAS, visual analog scale.

FCs after sham acupuncture were found in FC between the SN-SMN and SN-DMN and within the DPMS. In general, real acupuncture modulated the DPMS-associated brain network more, while sham acupuncture modulated the SN-related brain network more. These results indicated that real acupuncture could modulate widespread brain networks associated with the DPMS, while sham acupuncture had a more focused modulation in the salience network associated with analgesia in patients with PDM. Furthermore, MANCOVA was used to detect the different brain functional modulatory mechanisms between real and sham acupuncture treatment in patients with PDM.

We found eight functional couplings that showed differences in alterations after real and sham treatment of PDM. As illustrated in **Figure 3**, real acupuncture increased but sham acupuncture decreased FC between the DPMS and SMN and FC within the DPMS (RVM-PAG), while real acupuncture reduced but sham acupuncture enhanced FC between the SN and SMN, and the SN and DMN. Our study was consistent with previous findings of different modulatory effects in the central nervous system between real and sham acupuncture in a healthy population. Cao et al. found that real acupuncture could increase pain intensity thresholds, which were associated with increased neural activity in the insula (Cao et al., 2018). A recent neuroimaging meta-analysis about acupuncture effects in the brain in healthy individuals found that in 29 out of 33 studies, real acupuncture, compared to sham acupuncture, resulted in more/different modulatory effects on neurological components measured by neuroimaging, including somatosensory, affective, and cognitive aspects (Scheffold et al., 2015). The dlPFC is known to be associated with center control and top-down processes for pain control (Wager et al., 2004; Kong et al., 2006), as well as anticipation of pain relief and expectation-related placebo analgesia (Krummenacher et al., 2010). The SMN represents the major ascending pathways of pain (Tracey and Mantyh, 2007; Davis et al., 2017), while the DMN is involved in the self-regulation of pain, such as mind wandering (Kucyi et al., 2013; Kucyi and Davis, 2015). Multiple neuroimaging studies have suggested that acupuncture may achieve analgesic effects by modulating the SN, DMN and SMN (Chen et al., 2015; Shi et al., 2015; Lee et al., 2019; Zhang et al., 2019). The DPMS is considered the key system for pain modulation (Millan, 2002) and has also been found to be modulated by acupuncture treatment in migraine (Li et al., 2016). The different modulatory effects of real and sham acupuncture in the pain-associated brain networks indicated different and complex neural mechanisms of real and sham acupuncture in patients with PDM.

The different alterations in these pain-related FCs resulted in different treatment responses, which also indicated the potential use of these FCs as predictors for acupuncture treatment response in PDM patients.

Our research is in line with the growing interest in predictive modeling using neuroimaging and machine learning methods (Janssen et al., 2018; Keshavan et al., 2020). Most machine learning studies have focused on differential diagnosis, identifying brain signatures that discriminate patients from healthy controls, and further established objective signs of disease pathology (Lai, 2019). Another important application of machine learning has been the use of brain characteristics to predict therapeutic outcomes and offer personalized tailoring of interventions (Gao et al., 2018; Leaver et al., 2018; Mithani et al., 2019). Here, using MVPA (Yang et al., 2012), a widely applied machine learning approach, we found that several pain-related FC patterns at baseline could be a useful predictor for acupuncture treatment response (both VAS changes and the change rate) in PDM patients. Recently, Tu et al. also used MVPA-based rs-FCs to predict real and sham acupuncture treatment responses in chronic LBP, and the rs-FC characteristics were significantly predictive of the differential responses to real and sham treatment in LBP (Tu et al., 2019a). In our study, the predictive power and strength of the MVPA approach was validated in several ways. First, we found that baseline clinical or demographic features were unable to predict the outcome responses of the PDM patients. Second, the traditional univariate correlations between FCs and VAS changes were not significant. Given the nature of MVPA approaches, these techniques provide improved sensitivity to subtle and spatially distributed brain differences that would likely remain undetected with the use of conventional univariate approaches (Fan et al., 2006; Haxby, 2012). Therefore, we demonstrated the feasibility and reliability of the MVPA model for predicting clinical symptom changes after acupuncture treatment in patients with PDM.

LIMITATIONS

The present study also has several limitations. First, the sample size in this study was small, especially in the sham acupuncture group, and previous studies have also found a significant analgesic effect of sham acupuncture (Tu et al., 2019a). Our study found that the VAS change in the sham acupuncture group was nearly significant ($P = 0.06$), and further studies with larger sample sizes are needed to address this point. Second, as the PDM is a cyclic chronic pain, the present study only explored the "trait pain" (average pain experienced over time) in the patients with PDM, and future studies should also explore the mechanism of the acupuncture-related analgesic effect on "state pain" (pain at the menstrual period) in PDM patients (Low et al., 2018). Third, to increase the statistical power, we combined the two treatment groups as a pooled group in the MVPA analysis after controlling for the effect of treatment. Nevertheless, we did not find that baseline FCs predicted the treatment response in the single groups. Further studies with larger sample sizes are needed to further detect the different predictors for real and sham treatment effects in PDM.

CONCLUSION

The present study verified the different brain mechanisms underlying real and sham acupuncture in PDM patients. In addition, the pain-related FC patterns at baseline could predict the acupuncture treatment response for patients with PDM. The findings supported the use of neuroimaging biomarkers for individual-based precise acupuncture treatment in patients with PDM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Chengdu University of Traditional Chinses Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JY and FL designed the study. SL, WW, XG, JT, QZ, and MX collected the data. SY and MX analyzed the data. SY contributed to the original draft. JY, FZ, and FL reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.559191/full#supplementary-material

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frontiers in Pharmacology

BRIEF RESEARCH REPORT
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Intrathecal Oxytocin Improves Spontaneous Behavior and Reduces Mechanical Hypersensitivity in a Rat Model of Postoperative Pain

*Antonio Espinosa De Los Monteros-Zúñiga, Guadalupe Martínez-Lorenzana, Miguel Condés-Lara and Abimael González-Hernández**

Departamento de Neurobiología del Desarrollo y Neurofisiología, Instituto de Neurobiología, Universidad Nacional Autónoma de México, Queretaro, Mexico

The first few days post-surgery, patients experience intense pain, hypersensitivity and consequently tend to have minor locomotor activity to avoid pain. Certainly, injury to peripheral tissues produces pain and increases sensitivity to painful (hyperalgesia) and non-painful (allodynia) stimuli. In this regard, preemptive pharmacological treatments to avoid or diminish pain after surgery are relevant. Recent data suggest that the neuropeptide oxytocin when given at spinal cord level could be a molecule with potential preemptive analgesic effects, but this hypothesis has not been properly tested. Using a validated postoperative pain model (*i.e.* plantar incision), we evaluated in male Wistar rats the potential preemptive antinociceptive effects of intrathecal oxytocin administration measuring tactile hypersensitivity (across 8 days) and spontaneous motor activity (across 3 days). Hypersensitivity was evaluated using von Frey filaments, whereas spontaneous activity (total distance, vertical activity episodes, and time spent in the center of the box) was assessed in real time using a semiautomated open-field system. Under these conditions, we found that animals pretreated with spinal oxytocin before plantar incision showed a diminution of hypersensitivity and an improvement of spontaneous behavior (particularly total distance and vertical activity episodes). This report provides a basis for addressing the therapeutic relevance of oxytocin as a potential preemptive analgesic molecule.

Keywords: hyperalgesia, oxytocin, postoperative pain, evoked pain, spontaneous pain, allodynia, anxiety

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University of Genoa, Italy

***Correspondence:**
Abimael González-Hernández
abimaelgh@comunidad.unam.mx

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INTRODUCTION

Every year, ∽240 million people undergo some type of surgery (Weiser et al., 2008; Weiser et al., 2015; Weiser et al., 2016), which causes postoperative pain and distress (*e.g.* anxiety), and in most cases impairs the patient's quality life for a brief period (Kehlet and Dahl, 2003). Aside from postsurgical pain and hypersensitivity, patients submitted to surgical procedures experienced an increased risk (10%–50%) of developing chronic pain (Perkins and Kehlet, 2000; Chapman and Vierck, 2017). Despite the arsenal of drugs to ameliorate pain during and after these procedures

(Chaparro et al., 2013; Reddi, 2016), treatments that prevent (namely preemptive analgesia) the establishment of central sensitization before the surgical incision seem to be a better strategy to ameliorate postsurgical pain (Kehlet et al., 2006; Kehlet, 2018). Certainly, as discussed by Katz and Seltzer (2009), if acute postoperative pain is reduced, so is the risk of chronic pain. This idea points to the relevance of preemptive (or perioperative) analgesia in the management of postsurgical pain.

In this context, some studies suggest that spinal oxytocin could have preemptive analgesic effects. Briefly, in preclinical experiments using female rats, Gutierrez et al. (2013) showed that after the postpartum period, endogenous spinal oxytocin release seems to diminish the hypersensitivity induced by spinal nerve ligature. Indeed, clinical data showed that the development of chronic pain in women submitted to cesarean is minor in comparison with other non-obstetric interventions (Eisenach et al., 2013). These data, coupled to fact that spinal oxytocin prevents spinal long-term potentiation (LTP) (DeLaTorre et al., 2009), a key process in the development of central sensitization leading to persistent pain (Ruscheweyh et al., 2011), point out the potential role of oxytocin as a preemptive analgesic. In the last 15 years, studies on the antinociceptive effects of spinal oxytocin have been published (for refs. See Poisbeau et al., 2018). Briefly, intrathecal (i.t.) oxytocin has been shown to reduce thermal and mechanical hyperalgesia or mechanical allodynia in neuropathic (Miranda-Cardenas et al., 2006; Sun et al., 2018; González-Hernández et al., 2019) and inflammatory pain models (Yu et al., 2003; Reeta et al., 2006). Most importantly, epidural oxytocin relieves refractory pain in humans (Condés-Lara et al., 2016).

Despite the above data suggesting the potential effect of spinal oxytocin as a preemptive analgesic molecule, this hypothesis has not been properly tested in a postoperative experimental pain condition. The present study was designed to preclinically test the preemptive antinociceptive effect of i.t. oxytocin pretreatment before a surgical procedure in a well-established model of postoperative pain. Hence, using the plantar incision model, we analyzed the effect of oxytocin on spontaneous behavior (as a surrogate model of spontaneous pain) or on evoked nociception by von Frey filaments. We found that i.t. pretreatment with oxytocin i) improves spontaneous behavior, ii) improves the recovery time, and iii) diminishes evoked hypersensitivity.

MATERIAL AND METHODS

Experimental Animals

Experiments were performed on 48 adults male Wistar rats weighing 180–220 g. The rats were provided by the bioterium of the Instituto de Neurobiología of the Universidad Nacional Autónoma de Mexico. The animals with free access to food and drinking water were housed in pairs in plastic cages with wood-based bedding, in a controlled temperature (22 ± 2°C) and humidity (50%) room under a 12:12 h light/dark cycle (light beginning at 7:00 h). For evoked pain measurements (von Frey filaments), the experiments were performed between 13:00–15:00 h; for spontaneous behavior, the experiments were performed between 17:00–09:00 h. At the end of the experiments, the animals were halted in a CO_2 chamber.

General Procedures

Plantar Incision

As previously described by Brennan et al. (1996), gaseous anesthesia with sevoflurane (4–5%; enriched with ¾ N_2O and ¼ O_2) was delivered with a mask and an incision of the hind paw was performed. Briefly, the plantar aspect of the left hind paw was prepared, and a 1 cm longitudinal incision was made through skin, fascia, and muscle. Under these conditions, the flexor *digitorum brevis* muscle was elevated and incised. In all cases the surgery was performed between 16:00–16:30 h. The incision started 12 mm distal from the edge of the heel and the skin was closed using 4–0 nylon sutures. Naïve rats underwent a procedure that included anesthesia and sterile preparation of the plantar area, but no incision. Immediately after surgery, the animals were divided into two main sets to evaluate the effects of oxytocin in two behavioral paradigms: i) evoked nociception induced by von Frey filaments and ii) spontaneous behavior using a semiautomated open-field system.

Cutaneous Evoked Nociceptive Responses (von Frey Filaments)

The von Frey filaments were used to measure the magnitude of mechanical hypersensitivity induced by the plantar incision. To assess baseline pain behavior, rats were placed individually on an elevated plastic mesh floor covered with a clear plastic cage top and allowed to acclimate. All rats were pre-tested (1 day before surgery) and tested at 1, 2, 3, 4, 5, 6, 7, and 8 days post-surgery for the response to the withdrawal threshold to von Frey filaments. Briefly, as previously described (Brennan et al., 1996), primary withdrawal to punctate stimulation was tested by applying calibrated nylon von Frey monofilaments (Touch-Test™ Sensory Evaluators, North Coast Medical, Inc., CA, USA) to an area adjacent to the incision (near the calcaneus). Each von Frey filament (14, 20, 39, 59, 78, 98, 147, 255, and 588 mN) was applied once, beginning with 14 mN until a withdrawal response occurred, if there was no paw withdrawal, 588 mN was recorded. The withdrawal threshold was calculated from the averaged of three tests.

Assessment of Spontaneous Activity

The rats were behaviorally tested on days 0 (basal), 1 (the same day of surgery), 2, and 3 post-surgical incision. In this case, the surgical incision was made on day 1 and the spontaneous behavior was assessed using the open-field system with arrays of photo-beam sensors to determine the location of the animal in real-time (Omnitech Electronics, Inc., OH, USA). Briefly, animals were placed individually in plexiglass boxes (16 × 16 × 11.75 inches) containing the photo-beam sensors. Locomotor activity and rearing episodes were assessed. The raw data were stored on a computer disk for off-line analyses using the Fusion v5.3 SuperFlex software (Omnitech Electronics, Inc., OH, USA). Under these conditions, spontaneous activity episodes were

monitored for 16 h (starting at 17:00 h and ending at 9:00 h the following day) for 4 days. The spontaneous behavior was analyzed during the light phase (17:00–18:00 h) and the dark phase (19:00–07:00 h). In all cases we measured the horizontal activity (total distance), vertical episodes (rearing), and time (seconds) spent in the center of the open-field system. Indeed, horizontal and vertical activity have been proposed as an endpoint to indirectly analyze potential analgesic compounds (Imanaka et al., 2008; Majuta et al., 2017a), whereas time spent in the center has been used as an endpoint to analyze anxiety-related effects (File, 1980; Castanheira et al., 2018), a key issue after postoperative pain (Carr and Goudas, 1999; Kehlet and Dahl, 2003; Kouya et al., 2015).

Drug Treatment

Under anesthesia (see above), oxytocin (10 nmol; Sigma-Aldrich, CAS: 50-56-6, base free) or isotonic saline solution (0.9% NaCl) were intrathecally (i.t.) injected in a volume of 20 µl 10 min before the plantar incision. The dose of oxytocin was chosen from previous reports showing that intrathecal oxytocin inhibits nociception in a range of 0.1–10 nmol (DeLaTorre et al., 2009; Gutierrez et al., 2013; Chow et al., 2016). The i.t. oxytocin or vehicle administration were given in a blinded manner by direct lumbar puncture (22 G) in the L5-L6 intervertebral space following the method described by Mestre et al. (1994).

Statistical Analysis

In all cases, statistical difference was considered at $p \leq 0.01$. The data from the von Frey filaments is represented using box and whisker plots or bar graphs [means ± standard error (SE); **Figure 1**] and were analyzed using the non-parametric Friedman test (**Figures 1A–C**) or Kruskal-Wallis test (**Figure 1D**). Furthermore, to compare the global effect of the different treatments, the area under the curve was calculated and analyzed using the parametric one-way analysis of variance (1W-ANOVA; **Figure 1E**). In the case of spontaneous activity (**Figure 3**) a Grubb's test was initially performed to exclude outliers and we graphed the data as means ± SE. To analyze the

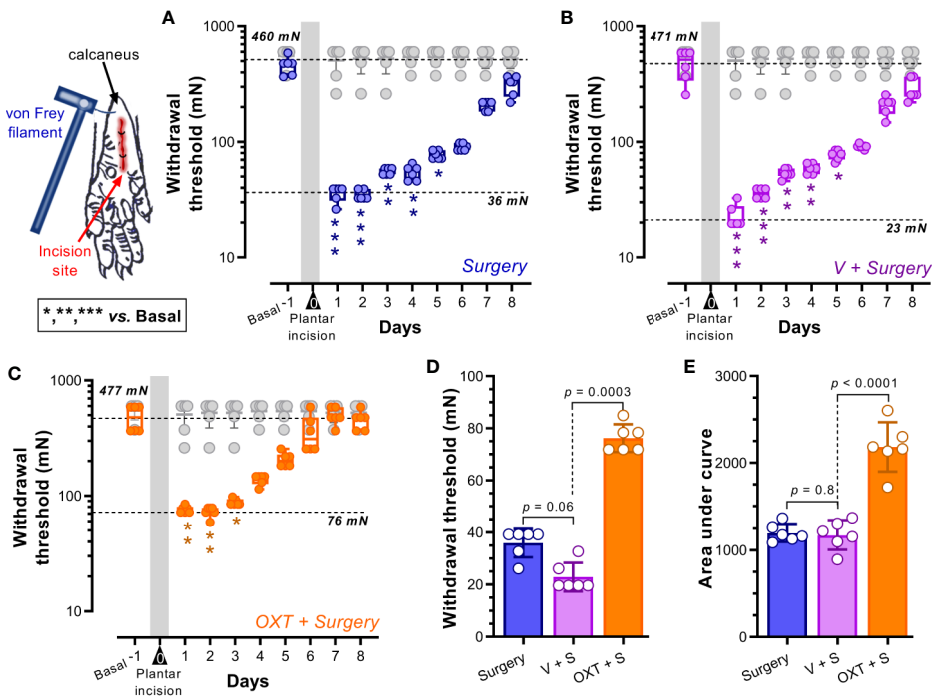

FIGURE 1 | Intrathecal oxytocin (OXT) diminished the evoked pain (mechanical hypersensitivity) in a postoperative pain model. **(A–C)** show the box-plots of the primary punctate withdrawal threshold before (Basal) and after (starting 1 day after surgery for 8 days) plantar incision in animals without pretreatment (*Surgery* alone; blue) or pretreated with vehicle (*V + Surgery*; magenta) or 10 nmol OXT (*OXT + Surgery*; orange); furthermore we insert in these panels the data from naïve rats (see gray box-plot). Vehicle or OXT were given intrathecally 10 min before the plantar incision under anesthesia. The forces applied by von Frey filaments in the plantar area of the rat's hind paw are expressed in newtons (mN). In all cases, the means of the basal withdrawal threshold were similar (≥ 460 mN), and a clear reduction of this value was induced one day after surgery (≤ 76 mN) (see the dotted lines in the panels); these data indicate that plantar incision induces mechanical hypersensitivity. Furthermore, the time necessary to find no difference in the withdrawal threshold to von Frey filaments was minor in the animals pretreated with OXT (3 days *versus* 5 days), suggesting an improvement in the recovery times. Asterisks *, **, *** indicate $p < 0.01$, 0.001, 0.0001 vs Basal value. **(D)** shows the means ± standard errors (SE) of the withdrawal thresholds of the different groups one day after surgery; note that the hypersensitivity in animals pretreated with OXT seems to be minor than that of *Surgery* or *V + Surgery* groups. In addition, **(E)** shows the global effect of treatments as the means of the areas under the curves (AUC) ± SE; since the AUC of animals pretreated with oxytocin is higher than that of the other two groups, we infer that oxytocin has an analgesic effect in a postoperative pain model. For statistical details, see **Table 1**.

spontaneous behavior before (basal) and after (day 1) plantar incision, we performed a paired t-test (**Figure 3A**) for each treatment. In addition, to compare the effect of different treatments for 3 days (**Figures 3B–G**), the differences were computed by the parametric two-way repeated measures analysis of variance (2W-RM-ANOVA) (in this case sphericity was not assumed). Except for the paired t-test, in all cases, to control the false discovery rate (q = 0.05) for multiple testing, we used the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. The statistical analysis results are detailed in **Table 1**.

RESULTS

The Postoperative Pain Model and Evoked Pain

Postoperative pain was induced in animals by plantar incision of the hind paw. **Figure 1A** shows the primary mechanical withdrawal threshold before (basal; 460 mN) and after plantar incision (for 8 days); note that one day after surgery the withdrawal threshold diminished (36 mN) ($p < 0.0001$). This hypersensitivity to von Frey filaments remains significant until day 5 post-surgery ($p = 0.01$). Similar results were obtained in animals pretreated with vehicle (**Figure 1B**). In contrast, as **Figure 1C** shows, when the animals received oxytocin (10 nmol, i.t.), the hypersensitivity to von Frey filaments remained significant until day 3 post-surgery ($p = 0.01$). Note that the withdrawal threshold of naïve animals was not modified during the test days. Furthermore, when comparing the withdrawal threshold of animals pretreated with oxytocin *versus* untreated animals 1 day after surgery (**Figure 1D**), we found that the withdrawal threshold of oxytocin-treated rats was higher than that in untreated rats; this result implies a preemptive antinociceptive effect. Similarly, upon analyzing the global effects as area under the curve (AUC) (**Figure 1E**), we found that oxytocin pretreatment increases the AUC, suggesting that this neuropeptide decreases the evoked hypersensitivity.

Postoperative Pain Model and Spontaneous Behavior

Figure 2 shows the actograms and representative heat map of locomotor activity from naïve (**Figure 2A**), surgery untreated

TABLE 1 | Statistical data with their respective *post hoc* comparison for each figure panel.

Figure	Test	*Post hoc* comparison
1	Friedman test for multiple comparisons	Benjamini, Krieger and Yekutieli
1A	$\chi^2 = 47.31$, n = 6 rats, $p < 0.0001$	Basal vs day (D): D1 (**<0.0001**); D2 (**<0.0001**); D3 (**0.0006**); D4 (**0.0004**); D5 (**0.01**); D6 (0.05)
1B	$\chi^2 = 47.69$, n = 6 rats, $p < 0.0001$	Basal vs day (D): D1 (**<0.0001**); D2 (**<0.0001**); D3 (**0.0002**); D4 (**0.001**); D5 (**0.01**); D6 (0.06)
1C	$\chi^2 = 45.41$, n = 6 rats, $p < 0.0001$	Basal vs day (D): D1 (**0.001**); D2 (**0.0003**); D3 (**0.01**); D4 (0.1); D5 (0.6); D6 (>0.99)
1	Kruskal-Wallis	Benjamini, Krieger and Yekutieli
1D	$\chi^2 = 14.77$, n = 6 rats, $p < 0.0001$	OXT+Surgery vs V+Surgery, $p <$ **0.0003**
1	One-way ANOVA	Benjamini, Krieger and Yekutieli
1E	Treatment effect: $F_{(2, 15)} = 50.21$; $p < 0.0001$	OXT+Surgery vs V+Surgery, $p <$ **0.0001**
3	Two-way RM ANOVA	Benjamini, Krieger and Yekutieli
3B	Interaction: $F_{(9, 60)} = 2.272$; $p = 0.0290$	Naïve vs Surgery: p at day 1 = **0.003**; day 2 < **0.0001**; day 3 < **0.001**
	Time effect: $F_{(1.371, 27.42)} = 37.18$; $p < 0.0001$	Naïve vs V+Surgery: p at day 1 = **0.006**; day 2 = **0.0004**; day 3 = **0.002**
	Treatment effect: $F_{(3, 20)} = 8.851$; $p = 0.0006$	Naïve vs OXT+Surgery: p at day 1 = **0.008**; day 2 = **0.0001**; day 3 = **0.002**
		V+Surgery vs OXT+Surgery: p at day 1 = 0.429; day 2 = 0.989; day 3 = 0.616
3C	Interaction: $F_{(9, 60)} = 1.789$; $p = 0.0891$	Naïve vs Surgery: p at day 1 = **0.001**; day 2 = **0.01**; day 3 = **0.004**
	Time effect: $F_{(1.904, 38.02)} = 37.18$; $p < 0.0001$	Naïve vs V+Surgery: p at day 1 = **0.001**; day 2 = **0.009**; day 3 = **0.005**
	Treatment effect: $F_{(3, 20)} = 23.76$; $p < 0.0001$	Naïve vs OXT+Surgery: p at day 1 = 0.02; day 2 = 0.2; day 3 = 0.3
		V+Surgery vs OXT+Surgery: p at day 1 = **0.005**; day 2 = **0.004**; day 3 = **0.008**
3D	Interaction: $F_{(9, 60)} = 3.439$; $p = 0.0018$	Naïve vs Surgery: p at day 1 = **0.007**; day 2 = 0.02; day 3 = 0.07
	Time effect: $F_{(2.350, 47.01)} = 15.43$; $p < 0.0001$	Naïve vs V+Surgery: p at day 1 = **0.008**; day 2 = 0.03; day 3 = 0.1
	Treatment effect: $F_{(3, 20)} = 9.077$; $p = 0.005$	Naïve vs OXT+Surgery: p at day 1 = 0.02; day 2 = 0.4; day 3 = 0.09
		V+Surgery vs OXT+Surgery: p at day 1 = 0.19; day 2 = 0.031; day 3 = **0.001**
3E	Interaction: $F_{(9, 60)} = 4.778$; $p < 0.0001$	Naïve vs Surgery: p at day 1 = **0.009**; day 2 = **0.002**; day 3 = **0.006**
	Time effect: $F_{(1.930, 38.61)} = 17.70$; $p < 0.0001$	Naïve vs V+Surgery: p at day 1 = **0.01**; day 2 = **0.002**; day 3 = **0.006**
	Treatment effect: $F_{(3, 20)} = 25.14$; $p < 0.0001$	Naïve vs OXT+Surgery: p at day 1 = 0.05; day 2 = 0.2; day 3 = 0.1
		V+Surgery vs OXT+Surgery: p at day 1 = **0.01**; day 2 < **0.0001**; day 3 = **0.002**
3F	Interaction: $F_{(9, 60)} = 1.85$; $p = 0.07$	Naïve vs Surgery: p at day 1 = 0.224; day 2 = 0.225; day 3 = 0.135
	Time effect: $F_{(2.77, 55.40)} = 2.58$; $p = 0.067$	Naïve vs V+Surgery: p at day 1 = 0.206; day 2 = 0.221; day 3 = 0.175
	Treatment effect: $F_{(3, 20)} = 1.32$; $p = 0.29$	Naïve vs OXT+Surgery: p at day 1 = 0.393; day 2 = 0.789; day 3 = 0.246
		V+Surgery vs OXT+Surgery: p at day 1 = 0.25; day 2 = 0.17; day 3 = 0.60
3G	Interaction: $F_{(9, 60)} = 1.073$; $p = 0.396$	Naïve vs Surgery: p at day 1 = 0.394; day 2 = 0.326; day 3 = 0.752
	Time effect: $F_{(2.324, 46.48)} = 0.864$; $p = 0.442$	Naïve vs V+Surgery: p at day 1 = 0.647; day 2 = 0.631; day 3 = 0.234
	Treatment effect: $F_{(3, 20)} = 8.137$; $p = 0.001$	Naïve vs OXT+Surgery: p at day 1 = **0.01**; day 2 = 0.06; day 3 = 0.02
		V+Surgery vs OXT+Surgery: p at day 1 = **0.01**; day 2 = 0.02; day 3 = **0.003**

In all cases we considered $p \leq 0.01$ (underline and bold) for significance.

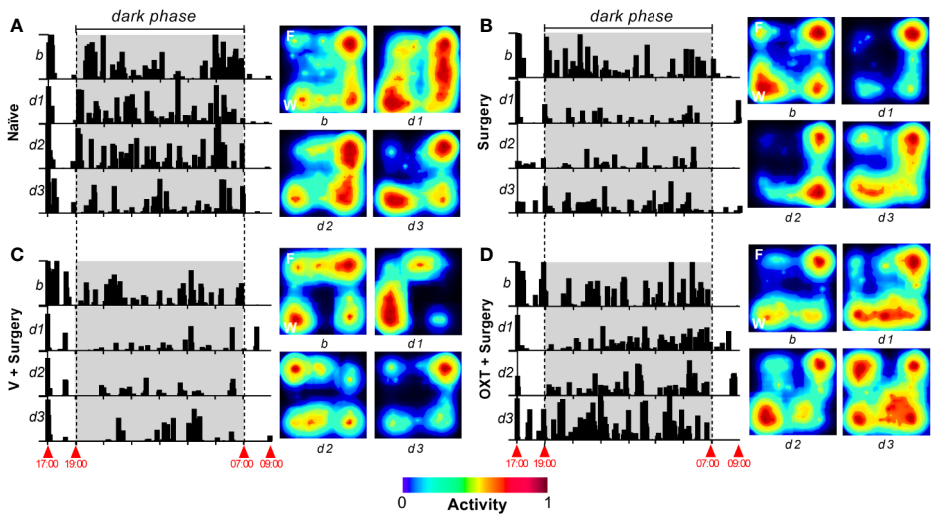

FIGURE 2 | Appearance of ambulatory activity and representative heat maps of different treatments before and after the surgical procedure. In all panels, to the left, a raster plot of the ambulatory activity is shown; the data was obtained through a 16-h recording between 17:00–09:00 h for 4 days, where *b* represents the basal value, and *d1*, *d2*, and *d3* represent day 1, day 2, and day 3, respectively, after the incision of the hind paw. To the right of each panel, a representative heat map of horizontal locomotor activity is depicted, where F and W represent the site in the open-field system where the food and water were presented. In the case of naïve animals **(A)**, the actograms and heat map are similar across test days; note that, according to the heat maps, naïve animals move around the open-field system in a similar pattern across all test days. Comparable results were obtained in basal condition in all animal groups. When the animals were subjected to an incision of the hind paw [**(B–D)**], activity was clearly impaired at *d1*. Furthermore, at *d3* the spontaneous activity was partially recovered (compared to basal condition) in the *Surgery* and *Vehicle + Surgery* groups. In the case of animals pretreated with 10 nmol oxytocin (OXT) **(D)**, a minimal impact in the activity was recorded at *d1*, and a complete recovery in the activity was achieved at *d3*.

(**Figure 2B**), vehicle + surgery (**Figure 2C**) and oxytocin + surgery (**Figure 2D**) rats obtained during the 16 h of recording before (basal activity, B) and after (day 1, 2, and 3) surgery. As shown in the actograms, when the animals were first placed in the activity boxes during the light phase (at 17:00 h), they actively explored the novel environment; after this initial exploration, the animals remained with low activity (between 18:00–19:00 h) until the start of the dark phase (lights off at 19:00 h). In the naïve group (**Figure 2A**), an increase in locomotor activity during the dark phase was clearly observed; this spontaneous activity diminished after surgery in the surgery untreated (**Figure 2B**) and surgery + vehicle (**Figure 2C**) groups. Indeed, a visual reduction in the intensity of actograms and heat maps was achieved after surgical incision (see subpanels *d1*). In contrast, the animals pretreated with oxytocin (**Figure 2D**) showed a minor impairment of the spontaneous locomotor activity; in fact, at day 3 post-surgery, the locomotor activity and heat map were similar to the basal response.

As shown in **Figure 3A**, after plantar incision, the total distance traveled and vertical activity tend to diminish in the surgery, vehicle + surgery and oxytocin + surgery groups; this effect is not observed in the naïve animals ($p > 0.2$). Although similar results were obtained in the time spent in the center during the exploratory hour for naïve, surgery and vehicle + surgery groups, no difference was observed before and after oxytocin treatment ($p = 0.929$). Also, during the dark phase the probability of finding a difference in the time spent in the center before and after surgery was low in all groups ($p > 0.2$); nevertheless, the rats receiving oxytocin tended to spend more time in the center ($p = 0.073$).

When we analyzed the impact of oxytocin treatment in comparison with the other groups, we found that after plantar incision (day 1), the *total distance traveled* diminished for the first hour of exploratory behavior and for the 12 h of nighttime activity (**Figures 3B, C**). This impairment was observed throughout the 3 days in the case of surgery and vehicle + surgery group. In contrast, in animals pre-treated with oxytocin, the activity during the dark phase was not statistically different from that of the naïve group (**Figure 3C**; $p \geq 0.02$), even though after surgery a decrease in the distance traveled was found during the first hour of exploratory behavior throughout the 3 days (**Figure 3B**; $p \leq 0.008$).

In the case of *vertical activity* (**Figures 3D, E**), at day 1 during light and dark phase, the number of rearing episodes (where the rat stands on its hind legs) was diminished ($p \leq 0.01$) only in the groups with surgery or vehicle + surgery but not in the animals treated with oxytocin ($p \geq 0.02$). Furthermore, during the dark phase, the vertical activity of the animals without oxytocin was clearly impaired on days 2 and 3 ($p \leq 0.006$); interestingly, during the light phase of these days we did not find any statistical differences ($p \geq 0.02$) despite the diminution of vertical activity. In animals treated with oxytocin, the number of vertical episodes displayed on days 2 and 3 was similar to that of the naïve group during the light and dark phase ($p \geq 0.09$).

Finally, in the case of time spent in the center (**Figures 3F, G**), the probability that oxytocin affected this variable in comparison

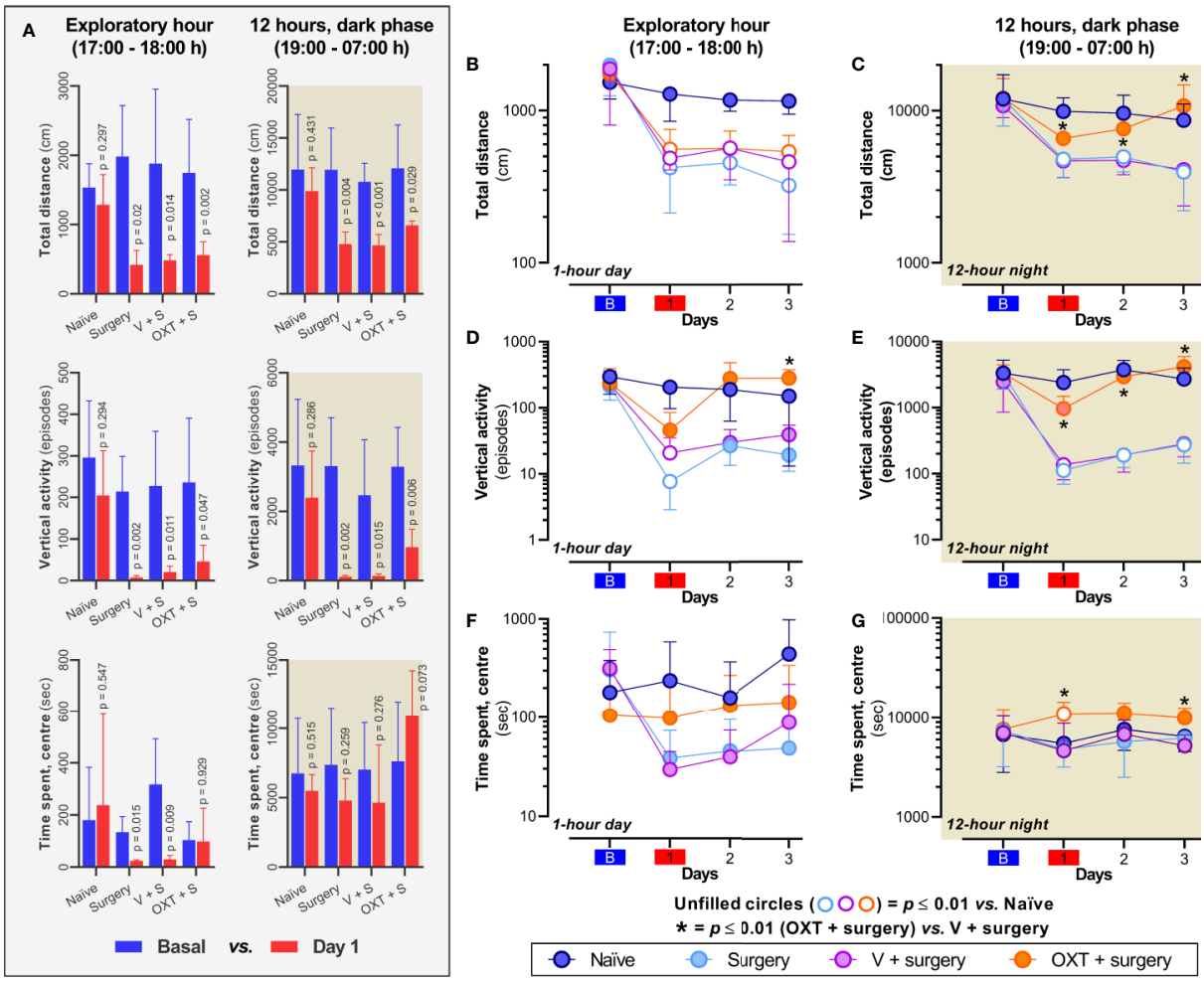

FIGURE 3 | Intrathecal oxytocin (OXT; 10 nmol) consistently improves spontaneous behavior during the dark phase. **(A)** depicts the data of spontaneous behavior before (basal) and after plantar incision in the different experimental groups; note that plantar incision impairs the *total distance traveled* and *vertical activity* in all groups, and the *time spent in the center* only was impaired during the exploratory hour in surgery and vehicle + surgery animals. **(B–G)** show the changes in *total distance*, *vertical activity*, and *time spent in the center* across four days in naïve rats and rats with: i) surgery (light blue circle); ii) vehicle (V) + surgery (magenta circle); and iii), OXT + surgery (orange circle). In surgery and V + surgery groups, incision of the hind paw induces a marked decline in the total distance [**(B, C)**] and vertical rearing activity [**(D, E)**] during the exploratory and 12-h dark phase when the rats are most active. These declines peak at day 1 in the exploratory hour and remained throughout the post-surgery test days in the dark phase. Furthermore, although no effect was observed in the group of animals that were intrathecally administered with 10 nmol OXT regarding total distance during the exploratory hour, a clear improvement in this measure was induced in the dark phase; similar results were obtained upon measuring vertical activity. Panel **(F)** showed that although a diminution in the time spent in the center was achieved after surgery in all animals, no statical difference was observed. Nevertheless, as shown in panel **(G)** an slight increase in the time spent in the centre was observed in animals treated with oxytocin. In these panels, unfilled circles represent a significant difference ($p < 0.01$) between "OXT + surgery" vs "naïve" animals, whereas (*) represent a $p < 0.01$ between "OXT + surgery" vs "V + surgery". For statistical details, see **Table 1**.

with the surgery untreated animals was low on the test days during the exploratory hour ($p > 0.2$). However, during the dark phase, the animals treated with oxytocin tended to spend more time in the center of the open field, particularly on days 1 and 3 post-surgery.

DISCUSSION

Using a surrogate model of postoperative pain, this study showed that spinal oxytocin pretreatment induces not only a diminution in the evoked mechanical hypersensitivity (**Figure 1**) but also an improvement of spontaneous activity (**Figures 2**, **3**). Apart from the implications discussed below, these data support our contention that oxytocin could have a potential effect as a preemptive analgesic drug. Furthermore, despite studies about the antinociceptive effect of spinal oxytocin, with perspectives ranging from neuropathic to inflammatory and from behavioral to electrophysiological (see Poisbeau et al., 2018 for refs.), we must acknowledge that no study has been performed using a postoperative pain model. This is a key point considering that each paradigm tested in previous reports has specific outcomes that are not interchangeable (Deuis et al., 2017). Specifically,

postsurgical pain involves lesion of the peripheral tissue, inflammatory process and injury of isolated nerves; hence, the pain induced by surgery is considered an entity apart from other types of pain (Brennan, 2011; Reddi, 2016).

As previously demonstrated (Brennan et al., 1996; Pogatzki et al., 2002; Pogatzki and Raja, 2003), paw incision has a remarkable effect on the punctate mechanical induced-hypersensitivity, an effect associated with the sensitization of spinal dorsal horn cells in response to the spontaneous activity of Aδ- and C-fibers (Vandermeulen and Brennan, 2000; Hämäläinen et al., 2002). Indeed, as shown in **Figure 1**, allodynia towards mechanical stimuli occurred after plantar incision, but in animals pretreated with oxytocin (see **Figures 1C, D**), the impact on the withdrawal threshold on day 1 after surgery was minor, suggesting a preemptive analgesic effect. Specifically, the withdrawal threshold of animals pretreated with this neuropeptide (**Figure 1C**) was non-statistically different ($p = 0.1$) to the basal value after four days, whereas the threshold value of untreated animals was not statistically different until day 6 post-surgery (**Figures 1A, B**; $p > 0.05$), suggesting that apart from the protective effect observed on day 1, oxytocin also decreases the recovery time. These data correlate with a previous electrophysiological report showing that spinal oxytocin prevents the spinal LTP (DeLaTorre et al., 2009), one of the mechanisms whereby acute pain (in this case skin incision) induces central sensitization and consequently long-term hypersensitivity (Ruscheweyh et al., 2011). We need to mention that the spinal LTP was measured as an increase in the activity of nociceptive Aδ- and C-fibers. Hence, the electrophysiological data about the preemptive antinociceptive effect of oxytocin was translatable to our behavioral experiments measuring mechanical hypersensitivity.

Certainly, to measure allodynia that reflects a better clinical condition, we used the von Frey filaments considering that after surgery, non-noxious stimuli could be the main source of evoked pain (Jensen and Finnerup, 2014). Nevertheless, although evoked nociceptive tests are useful to evaluate potential analgesics drugs, the measurement of spontaneous behavior is crucial because provides insight into the pain process under "normal" environmental conditions (Matson et al., 2007; Gould et al., 2009; Urban et al., 2011; Cho et al., 2013; Majuta et al., 2017b; Castel et al., 2018). In humans, spontaneous pain can be quantified by asking them (Gaston-Johansson et al., 1990), whereas in rodents the locomotor activity can be analyzed as a surrogate model of this outcome (Roughan et al., 2009; Majuta et al., 2018). In addition, the classical stimulus-evoked pain-like behaviors are mainly performed during the light phase of the day, which is when rodents are less active (Yasenkov and Deboer, 2012; Frank et al., 2017).

In this sense, using an automated system we measured locomotor activity in real time for 16 h, from 17:00 to 09:00 h. When the animals were placed in the activity boxes during the light phase (17:00 h), they actively explored the new environment for approximately 1 h. After initial exploration, most of the animals remained at rest or markedly reduced their activity until the beginning of the dark phase (19:00 h) (for examples see **Figure 2**). At this point, the animals showed a significant increase in locomotor activity during the night hours (19:00–07:00 h) which decreased once the room light was turned on again (07:00 h). The increase in activity during the day (17:00–18:00 h) is consistent with exploratory behavior in search of possible threats within a new environment (Seibenhener and Wooten, 2015), while the increase in activity immediately after the room darkened (19:00–7:00 h) is consistent with the activity related to drinking, nesting, and exploring the environment to search for food. These observations and studies from several groups (Yehuda et al., 1986; Imanaka et al., 2008; Parent et al., 2012; Majuta et al., 2017a) demonstrate that spontaneous activity is reduced when there is a lesion in the body. Certainly, as discussed by Majuta et al. (2017a, 2017b, 2018), monitoring day/night activity provides an unbiased assessment of discomfort induced by paw incision and, although the common endpoint used to evaluate behavioral hypersensitivity in rodents is mechanical punctate hypersensitivity, few human studies use skin hypersensitivity as a primary endpoint. In this sense, our data using the spontaneous behavior showed that plantar incision decreases the spontaneous behavior (**Figures 2**, **3**).

As previously reported (Brennan et al., 1996; Pogatzki et al., 2002; Pogatzki and Raja, 2003), the total distance traveled, and vertical activity were reduced after paw incision in treated and untreated animals (**Figure 3A**; superior and middle panels). However, when we compared the effect of treatments on the 3 days post-surgery, we found that oxytocin clearly improved the aforementioned variables, particularly during the dark phase (**Figures 3B–E**; see **Table 1** for statistical details). Together, these data support our contention that this neuropeptide could have preemptive analgesic properties, probably by interruption of the spinal LTP (DeLaTorre et al., 2009) elicited by the plantar incision.

Considering that a previous report (Kouya et al., 2015) suggested that after plantar incision the animals develop anxiety-like behaviors (using escape-avoidance tests), we decided to analyze the time spent in the center of the cage before and after surgery. As shown in **Figure 3A** (inferior panels), we found that during the exploratory hour, untreated surgery animals spent less time ($p \leq 0.015$) in the center of the cage, but this behavior was not observed in animals treated with oxytocin ($p = 0.929$). Certainly, under light conditions, anxious animals tend to exhibit minimal exploratory behavior; thus, spending less time in the center is predictable (Castanheira et al., 2018). Accordingly, during the dark phase we did not find a substantial statistical effect before and after surgery, although a slight increase in this variable was observed in the oxytocin-treated animals ($p = 0.073$). However, when we compared the effect of treatments for 3 days post-surgery, we observed an increase in the time spent ($p \leq 0.01$) in the center during the dark phase in the oxytocin-treated animals.

Collectively, these data suggest that intrathecal oxytocin seems to induce anxiolytic effects, and current evidence shows that oxytocin induces anxiolytic-like effects when given at supraspinal levels (Ring et al., 2006; Neumann and Slattery, 2016). Therefore, the question remains as to how intrathecal oxytocin induces anxiolytic effects. Although the validation of this hypothesis requires additional experiments that fall beyond the scope of the present study, we propose that intrathecal

oxytocin could reach supraspinal levels using the cerebrospinal fluid dynamics, thus exerting its anxiolytic effects.

Finally, and considering that we injected oxytocin 10 min before the paw incision, we must to acknowledge that oxytocin when given at central level exerts antinociception between 10 and 90 min after injection (DeLaTorre et al., 2009; Sun et al., 2018; Taati and Tamaddonfard, 2018; González-Hernández et al., 2019). Certainly, the half-life of this neuropeptide at the central nervous system seems to be nearly 20 min (Mens et al., 1983), which is enough to engage intracellular mechanisms that, in turn, block neuronal sensitization. Although in our study we did not analyze the mechanisms involved in the preemptive effect, evidence has shown that at spinal level, oxytocin blocks the neuronal activity of nociceptive Aδ/C-fibers, an action blocked by oxytocin receptor antagonists (Miranda-Cardenas et al., 2006; Condés-Lara et al., 2009; Eliava et al., 2016). Furthermore, as reviewed by Poisbeau et al. (2018) and González-Hernández and Charlet (2018), at spinal dorsal horn level, this neuropeptide recruits mechanisms/circuits associated with a diminution of nociception, for example: i) inhibition of transient potassium current (I_A), ii) recruitment or enhancement of GABAergic transmission, and iii) desensitization of spinal TRPV1 channels.

In summary, this study showed that i.t. oxytocin pretreatment given before the incision of the hind paw not only reduces evoked pain-like behaviors but also improves spontaneous behaviors. Taken together, these data support our contention that preoperative i.t. oxytocin administration may be an alternative to provide preemptive analgesia. Further studies focusing on the translational aspects of spinal cord delivery are needed, as well as research about the precise pharmacodynamic/pharmacokinetics aspects of oxytocin effects.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Instituto de Neurobiología (UNAM).

AUTHOR CONTRIBUTIONS

AE, GM-L, MC-L, and AG-H designed the experiments. AE made a draft of the manuscript. AG-H was responsible for the overall direction of the project and for editing the manuscript. All authors contributed to the article and approved the submitted version.

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Contribution of Histamine to Nociceptive Behaviors Induced by Intrathecally Administered Cholecystokinin-8

Takafumi Hayashi[1], *Chizuko Watanabe*[2], *Soh Katsuyama*[3], *Yasuyuki Agatsuma*[1], *Damiana Scuteri*[4], *Giacinto Bagetta*[4], *Tsukasa Sakurada*[5]* and *Shinobu Sakurada*[2]*

[1]Laboratory of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai, Japan, [2]Department of Physiology and Anatomy, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai, Japan, [3]Center for Clinical Pharmacology and Pharmaceutics, Nihon Pharmaceutical University, Saitama, Japan, [4]Preclinical and Translational Pharmacology, Department of Pharmacy, Health Science and Nutrition, University of Calabria, Cosenza, Italy, [5]Center for Supporting Pharmaceutical Education, Faculty of Pharmaceutical sciences, Daiichi University of Pharmacy, Fukuoka, Japan

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***Correspondence:**
Tsukasa Sakurada
tsukasa@daiichi-cps.ac.jp
Shinobu Sakurada
s-sakura@tohoku-mpu.ac.jp

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The involvement of spinal release of histamine in the nociceptive behaviors induced by cholecystokinin-8 (CCK-8) was investigated in mice. Intrathecal (i.t.) injection of CCK-8 elicited the nociceptive behaviors consisting of biting and licking. The nociceptive behaviors induced by i.t. treatment with CCK-8 showed two bell-shaped patterns. The histamine H_3 receptor antagonist significantly promoted the nociceptive behaviors induced by CCK-8 at doses of 1–100 fmol and 100 pmol. The nociceptive behaviors elicited by CCK-8 was inhibited by i.t. administration of the CCK-B receptor antagonist in a dose-dependent manner, but not by the CCK-A receptor antagonist. The nociceptive behaviors induced by CCK-8 were markedly suppressed by i.t. pretreatment with antiserum against histamine and were abolished in histidine decarboxylase-deleted gene mice. In histamine H_1 receptor-deleted gene mice, the nociceptive behaviors induced at both 10 amol and 10 pmol of CCK-8 were not affected. The tachykinin neurokinin-1 (NK_1) receptor antagonists inhibited CCK-8 (10 pmol)-induced nociceptive behaviors in a dose-dependent manner. CCK-8 (10 amol)-induced nociceptive behaviors was not antagonized by co-administration with the tachykinin NK_1 receptor antagonists. The nociceptive behaviors elicited by CCK-8 were inhibited by i.t. administration of the antagonist for the *N*-methyl-D-aspartate (NMDA) receptor in a dose-dependent manner. Our results suggest that the nociceptive behaviors induced by i.t. administration of CCK-8 (10 pmol) are mediated through the spinal release of histamine and are elicited via activation of the tachykinin NK_1 and NMDA receptors, whereas the nociceptive behaviors induced by i.t. administration of CCK-8 (10 amol) are mediated through the spinal release of histamine and elicited via NMDA receptor activation.

Keywords: cholecystokinin-8, nociceptive behaviors, histamine, spinal cord, tachykinin neurokinin-1 receptor, *N*-methyl-D-aspartate receptor

INTRODUCTION

Cholecystokinin belongs to the gastrin family of peptide groups. It is widely distributed in the central nervous system and mainly present as cholecystokinin-8 (CCK-8). The sulfated octapeptide cholecystokinin, CCK-8 (H-Asp-Tyr (SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) is present in the spinal dorsal horn and the primary sensory neurons in both humans and rodents (Hökfelt et al., 1985; Klein et al., 1992). Cholecystokinin A (CCK-A) receptors which are localized in the pancreatic acinar cells and cholecystokinin B (CCK-B) receptors in the stomach and brain areas, are functional membrane receptors involved in nociceptive modulation and have been identified as endogenous receptors of CCK-8 (Dufresne et al., 2006). Enhanced expression of CCK-8 mRNA in the dorsal root ganglion due to peripheral nerve injury sensitizes the primary sensory neurons, inducing nociceptive hypersensitivity (Xu et al., 1993). Moreover, CCK-8 contributes to nociceptive hypersensitivity by exciting the same neurons (Cao et al., 2012). CCK-8 exerts an antagonistic effect on opioids by decreasing morphine-induced antinociception (Noble et al., 1999), whereas morphine-induced antinociception is potentiated by pretreatment with the CCK-B receptor oligonucleotide antisense (Vanderah et al., 1994). Previous studies using a specific antagonist of the cholecystokinin receptors have shown that CCK-8 inhibited opioid antinociception through the CCK-B receptors (Dourish et al., 1990; Pu et al., 1994; Huang et al., 2007). Moreover, antagonism of the CCK-B receptor antagonists reduces burn-induced pain (Yin et al., 2016), and deletion of CCK-B receptor-deleted gene mice reduce the sensitivity of mechanical allodynia in neuropathic pain (Kurrikoff et al., 2004). Besides opioid antagonism of CCK-8, CCK-8 may have an important role as a pronociceptive peptide considering that CCK-8 has possibility to stimulate substance P-sensitive spinal neurons (Willetts et al., 1985). The above studies show that CCK-8 and cholecystokinin receptors may elicit nociceptive activation.

In the spinal cord, pain is transmitted by various nociceptive transmitters and modulators. It is well known that histamine, glutamate and substance P, and each receptor are nociceptive transmitter or modulator in the spinal cord and primary afferent neuron. Histaminergic neurons originating from the tuberomammillary nucleus in posterior hypothalamus project various brain regions, periaqueductal gray and the spinal dorsal horn, which regulate nociceptive information. (Panula et al., 1984; Panula et al., 1989; Watanabe and Yanai, 2001). These neurons are found in the superficial laminae of the spinal dorsal horn. The mRNA signals of histamine H_1 receptor genes in the lumbar dorsal root ganglia were found in substance P and calcitonin gene-related peptidergic neurons following peripheral nerve injury in rodent (Kashiba et al., 1999). The receptors for histamine are divided into four types: H_1, H_2, H_3, and H_4 receptors. H_1 and H_2 receptors are postsynaptic receptors distributed in many parts of the brain (Panula and Nuutinen, 2013), whereas the location of H_3 receptors are in the spinal cord and on the primary sensory neurons. H_3 receptors can act as autoreceptors coupled to Gi/Go protein and control histamine synthesis and release (Morisset et al., 2000; Haas and Panula, 2003; Lin et al., 2011). H_4 receptors are found mainly in the peripheral tissues involving in immune response and allergic reactions. Besides H_1, H_2, H_3, and H_4 receptors, histamine may modulate the response of the *N*-methyl-D-aspartate (NMDA) receptors in the central nervous system by a mechanism that does not involve H_1, H_2, H_3, and H_4 receptors (Williams, 1994; Brown et al., 2001). Histamine acting as a neurotransmitter or modulator within the brain and spinal cord, as well as on the primary neurons, can modulate pain transmission (Schwartz et al., 1991; Andrew and Craig, 2001; Sakurada et al., 2002; Hough and Rice, 2011). In behavioral research, intrathecal (i.t.) administration of histamine evoked nociceptive behaviors consisting of scratching, biting, and licking (Sakurada et al., 2003; Watanabe et al., 2008).

Glutamate receptors, both ionotropic and metabotropic, are expressed on presynaptic terminal of spinal cord, where they regulate neurotransmitter release. The spinal cord NMDA receptors play an important role in the nociceptive transmission (Dingledine et al., 1999; Sandkühler, 2000) and have attracted particular attention because of their involvement in the modulation of nociception. The primary afferent nociceptors primarily terminate in superficial laminae, whereas they connect to postsynaptic neurons in the spinal dorsal horn (Nagy et al., 2004). The NMDA receptors are heterotetrameric channels composed of seven subunits (NR1, 2A-D, and N3A + B) that form a glutamate-gated ion channel (Dingledine et al., 1999; Kew and Kemp, 2005; Ogden and Traynelis, 2011). The NR2 binding to glutamate activates a binding site to glutamate and opens the Ca^{2+}/Na^{+} channel, thus resulting in neural excitation. The opening channel plays an important role in nociceptive transmission in the spinal cord (Dickenson et al., 1997). The NR1 binding to glycine are ubiquitously distributed in all laminae of the spinal cord (Nagy et al., 2004; Ogden and Traynelis, 2011), whereas NR2A, NR2B, and NR2D subunits are detected mainly in the superficial dorsal horn of the spinal cord (Boyce et al., 1999; Nagy et al., 2004). Polyamines modulate functions of the NMDA receptors through the polyamine recognition site on the NR1 subunit of the NMDA receptor complex, whereas histamine modulates polyamine recognition near site, which is called histamine binding site of the NMDA receptor (Burban et al., 2010). Histamine can imitate the action of polyamines on the NR1 subunit of the NMDA receptor ion-channel complex (Chizh et al., 2001) Glutamate released from the primary sensory neurons sensitized by noxious stimuli (Miller et al., 2011), can interact with the NMDA receptors on the cell body of postsynaptic neurons for nociceptive transmission (Verkhratsky and Kirchhoff, 2007).

It is the first report to find that i.t. administered CCK-8 induces the nociceptive behaviors, therefore the mechanism of the CCK-8-induced nociceptive behaviors is unknown.

In the current study, we found that i.t. administration of CCK-8 at extremely low doses (1 zmol–100 pmol) elicited the nociceptive behavioral response consisting of biting and licking of the hind paw and the tail along with hindlimb scratching directed toward the flank which is similar to those induced by spermine, histamine, NMDA, and tachykinin NK_1 receptor agonists. Therefore, the mechanism of the nociceptive

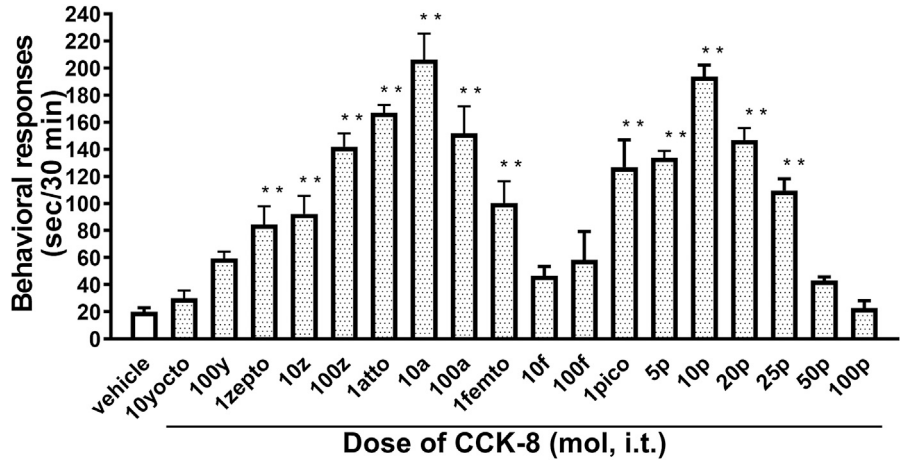

FIGURE 1 | Effect of varying doses of i.t. injected CCK-8. Groups of mice were administered CCK-8 i.t., and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. *F*-value of the one-way ANOVA is $F[18, 171] = 23$ ($p < 0.0001$). $^{**}p < 0.01$ when compared with vehicle-control.

behaviors induced by i.t. administration of CCK-8 was investigated using antagonists of the CCK-A, CCK-B, tachykinin NK_1, and NMDA receptors.

MATERIALS AND METHODS

Animals

The experiments were performed with the approval of the Committee of Animal Experiments in Tohoku Medical and Pharmaceutical University and conformed to their guidelines. Male ddY mice (Japan SLC, Inc., Hamamatsu, Japan), histidine decarboxylase-deleted gene mice (Ohtsu et al., 2001), histamine H_1 receptor-deleted gene mice (Inoue et al., 1996), and the respective wild-type mice weighing 20–24 g were used. Histidine decarboxylase-deleted gene mice and histamine H_1 receptor-deleted gene mice were previously reported in detail (Mobarakeh et al., 2000; Yanai et al., 2003; Yoshida et al., 2005). Histidine decarboxylase-deleted gene mice and histamine H_1 receptor-deleted gene mice were analyzed by PCR of genomic DNA from tail biopsies in order to verify whether the histidine decarboxylase or H_1 receptor were absent in mice. The wild-type mice corresponding to respective deleted gene mice were used as controls. They were housed in cages with 5–6 weight-matched animals and placed in a colony room. Animals were housed with free access to standard food (F-2, Funabasi Farm, Co., Funabashi, Japan) and tap water in an air-conditioned room under a constant 12:12 h light/dark cycle (light on at 7:00 AM and off at 7:00 PM) at 22–24°C and 50–60% relatively humidity. Animals were used after breeding in the examination room for at least 2 days.

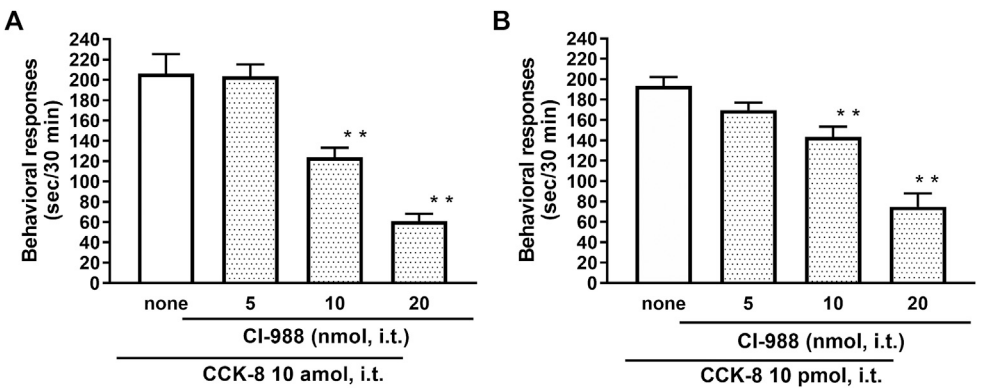

FIGURE 2 | Effect of CI-988 (CCK-B receptor antagonist) on CCK-8 (**(A)**: 10 amol **(B)**: 10 pmol)-induced behavioral responses. CI-988 was co-administered i.t. with CCK-8, and nociceptive behaviors were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. *F*-values of the one-way ANOVA are **(A)**; $F[3, 36] = 30.74$ ($p < 0.0001$) **(B)**; $F[3, 36] = 25.96$ ($p < 0.0001$). $^{**}p < 0.01$ when compared with CCK-8 alone.

TABLE 1 | Effect of SR27897 (CCK-A receptor antagonist) on CCK-8-induced nociceptive behaviors in mice.

Treatment	Mean ± S.E.M.
CCK-8 (10 amol)	206.3 ± 19.1
+ SR-27897 (10 nmol)	178.9 ± 14.4
CCK-8 (10 pmol)	193.6 ± 8.6
+ SR-27897 (10 nmol)	175.2 ± 15.5

The nociceptive behaviors induced by i.t. injection of CCK-8 was observed for 30 min. SR27897 was co-administered i.t. with CCK-8 (10 amol or 10 pmol). The total time spent in nociceptive behaviors (s) for 30 min is represented as the mean ± S.E.M.

Drugs and Treatment

The drugs used were CCK-8 (cholecystokinin octapeptide (sulfated) ammonium salt, Bachem, Bubendorf, Switzerland), 2-[[[4-(2-chlorophenyl)-2-thiazolyl]amino]carbonyl]-1*H*-indole-1-acetic acid (SR27897, Tocris Bioscience, Bristol, United Kingdom), 4-[[(1*R*)-2-[[(2*R*)-3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo [3.3.1.13,7] dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxobutanoic acid (CI-988, Tocris Bioscience), histamine monoclonal (mouse) antibody (Bertin Bioreagent, Montigny le Bretonneux, France), thioperamide (Sigma-Aldrich, St. Louis, MO, United States) (2*S*,3*S*)-3-(2-methoxybenzylamino)-2-phenylpiperidine dihydrochloride (CP-99994, Sigma-Aldrich) [Tyr^6, D-Phe^7, D-His^9]-substance P (6–11) (sendide, Enzo Life Sciences, Farmingdale, NY, United States), *N*-(Bz)Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 (GR94800, Tocris Bioscience), agmatine sulfate (Tocris Bioscience), arcaine (Tocris Bioscience) (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [*a*,*d*]cyclohepten-5,10-imine maleate (MK-801, Tocris Bioscience), D-(-)-2-amino-5-phosphonopentanoic acid (D-APV, Tocris Bioscience), and (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, Tocris Bioscience). All other drugs were dissolved in artificial cerebrospinal fluid (aCSF, Tocris Bioscience). Intrathecal injection (i.t.) was performed following the method described by Hylden and Wilcox (Hylden and Wilcox, 1980). The volume injected was 5 μL.

Behavioral Procedures

To reduce of variability, mice were acclimated to individual plastic observation cages (22.0 × 15.0 × 12.5 cm) for approximately 1 h before i.t. injection. Immediately after the i.t. injection, the mice were placed in the transparent cage and the accumulated response time of biting and/or licking of the hindpaw and the tail, and along with reciprocal hindlimb scratching, was measured for 30 min at 5 min intervals using a stopwatch. The total response times of these nociceptive behaviors were pooled and recorded as single value for each animal.

Statistical Analysis

The time of nociceptive behaviors for each group was presented as the mean ± S.E.M. Each data was calculated with a computer-associated curve-fitting program (GraphPad Prism version 8.4.3; GraphPad Software, San Diego, CA, United States). Statistical significance of the differences between the groups was established using Dunnett's test for multiple comparisons after analysis of variance (ANOVA).

RESULTS

Cholecystokinin-8-Induced Nociceptive Behaviors and Effects of Antagonists for the Cholecystokinin-A and Cholecystokinin-B Receptors on Nociceptive Behaviors Induced by Cholecystokinin-8

The nociceptive behaviors induced by treatment with CCK-8 were observed in mice. I.t. injection of CCK-8 elicited the nociceptive behaviors consisting of biting and licking, and a little scratching in mice. The nociceptive behaviors induced by CCK-8 were measured for 30 min. The CCK-8-induced nociceptive behaviors was evoked significantly 5–10 min after

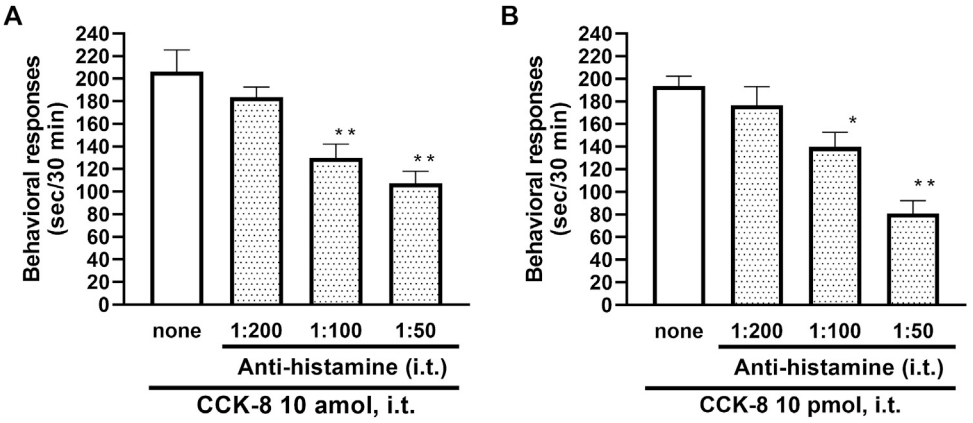

FIGURE 3 | Effect of histamine antibody (anti-histamine) on CCK-8 (**(A)**: 10 amol **(B)**: 10 pmol)-induced behavioral responses. Anti-histamine was treated i.t. 5 min prior to CCK-8 treatment, and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. *F*-values of the one-way ANOVA are **(A)**; F [3, 36] = 11.87 ($p < 0.0001$) **(B)**; F [3, 36] = 15.52 ($p < 0.0001$). $^{**}p < 0.01$ and $^{*}p < 0.05$ when compared with CCK-8 alone.

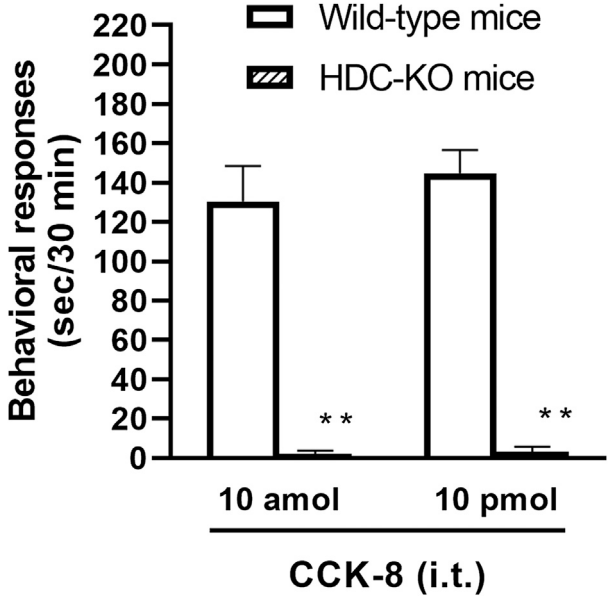

FIGURE 4 | CCK-8 (10 amol, 10 pmol)-induced nociceptive behaviors in histidine decarboxylase-deleted gene mice (HDC-KO) and their wild-type mice. Groups of mice were administered CCK-8 i.t., and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. $^{**}p < 0.01$ when compared with wild-type mice.

i.t. injection and reached a maximum at 20–25 min. CCK-8-induced nociceptive behaviors showed bell-shaped patterns with two peaks from 1 zmol to 1 fmol and from 1 to 25 pmol, while the maximum effect of CCK-8 was observed at 10 amol and 10 pmol (**Figure 1**). The nociceptive behaviors elicited by CCK-8 (10 amol and 10 pmol) was inhibited by i.t. administration of the CCK-B receptor antagonist CI-988 in a dose-dependent manner (**Figure 2**). The CCK-A receptor antagonist, SR-27897, at a dose of 10 nmol had no effect on the response elicited by CCK-8 (**Table 1**).

Involvement of Histamine Release on Cholecystokinin-8-Induced Nociceptive Behaviors

To clarify the involvement of spinal release of histamine on nociceptive behaviors induced by CCK-8, the effect of an antiserum against histamine on the CCK-8-induced nociceptive behaviors was determined. Groups of mice were pretreated with i.t. antiserum against histamine (1:200–1:50 dilution) 5 min prior to i.t. treatment with CCK-8 (10 amol or 10 pmol) and the nociceptive behaviors induced by CCK-8 were measured for 30 min. I.t. pretreatment with an antiserum against histamine suppressed the CCK-8 (10 amol and 10 pmol)-induced nociceptive behaviors in a dilution-dependent manner (**Figure 3**). The involvement of endogenous spinal histamine in the nociceptive behaviors induced by CCK-8 was confirmed using histidine decarboxylase-deleted gene mice. The nociceptive behaviors induced at both 10 amol and 10 pmol of i.t. administered CCK-8 were abolished in histidine decarboxylase-deleted gene mice, compared to wild-type mice (**Figure 4**).

Involvement of the Histamine Receptors on Cholecystokinin-8-Induced Nociceptive Behaviors

The involvement of the histamine H_1 receptors in CCK-8-induced nociceptive behaviors were determined using histamine H_1 receptor-deleted gene mice. The nociceptive behaviors induced by i.t. administered CCK-8 at both 10 amol and 10 pmol were not affected in histamine H_1 receptor-deleted gene mice, compared to wild-type mice (**Figure 5**).

The involvement of the histamine H_3 receptors in CCK-8-induced nociceptive behaviors were determined in ddY strains of mice. Under the presence of the histamine H_3 receptor antagonist, thioperamide at a dose of 6 nmol, CCK-8 produced similar degrees of the nociceptive behaviors at any doses (**Figure 6**).

Involvement of Tachykinin Neurokinin$_1$ and Neurokinin$_2$ Receptor Antagonists on Cholecystokinin-8-Induced Nociceptive Behaviors

The involvement of the tachykinin NK_1 receptors on the nociceptive behaviors induced by CCK-8 were examined in mice. Groups of mice were i.t. co-administered a tachykinin NK_1 receptor antagonist CP-99994 (0.25–2 nmol) or sendide (0.25–2 pmol) with CCK-8 (10 amol or 10 pmol). Sendide, a

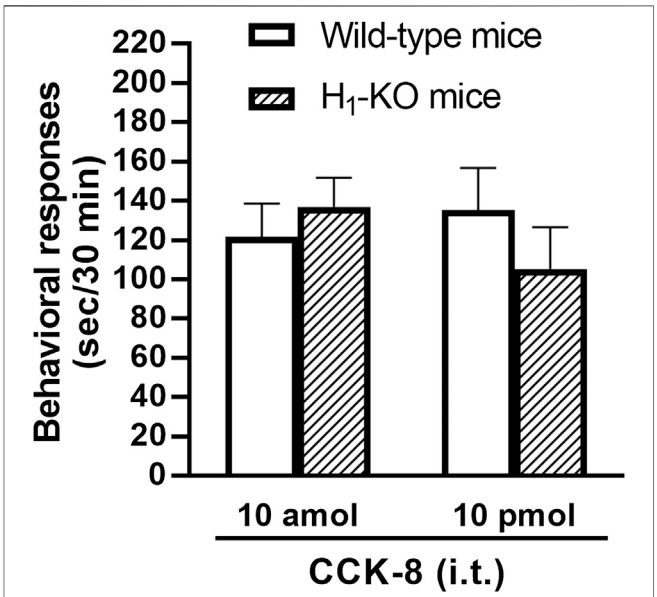

FIGURE 5 | CCK-8 (10 amol, 10 pmol)-induced nociceptive behaviors in histamine H_1 receptor-deleted gene mice (H_1-KO) and their wild-type mice. Groups of mice were administered i.t. CCK-8, and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group.

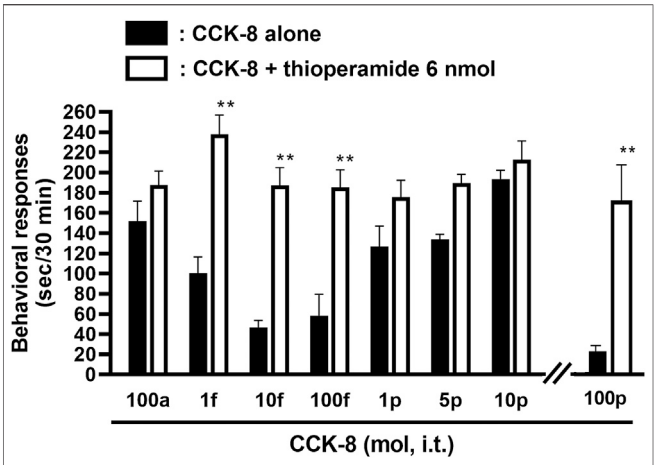

FIGURE 6 | Effect of thioperamide (histamine H_3 receptor antagonist) on CCK-8-induced behavioral responses. Thioperamide was co-administered i.t. with CCK-8, and nociceptive behaviors were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. $^{**}p < 0.01$ when compared with each dose of CCK-8 alone.

tachykinin NK_1 receptor antagonist, can inhibit the substance P-induced nociceptive behaviors without affecting the nociceptive behaviors produced by the tachykinin NK_2 and NK_3 receptor agonists (Sakurada et al., 1992). Co-administration with CP-99994 or sendide eliminated the nociceptive behaviors induced by 10 pmol of CCK-8 (**Figures 7B,D**). No significant reduction of the CCK-8 (10 amol)-induced nociceptive behaviors was detected with CP-99994 or sendide (**Figures 7A,C**). No significant reduction of the CCK-8 (10 amol or 10 pmol)-induced nociceptive behaviors was detected on co-administration with GR94800, a selective tachykinin NK_2 receptor antagonist (**Figures 7E,F**).

Involvement of Antagonist for *N*-methyl-D-aspartate Receptor on Cholecystokinin-8-Induced Nociceptive Behaviors

The NMDA receptors have multiple ligand-binding sites, having a binding site to glutamate, a binding site to glycine, an ion channel modulator-binding site, and a polyamine binding site. Therefore, the involvement of NMDA receptors on nociceptive behaviors induced by CCK-8 was examined in mice. The nociceptive behaviors induced by CCK-8 at both 10 amol and 10 pmol were attenuated by the i.t. co-administration of agmatine (0.3125–20 pmol) (**Figures 8A,B**) and arcaine (3.25–120 pmol) (**Figures 8C,D**), antagonist for NMDA receptor polyamine-binding site, in a dose-dependent manner. Co-administered MK-801 (0.078125–10 nmol) (**Figures 9A,B**), an antagonist for the ion channel modulator-binding site, D-APV (6.25–1,000 pmol) (**Figures 9C,D**) or CPP (3–30 pmol) (**Figures 9E,F**), antagonists for the glutamate binding site of the NMDA receptors eliminated the nociceptive behaviors induced by CCK-8 (10 amol or 10 pmol).

DISCUSSION

CCK-8 has been identified in the central nervous system of mammalian species. CCK-8 is distributed throughout the periaqueductal gray, ventromedial thalamus, and spinal dorsal horn, which are areas known to be associated with the nociceptive information (Baber et al., 1989). In the present study, the mechanism of the CCK-8-induced nociceptive behaviors was investigated in the mouse spinal cord. We found that i.t. administration of the CCK-8 induced nociceptive behaviors, mainly consisting of biting and licking, which are similar to that seen after i.t. injection of nociceptin or spermine (Tan-No et al., 2000; Sakurada et al., 2004; Mizoguchi et al., 2017), and i.t. administration of CCK-8 from 1 zmol to 1 fmol and from 1 pmol to 25 pmol each elicited the nociceptive behaviors in mice in two bell-shaped patterns (**Figure 1**). Surprisingly, 1 zmol, a minimum dose of CCK-8, significantly induced the nociceptive behaviors (**Figure 1**). It may be the lowest dose of any drug or compound to elicit pharmacological effect. To consider the mechanism of the CCK-8-induced nociceptive behaviors, the present study was done using 10 amol (a low dose) and 10 pmol (a high dose) of CCK-8. The nociceptive behaviors induced by i.t. administration of CCK-8 at both 10 amol and 10 pmol were antagonized by co-administered CI-988, a CCK-B receptor antagonist, and had no effect with SR27987, a CCK-A receptor antagonist (**Figure 2**; **Table 1**). Our results showed that the nociceptive behaviors induced by CCK-8 in the spinal cord were mediated by the spinal CCK-B receptor.

The involvement of the spinal release of histamine in nociceptive behaviors induced by CCK-8 was examined in mice. We found that i.t. administration of CCK-8 at both 10 amol and 10 pmol decreased nociceptive behavior after pretreatment with intrathecally administered antiserum against histamine (**Figure 3**). Our results showed that the nociceptive behaviors induced by i.t. administration of CCK-8 was mediated by the spinal release of histamine. This result was reinforced by the fact that the nociceptive behaviors induced by i.t. administration of CCK-8 at both 10 amol and 10 pmol was completely abolished in histidine decarboxylase-deleted gene mice (**Figure 4**). This finding shows that the CCK-8-induced nociceptive behaviors are caused through histamine released in the spinal cord. The nociceptive behaviors induced by intrathecally administered CCK-8 at both 10 amol and 10 pmol was not abolished in histamine H_1 receptor-deleted gene mice (**Figure 5**). In the histamine receptor subtypes, H_1, H_2, H_3, and H_4 receptors, in the central nervous system, the histamine H_3 receptor is expressed as a presynaptic autoreceptor (Schwartz et al., 1991; Arrang, 1994). Antagonism of thioperamide, a histamine H_3 receptor antagonist, promotes the release of histamine (Itoh et al., 1991; Mochizuki et al., 1991; Barke and Hough, 1994; Jansen et al., 1998; Hough and Rice, 2011). Thioperamide significantly promoted the nociceptive behaviors induced by CCK-8 (1 fmol, 10 fmol, 100 fmol, and 100 pmol) (**Figure 6**). Our results show that the increase in the release of histamine induced by co-administered thioperamide could lead to enhance the CCK-8-induced nociceptive behaviors.

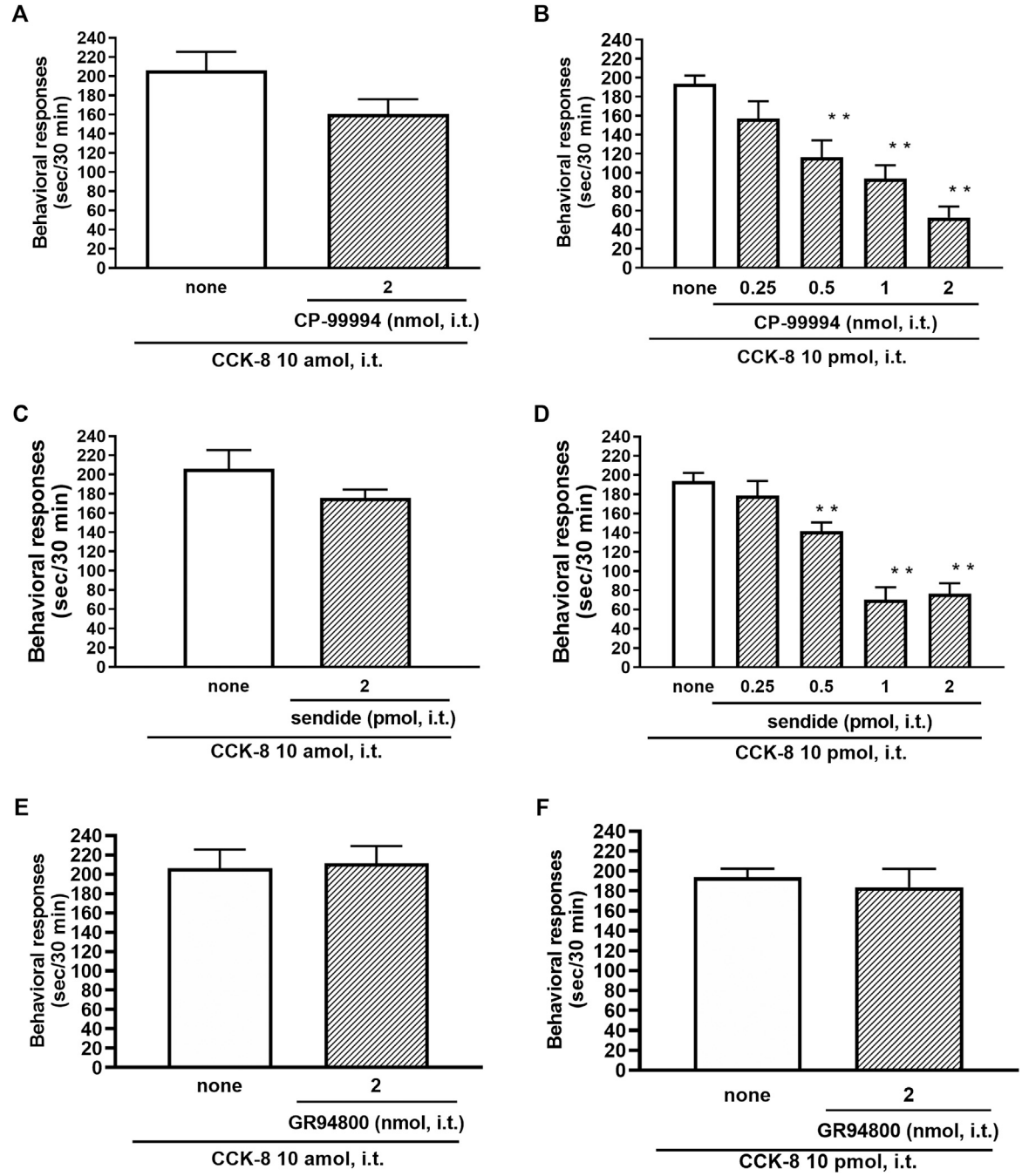

FIGURE 7 | Effect of CP-99994 **(A,B)** and sendide **(C,D)** (tachykinin NK_1 receptor antagonists), and GR94800 (tachykinin NK_2 receptor antagonist) **(E,F)** on CCK-8 (10 amol or 10 pmol)-induced behavioral responses. CP-99994, sendide, or GR94800 was co-administered i.t. with CCK-8, and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. *F*-values of the one-way ANOVA are **(B)**; F [4, 45] = 14.16 ($p < 0.0001$) **(D)**; F [4, 45] = 24.03 ($p < 0.0001$). $^{**}p < 0.01$ when compared with CCK-8 alone.

In this study, the nociceptive behaviors induced by i.t. administration of 10 pmol of CCK-8 were reduced by the co-administered tachykinin NK_1 receptor antagonists CP-99994 and sendide in a dose-dependent manner (**Figures 7B,D**). In contrast to the nociceptive behaviors induced by i.t. administration of 10 pmol of CCK-8, the nociceptive behaviors induced by i.t. administration of 10 amol of CCK-8 were not affected by the co-administered CP-99994 at a dose of 2 nmol or sendide at a dose of 2 pmol of CCK-8 (**Figures 7A,C**). The histamine-induced nociceptive behaviors were inhibited by the tachykinin NK_1 receptor antagonists (Sakurada et al., 2003). These results suggested that the nociceptive behaviors associated with i.t.

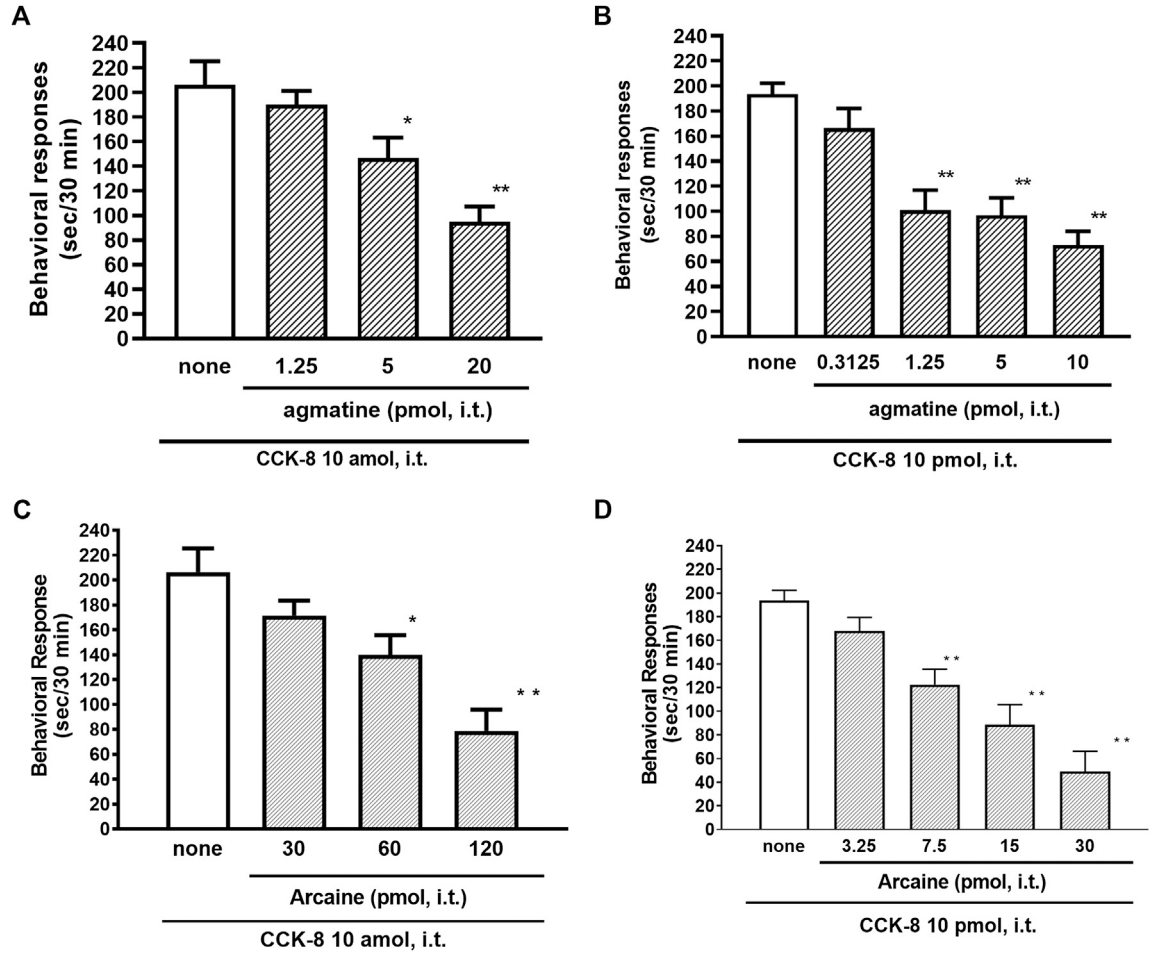

FIGURE 8 | Effect of agmatine **(A,B)** and arcaine **(C,D)** on CCK-8 (10 amol or 10 pmol)-induced behavioral responses. Agmatine or arcaine was co-administered i.t. with CCK-8, and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. *F*-values of the one-way ANOVA are **(A)**; F [3, 38] = 11.11 ($p < 0.0001$) **(B)**; F [4, 49] = 13.29 ($p < 0.0001$) **(C)**; F [3, 36] = 11.07 ($p < 0.0001$) **(D)**; F [4, 45] = 17.74 ($p < 0.0001$). $^{**}p <$ 0.01 and $^{*}p < 0.05$ when compared with CCK-8 alone.

administration of 10 pmol of CCK-8 were due to the release of substance P, which bind to the tachykinin NK_1 receptors on the dorsal spinal cord. The nociceptive behaviors associated with i.t. administration of nociceptin or spermine are due to the spinal release of histamine, which are mediated by the activation of the histamine H_1 receptors located on the primary afferent nerves and lead to the release of substance P which bind to the tachykinin NK_1 receptors on the dorsal spinal cord (Sakurada et al., 2004; Mizoguchi et al., 2017). However, the above results demonstrated that the nociceptive behaviors induced by i.t. administration of CCK-8 at both 10 amol and 10 pmol did not involve the histamine H_1 receptors (**Figure 5**).

The pharmacological similarity between substance P and CCK-8 is known on the nociceptive behaviors. CCK-8 and substance P, which are present in the capsaicin-sensitive neurons in the spinal cord, co-exist within the same fibers in the spinal dorsal horn (Dalsgaard et al., 1982; Zouaoui et al., 1990). Moreover, Willetts et al. demonstrated that spinal dorsal neurons sensitive to substance P can be excited by CCK-8 conceivably representing a pivotal nociceptive peptide in the primary sensory neuron (Willetts et al., 1985). CCK-8 was released from the spinal cord by the nociceptive stimulation (Yaksh et al., 1982). Taken together with the above reports, our current findings indicate the neuronal correlation between CCK-8 and substance P in the dorsal horn of the spinal cord. In guinea pig ileal longitudinal muscle, which mediate contractions via neurons, the CCK-8-induced contractions were mediated by the release of substance P, since a substance P antagonist reduced the maximal contraction induced by CCK-8 (Lucaites et al., 1991). Although there is no direct evidence to demonstrate that CCK-8 releases substance P, our results present the possibility that the nociceptive behaviors induced by i.t. administration of CCK-8 (10 pmol) is mediated by substance P neurons in the dorsal horn of the spinal cord, since tachykinin NK_1 receptor antagonists reduced the CCK-8-induced nociceptive behaviors.

The NMDA receptor is a heterotetrameric protein complex consisting of seven NMDA receptor subunits (NR1, NR2A-D,

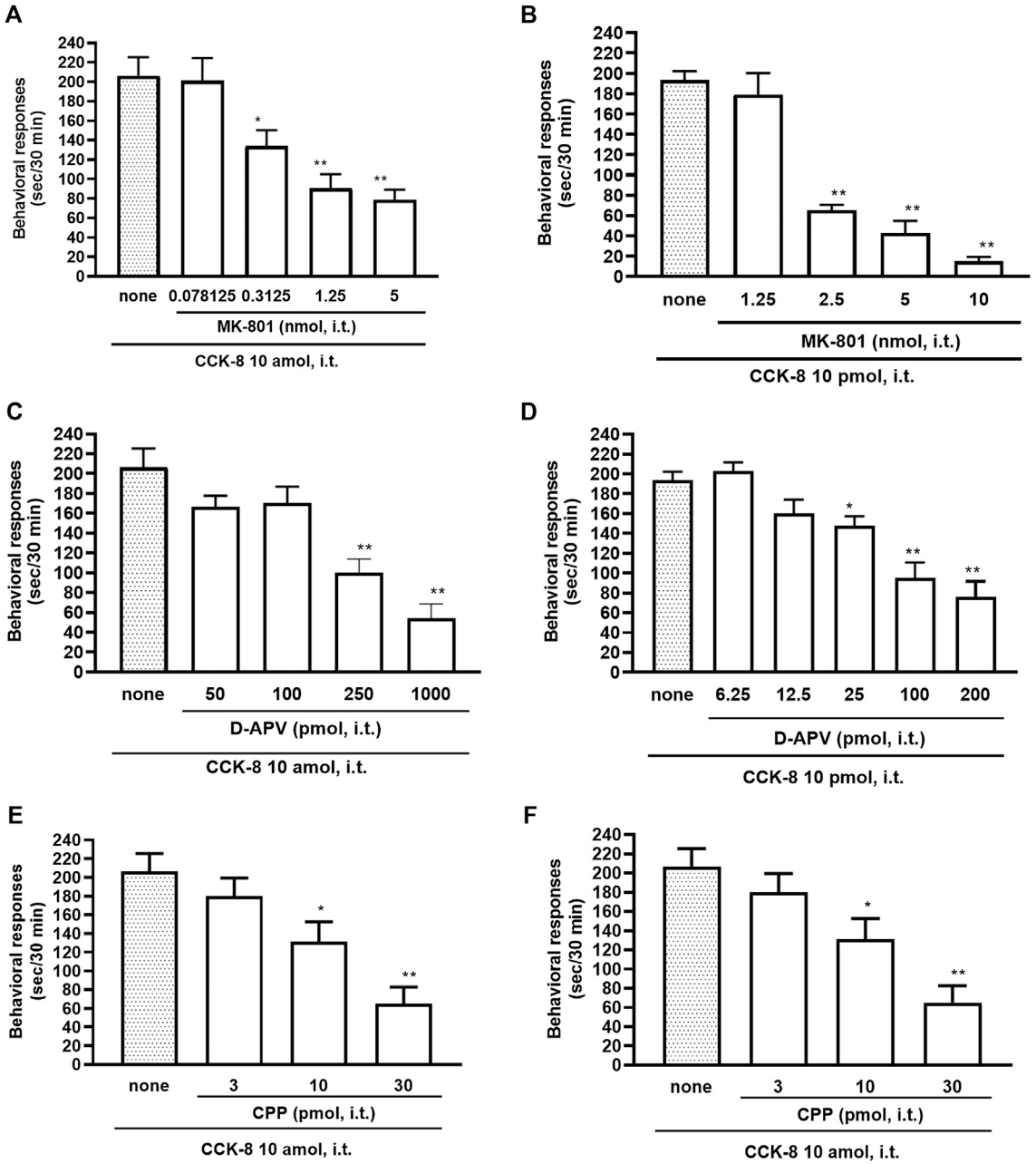

FIGURE 9 | Effect of MK-801 **(A,B)**, D-APV **(C,D)**, and CPP **(E,F)** on CCK-8 (10 amol or 10 pmol)-induced behavioral responses. MK-801, D-APV, and CPP each were co-administered i.t. with CCK-8, and nociceptive behaviors induced by CCK-8 were observed for 30 min. Each value represents the mean ± S.E.M. of 10 mice in each group. *F*-values of the one-way ANOVA are **(A)**; F [4, 52] = 12.08 ($p < 0.0001$) **(B)**; F [4, 47] = 43.75 ($p < 0.0001$) **(C)**; F [4, 49] = 15.68 ($p < 0.0001$) **(D)**; F [5, 60] = 15.88 ($p < 0.0001$) **(E)**; F [3, 36] = 10.36 ($p < 0.0001$) **(F)**; F [3, 36] = 10.16 ($p < 0.0001$). $^{**}p < 0.01$ and $^{*}p < 0.05$ when compared with CCK-8 alone.

and NR3A + B) that form a glutamate-gated ion channel, Ca^{2+}/Na^{+} channel (Dingledine et al., 1999; Kew and Kemp, 2005; Ogden and Traynelis, 2011). The NMDA receptors have multiple ligand-binding sites, having a binding site to glutamate, a binding site to glycine, an ion channel modulator-binding site, and a polyamine-binding site. Therefore, the involvement of the NMDA receptors in the CCK-8-induced nociceptive behaviors was determined by blockers of multiple ligand-binding sites of NMDA receptor. In the present study, the nociceptive behaviors induced by i.t. administration of CCK-8 at both 10 amol and 10 pmol were dose-dependently reduced by the co-administration of antagonists for the polyamine binding site, agmatine or arcaine (**Figures 8A,B**), by the co-administration of an antagonist for ion channel modulator-binding site of the NMDA receptors, MK-801 (**Figures 9A,B**), by the co-administration of antagonists for binding site to glutamate,

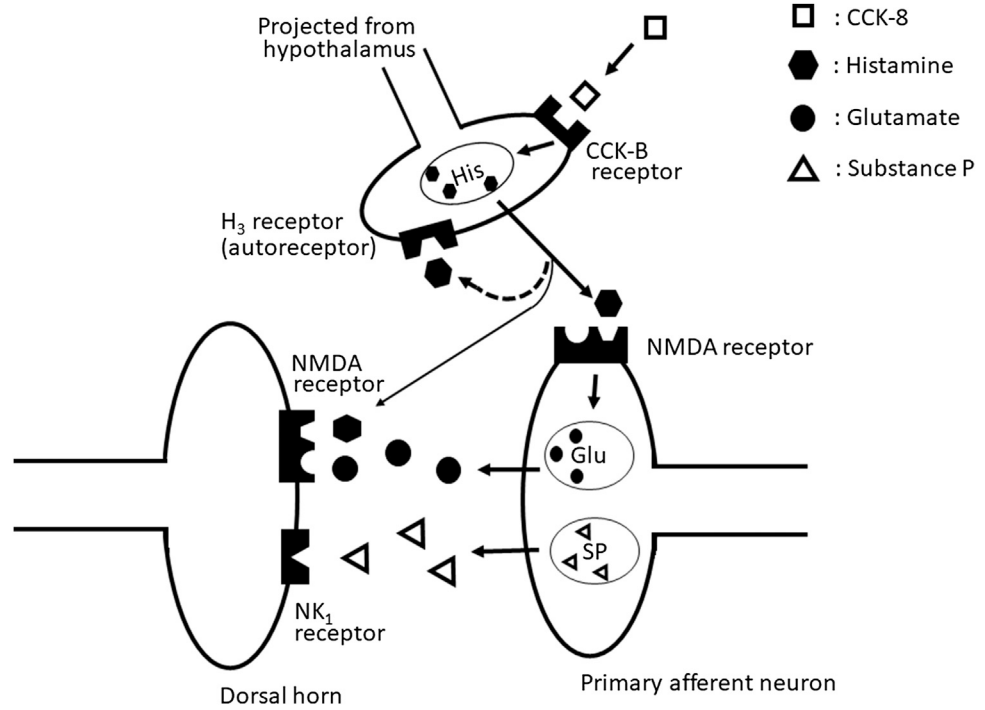

FIGURE 10 | The schematic circuit diagram of the spinal dorsal horn with speculative location of the NMDA, tachykinin NK_1, histamine H_3, and CCK-B receptors, and their transmitter containing neuron.

D-APV (**Figures 9C,D**) or CPP (**Figures 9E,F**). These results clearly show that the nociceptive behaviors associated with i.t. administration of CCK-8 at both 10 amol and 10 pmol are due to the spinal release of histamine, which leads to the release of glutamate then binding to the NMDA receptors on the dorsal horn of the spinal cord. The nociceptive behaviors induced by CCK-8 at both 10 amol and 10 pmol were strongly suppressed by agmatine and arcaine, which are antagonists for the NMDA receptor polyamine-binding site (**Figure 8**). I.t. high-dose histamine elicits the nociceptive behavior through the polyamine-binding site, ion channel modulator-binding site and binding site to glutamate on the NMDA receptors, but not H_1 and H_2 receptors, or tachykinin NK_1 receptors (Watanabe et al., 2008). The current findings indicate that pain transmission in the spinal cord involves a complicated mutual regulation between CCK-8 and histamine. The nociceptive behaviors associated with i.t. administration of CCK-8 at both 10 amol and 10 pmol may be due to the spinal release of histamine, which preferentially and directly stimulates the polyamine-binding site of the NMDA receptors, but not the histamine H_1 receptors, on the dorsal spinal cord.

Our results suggested that the nociceptive behaviors induced by i.t. administration of CCK-8 (10 pmol) are mediated through the spinal release of histamine and elicited by activating the tachykinin NK_1 and NMDA receptors, whereas it is suggested that nociceptive behaviors induced by i.t. administration of CCK-8 (10 amol) are mediated through the spinal release of histamine and are elicited by activating the NMDA receptors (**Figure 10**).

CONCLUSION

In conclusion, the nociceptive behaviors induced by i. t. administration of CCK-8 are mediated by the spinal release of histamine as the deletion of histidine decarboxylase gene eliminates symptom of nociceptive behavior induced by CCK-8. The released histamine directly stimulates the polyamine-binding site of the NMDA receptors as histamine acts on the polyamine-binding site of the NMDA receptors. Therefore, histamine indirectly activates the tachykinin NK_1 and NMDA receptors via the spinal release of substance P and glutamate, respectively.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

TH, TS, and SS were involved in protocol and project development, collected, and analyzed the data, and wrote the manuscript. CW and SK collected and analyzed the data. YA, DS, and GB were involved in protocol and project development.

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Pain in Older Adults With Dementia: A Survey in Spain

Lydia Giménez-Llort[1,2], Maria Luisa Bernal[3,4], Rachael Docking[5], Aida Muntsant-Soria[1,2], Virginia Torres-Lista[1,2], Antoni Bulbena[1] and Patricia A. Schofield[5,6]*

[1] Department of Psychiatry and Forensic Medicine, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain, [2] Institut de Neurociències, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain, [3] Department of Pharmacology and Physiology, University of Zaragoza, Zaragoza, Spain, [4] Instituto de Investigación Sanitaria de Aragón, Zaragoza, Spain, [5] Abertay University, Dundee, United Kingdom, [6] Sheffield Hallam University, Sheffield, United Kingdom

The risk of suffering pain increases significantly throughout life, reaching the highest levels in its latest years. Prevalence of pain in nursing homes is estimated to range from 40 to 80% of residents, most of them old adults affected with dementia. It is already known that pain is under-diagnosed and under-treated in patients with severe cognitive impairment and poor/absent verbal communication, resulting in a serious impact on their quality of life, psychosocial, and physical functioning. Under-treated pain is commonly the cause of behavioral symptoms, which can lead to misuse of antipsychotic treatments. Here, we present two Regional and National Surveys in Spain (2015–2017) on the current practices, use of observational tools for pain assessment, guidelines, and policies. Results, discussed as compared to the survey across central/north Europe, confirm the professional concerns on pain in severe dementia, due to poor standardization and lack of guidelines/recommendations. In Spain, observational tools are scarcely used because of their difficulty and low reliability in severe dementia, since the poor/absent verbal communication and comprehension are considered limiting factors. Behavioral observation tools should be used while attending the patients, in a situation including rest and movement, should be short (3–5 min) and scored using a numeric scale. Among the pain items to score, "Facial expression" and "Verbalization" were considered essential and very useful, respectively. This was in contrast to "Body movements" and "Vocalizations," respectively, according to the survey in central/north Europe. Scarce time availability for pain assessment and monitoring, together with low feasible and time-consuming tools, can make pain assessment a challenge. The presence of confounding factors, the low awareness and poor knowledge/education of specific tools for this population are worrisome. These complaints draw future directions to improve pain assessment. More time available, awareness, and involvement of the teams would also benefit pain assessment and management in cognitive impairment. The experiences and opinions recorded in these surveys in Spain and other E.U. countries were considered sources of knowledge for designing the "PAIC-15 scale," a new internationally agreed-on meta-tool for Pain Assessment in Impaired Cognition and the "Observational pain assessment" in older persons with dementia.

Keywords: pain, dementia, elderly, pain assessment, pain management, International "IR" framework, guidelines, impaired cognition

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****Correspondence:***
Lydia Giménez-Llort
lidia.gimenez@uab.cat

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INTRODUCTION

Pain is, in all cases, a threat to human dignity, and when it comes to avoidable pain in those who cannot properly think or speak for themselves, we find ourselves faced with an imperative to join efforts to improve the situation. The risk of suffering pain increases significantly throughout life, reaching the highest levels in its latest years. It is estimated that pain exists in 50% of community-dwelling older adults, while the prevalence in nursing homes is estimated to range from 40 to 80% of residents (1). These rates indicate a serious impact on the quality of life and psychosocial and physical functioning. Although pain is a frequent complaint in residents at nursing homes, 25% do not regularly receive pain-relieving drugs (2). The administration and prescription of pain treatment occur below the experts' recommendations (3). In the older population, factors of pain management that can explain this situation are five. First, less self-report of pain. Second, pain is often present along with multiple problems and comorbidities that complicate evaluation and treatment. Third, increased incidence of side effects. Fourth, a greater potential for adverse effects and complications secondary to treatment procedures. Finally, older adults and health professionals often assume that pain is part of aging.

While pain in older adults appears to be a challenging problem for many health care professionals, it seems that pain in older adults with dementia is even more so (4). Over 50–60% of the older population living in care homes in developed countries are affected by dementia (5), while the impact of dementia in low-to-middle income countries should not be underestimated [i.e., (6)]. About 50% of the 35 million people with dementia worldwide experience pain on a regular basis. The global burden associated with the aging population projected to happen in the coming decades demands efforts to counteract its socio-economic impact but, mostly, on the quality of life of patients, caregivers and families (7).

In fact, there is no evidence to suggest that older adults with dementia experience less pain than do those without dementia (8). Thus, pain in dementia patients is assumed to be at least as prevalent as in cognitively healthy individuals of the same age. The impact that dementia and other types of cognitive damage have on the perception and expression of pain has been scarcely studied, except in Alzheimer's disease (9, 10). In this common form of dementia, extensive evidence indicates that pain sensitivity is not only intact but may even be increased. In vascular dementia, the prevalence of pain may be even higher due to a promotion of neuropathic pain (11). However, the latest studies indicate that pain is underdiagnosed and under-treated in people with cognitive damage, especially dementia (12, 13). Thus, dementia patients receive even less pain treatment than individuals without cognitive impairment, as observed in many studies. The exact reasons for this poor treatment of dementia patients have yet to be determined (14). One of the most probable reasons for under detection of pain is because, nowadays, the diagnostic tools of pain, their classification, and evaluation, depend largely on self-reports that require the intact cognitive and communication skills. Both abilities are lost throughout the disease until they are completely absent in the more advanced stages. In this scenario, it is important to note that agitation, shouting, aggression, and increased irritability are common ways for patients with dementia to express discomfort and pain. If the latter is the case, the use of antipsychotics to manage the expression of these neuropsychiatric (NPS) or behavioral (BPSD) symptoms could be inappropriate. Atypical antipsychotics are associated with a significantly greater mortality risk than placebo (15). Increased mortality risk has also been described in cerebrovascular adverse events in elderly users of antipsychotics (16). Therefore, improvement in pain assessment in the elderly demented patients is important for the proper pharmacological pain management in this population (17, 18). Currently, the use of antipsychotics and opioids and in elderly with dementia to treat BPSD is an important topic in the pharmacological management of Alzheimer's disease (19).

In this regard, a series of specialized tools have been developed since the early 1990s to assess pain in community and nursing homes, particularly in patients with communication difficulties especially dementia (20). The pros and cons of these scales are already well known in the forums on this topic. However, due to the lack of internationally coordinated research to validate these scales and select the best available solutions, almost every hospital/clinic and each research center favors its scale. At best, the consistent practice has been developed at the national level and is only described in national guidelines (21). Since age is the main risk factor for dementia and pain, it is expected that the number of patients with dementia and so much pain will also grow. These combined circumstances are of great socio-health relevance since when dementia and pain concur, their individual and social impact multiply and require transnational solutions. It is not just that there is already evidence that pain is poorly treated in dementia. Other questions, which also require an urgent response, are those regarding neurophysiological aspects of pain in dementia and pain management throughout the disease, including end-of-life dementia stages. For now, the lack of validated pain assessment tools in older people with cognitive damage, especially dementia, has thus far impeded important advances.

The COST (European Cooperation in Science and Technology) Action TD1005 "Pain Assessment in Patients with Impaired Cognition, especially Dementia" led by Stephan Lautenbacher was established to address the issues related to pain assessment and dementia [(20), see also **Table 1**]. The major aim was to develop PAIC, a comprehensive and internationally agreed-on assessment toolkit for older adults targeting the various subtypes of dementia and various aspects of pain, including pain diagnostics, cognitive examination and guidelines for proper assessment. In this context, Working Group 2 (WG2) developed a survey that was designed to explore the existing use of pain assessment tools and guidelines and develop an understanding of the practical considerations required to facilitate their use within clinical settings. This survey was conducted across the central European participating countries, including questions about participants' knowledge and use of existing pain assessment guidelines, the usage of existing pain assessment (observational tools) and the experience health

TABLE 1 | State-of-art of challenges in pain assessment and management in the elderly and people with cognitive impairment, especially dementia.

Challenges pain management in aging and cognitive impairment
Conditionants of pain management in the elderly
1. Worse **self-report**
2. Pain is **comorbid** to pluripathology and comorbidities that complicate the evaluation and treatment
3. More incidence of **side effects** + more potential for **adverse effects** and **iatrogenesis** due to the procedure of treatments
High prevalence, needs and challenges pain diagnosis in cognitive impairment
1. **Epidemiology**: 80% in nursing homes, 50% at home are in pain
2. Cognitive decline associated to aging/MCI/Neurodegeneration
3. Pain = **Discomfort + Burden** physical-phychological-social levels
4. Infra-diagnosed
5. Infra-treated
6. Loss of **verbal communication**
7. Equal detection – pathology and stage of dementia influence the perception of pain and the pain related behavior
8. Higher tolerance
9. **NPS/BPSD** – pain manifests as agitation, shouting, aggression
10. **Scales** proxi **behavior of pain**: possible presence/absence, intensity, behavior frequency, six behavioral dimensions **FACS, vocalization, body movements, behavioral changes**, physiological changes, physical changes

Key words are indicated in bold. NPS/BPSD, Neuropsychiatric symptoms/Behavioral and Psychological Symptoms associated to Dementia; FACS, Facial Action Coding System.

care professionals have of implementing the tools. Recently, this survey results in central-north Europe have been reported (22), and here we present the results from southern Europe (Spain). The preliminary analysis on one of the "open questions" regarding the professional concerns on problems experienced in Spain and solutions for its improvement was presented for discussion with the scientific community of the International Psychogeriatric Association in our last IPA International Congress (23). In the present work, the results of that question have been further analyzed.

Therefore, the aims of the present study were three. First, to conduct a preliminary survey in Spain to estimate the implementation and use of pain scoring systems in old patients with dementia and their reliability. Second, to survey on the use of standards and guidelines for pain assessment in people with cognitive impairment/dementia in Spain and, particularly, of the use of behavioral assessment scales. Finally, to obtain a detailed analysis of the current problems that health/care staff encounter and, learning from their experience and opinion, what would help improve their management.

METHODS

In the Preliminary Regional Survey, a short online questionnaire consisting of the "General information about professionals and patients" of the PAIC study was sent to an opportunistic sample of healthcare professionals (nurses and physicians) from hospitals, nursing homes, and daycare centers in Catalonia, Aragón and Comunitat Valenciana, three neighboring Spanish autonomous communities.

After this preliminary survey, the self-administered "Questionnaire about the use of standards/guidelines, instruments and professional's profile" set up by the members of WG2 of the COST Action TD1005 (22) was translated into Spanish using a backward, forward procedure. Minor modifications were necessary to clarify aspects of the different levels of qualifications and healthcare settings.

The WG2 developed the survey questions in English. The survey's focus was to explore practitioners' current use and opinions on the usefulness and usability of existing tools to identify attitudes toward assessment tools and possible barriers to their implementation.

Together with sociodemographic data, the survey included both open-ended and multiple-choice questions about participants' knowledge, use of existing pain assessment tools, and the experience healthcare professionals have of using these tools in daily practice.

The instrument contained 36 questions. In Spain's final instrument, five more questions were added to record the level of confidence the professionals had on the scales and their ability to score pain in patients with no cognitive impairment, mild cognitive impairment or dementia, and moderate/severe cognitive impairment or dementia.

The National Survey was sent online to the sampling frame consisting of all the hospitals registered at the Spanish National Health System. An opportunistic sample of nursing homes and day care centers was also included. The survey was conducted between February 2015 and November 2017.

DATA COLLECTION

The National Survey used a probability sample of healthcare professionals. As in the previous study (22), a sample size calculation was not performed since the study aimed to describe the currently used guidelines or observational tools for pain assessment amongst older adults with cognitive impairment. The response rate was very low, with no answers from the southern part of the country. Three rounds of submissions were performed. Besides, targeted strategies were adopted to circulate the link for the web-based questionnaire. Thus, the survey was also announced on the COST-Action TD1005 website and distributed via the newsletter of other health and mental health professional groups: Sociedad Española de Infermería Geriátrica (Spanish Society of Geriatric Nursery), Master in Psychogeriatry UAB, CORE Salut Mental de Catalunya, Germanes Hospitalaries del Sagrat Cor de Jesús de Martorell, UVaMiD Unitat de Valoració de la Memòria i la Demència a Salt - Girona, and Instituto Aragonés de Salud. Respondents were not required to enter their names in the survey, and therefore it was completed anonymously. Respondents had the opportunity to stop completion of the survey at any moment, which could result in an incomplete survey.

ANALYSIS

Quantitative data were analyzed using SPSS version 25, and descriptive statistics were performed. For the multiple-choice answers, valid percentages were used given the variation in the

number of responses per question. The open-ended questions were analyzed using content analysis. The comments to the open-ended questions were analyzed using deductive classification and superordinate categories created with an open matrix (24). A consensual agreement was evaluated by having a peer group reviewing the data to verify the responses on the open-ended questions and categories. In case of disagreement between the two researchers, it was agreed upon to discuss the differences and seek for consensus.

RESULTS

Preliminary Regional Survey

In the preliminary regional survey, 64 professionals from Catalonia, Aragón, and Comunitat Valenciana answered the questionnaire; they were mostly women +30 years old, mainly physicians and nurses. Of both sexes, their patients were mostly +60 years old, diagnosed with Alzheimer's disease, vascular, or mixed dementias and + 3Reisberg GDS stage. The duration of acquaintance with the patients was high since most of them were institutionalized patients. The 51.6% of professionals asked scored their capacity to evaluate pain between 7 and 8, with nurses self-reporting a higher score than physicians. 59.7% of professionals use pain scale (mostly EVA and observational scales), being bit more used in nursing homes than in hospitals. Forty-five percentage of professionals used the pain instruments daily or at least once per week. Overall, the reliability given to the scales was 1 or 2 ranks lower than for their own professional ability to detect pain in the patients (**Figure 1**).

Participants in the National Survey

The results summarize the answers of 64 Spanish health professionals working with older people with cognitive impairment (dementia). Despite respondents having the opportunity to stop completing the survey at any moment, which could result in an incomplete survey, all the submissions were completed surveys. Noticeable, nor submissions were received from the southern area of Spain in any of the three rounds. **Figure 2** depicts the participants (**Figure 2A**), institutional (**Figure 2B**) and dementia wards (**Figure 2C**) profiles. The participant's age was normally distributed, from 21 to 65 years old, with 1/3 of the participants being 31–40 years old. Concerning their sex, the sample of participants was enriched in females (64%). There was a similar composition of registered nurses, medical practitioners, and therapeutic professions (physiotherapists, occupational therapy, psychologists). They worked in public and private hospitals, nursing homes, and day-care centers of 16 different regional areas of Spain (see map in **Figure 2C**). Most of the institutions had a dementia ward (60.9%), which was specialized in 43.8% of cases (**Figure 2C**). Several types of dementia were referred by participants as those requesting their professional attention, ranging from 56.1% of professionals taking care of Alzheimer's disease patients to 31.6% of them caring for people with Lewy body dementia.

Pain Education

Only 14% of participants have ever received post-registration education relating to pain assessment in people with cognitive impairment. Similarly, only 19% reported that case conferences or multidisciplinary team meetings are held about managing pain in cognitive impairment. Accordingly (see **Figure 2A**), 58% of professionals reported feeling very (32%) or slightly (27%) dissatisfied with their knowledge about this important issue, despite their efforts to do it well, paying special attention and taking into account the family and caregivers reports. Thus, there are few opportunities for multidisciplinary team meetings or specialized pain education, mostly thanks to pain committees and online courses of pain education for health professionals. However, health professionals that received them reported high satisfaction with their knowledge and management of pain.

Use of Standards/Guidelines

On the use of rules and guidelines, self-reporting pain assessment tools were the most recommended (50%) for people able to use self-report (i.e., with mild dementia/cognitive impairment) (**Figure 3A**). However, a high percentage (35.7%) of their institutions do not recommend a tool (**Figure 3A**); this increased to 59% for those unable to use self-report (**Figure 3B**). Interestingly, behavioral/observational instruments (e.g., Abbey, PainAD) were poorly recommended in people with dementia with mild (12.5%, **Figure 3A**) to moderate/severe conditions (17%, **Figure 3B**).

In fact, when asking about the source of recommendations, the participants informed that the use of national/international standards/guidelines in their institutions or the existence of local policies for pain assessment in people with cognitive impairment associated to dementia was poor (**Figure 4A**). Thus, more than half of professionals (65%) indicated no guidelines or local policy (48%) or did not know if their institution had any (17%), in contrast to that 35% of professionals that use a local policy and/or national/international guidelines (**Figure 4A**). The ratio of organizations reporting auditing pain assessment in older people with dementia was low, as it could only be confirmed by 9% of participants (**Figure 4A**).

Use of Observational Pain Assessment Tools

As shown in **Figure 4B** on the use of observational instruments, only 35.7% of participants use them for pain assessment in people with dementia in the current practice. In fact, EVA, PAINAD-sp and observational scales were the most commonly used pain assessment scales reported. Overall, the participants find these tools difficult (28%) or very difficult to use (10%), they have medium to high reliability, but none of the participants gave them a 10 out of 10. Thirty-two percent do not use observational tools.

Regarding the participants opinion about the clinical settings to use of observational instruments (**Figure 4C**), they informed that the behavioral pain assessment is mostly (80%) conducted while caring for a patient and over a period of time involving rest and movement (61%) or at rest (30%). When asking about

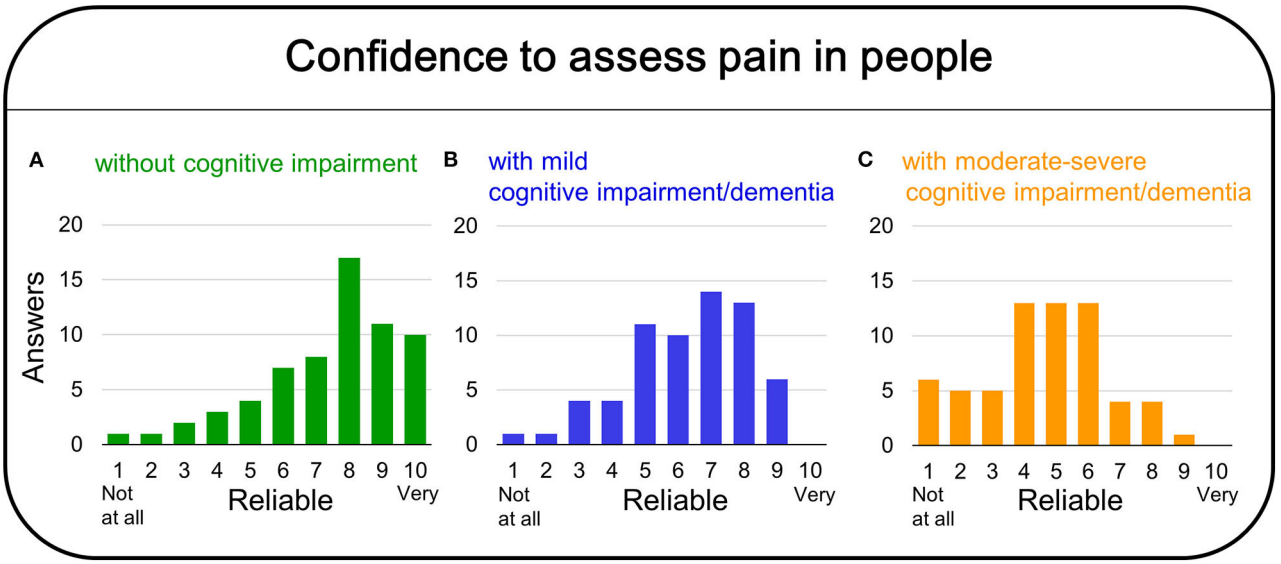

FIGURE 1 | Preliminary regional survey on the confidence on pain assessment for people without cognitive impairment **(A)**, mild cognitive impairment/dementia **(B)**, and moderate/severe cognitive impairment/dementia **(C)**.

preferences, the professionals considered it ideal for a behavioral pain assessment to take 3–5 min (43%) or 1–2 min (36%).

Regarding the utility of the pain items in the observational instruments (**Figure 5**), facial expression (41%) followed by changes in activity patterns or routines (28%) were considered by professionals as essential elements of a behavioral pain assessment. Verbalizations (40%), followed by vocalizations (34%), body movements and changes in interpersonal interactions (both 32%), and changes in mental status (27%) were considered as very useful (see **Figures 5A,B**).

In the different institutions, pain assessment amongst people with cognitive impairment is conducted mostly by registered nurses and medical practitioners (50%). It is recorded in the patients' clinical history and "nursery working sheets," and its mainly communicated to medical practitioners (59%). Registered nurses (43%), family members and other important persons apart from family (46%), as well as members of therapeutic professional groups (41%) are equally informed of the results of pain assessment. When using a behavioral pain assessment tool, the professionals would prefer to respond to items by rating items on a numeric scale (39%), or ticking a simple present/absent (32%) or selecting categories (i.e., slight moderate/a lot) (23%) (see **Figure 5C**).

Challenges and Future Directions

Figure 6 details the challenges reported by Spanish professionals in assessing pain in cognitive impairment (**Figure 6A**), and the solutions and recommendations for its improvement (**Figure 6B**). In an open question, there was a consensus among the different professionals that the difficulties were found at three levels: the patient, the professional knowledge and the tools. They pointed out pain management in severe cognitive impairment, due to its complexity and diverse etiology, like raising more concern. The difficulties are currently found in the poor or absent verbal communication and level of comprehension in such severe cognitive conditions (14%); the scarce time availability for pain assessment and monitoring confronted to low feasible and time-consuming tools (14%); the lack of specialized pain education and poor knowledge of specific tools for this population (12%); as well as the poor standardization (10%) and reliability (10%) of the tools, mostly for severe cognitive impairment. Also, the professionals referred to difficulties due to the presence of confounding factors, general lack of guidelines and recommendations, low awareness among the health professionals.

When the participants were asked what would help them improve pain assessment in people with cognitive impairment/dementia (**Figure 6B**), their answers focused on pain education specialized for this population (28%). They also stated the need for pain assessment tools to be: specific (24%), standardized (19%), easy and fast to use (17%), and reliable (10%). More time available (8%), awareness and involvement of the teams (5%) were also commonly referred to as aspects that would benefit pain management in cognitive impairment.

DISCUSSION/CONCLUSIONS

Pain assessment among older adults is often neglected or not done (21, 25, 26). Two-thirds of residents in long term care facilities have dementia (5). Pain identification, measurement, and management confront a series of difficulties due to the several forms of dementia, their different etiology, neurodegeneration processes, and worsening by the progressive loss of verbal communication and self-reports. Thus, these

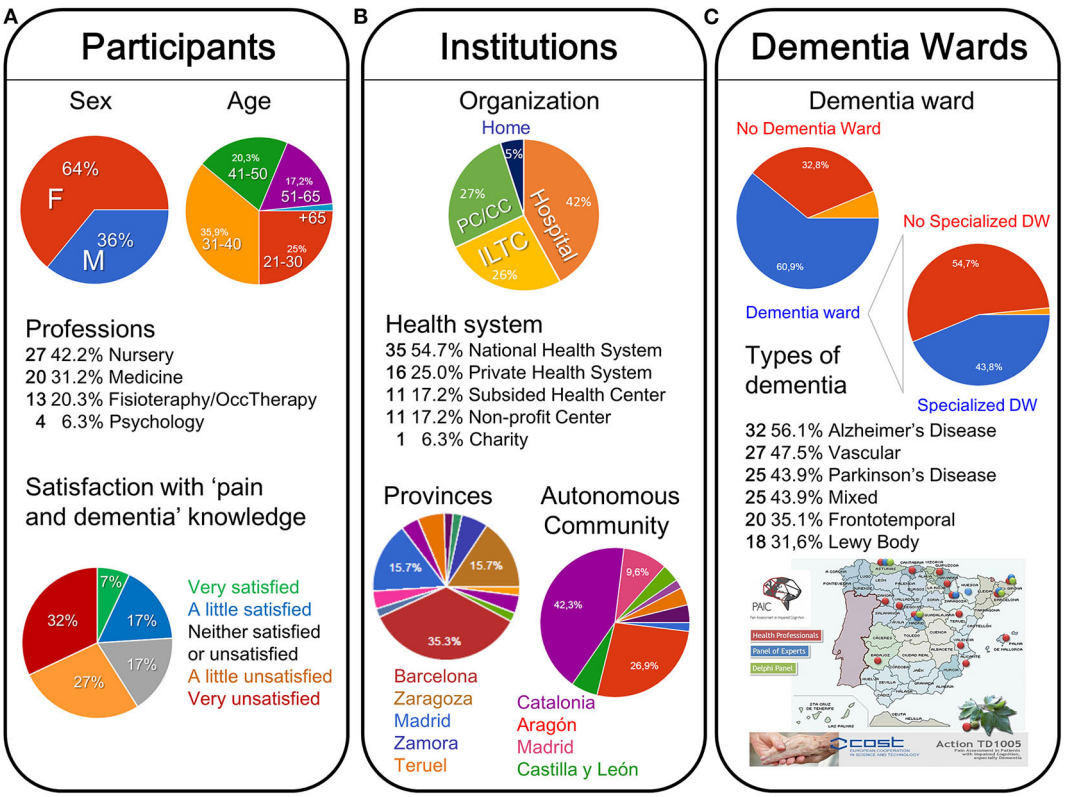

FIGURE 2 | Participants **(A)**, institutions **(B)**, and dementia wards **(C)** profiles in the National Survey and geographical distribution of the answers received and panels of professionals.

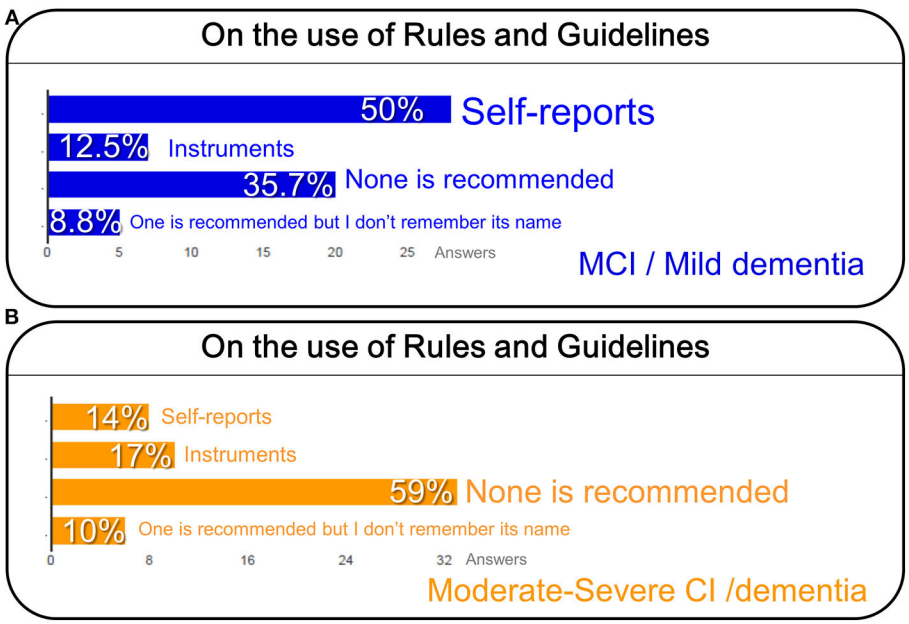

FIGURE 3 | On the use of rules and guidelines for pain assessment in people with mild cognitive impairment (MCI) or mild dementia **(A)**, and cognitive impairment (CI), and severe dementia **(B)**.

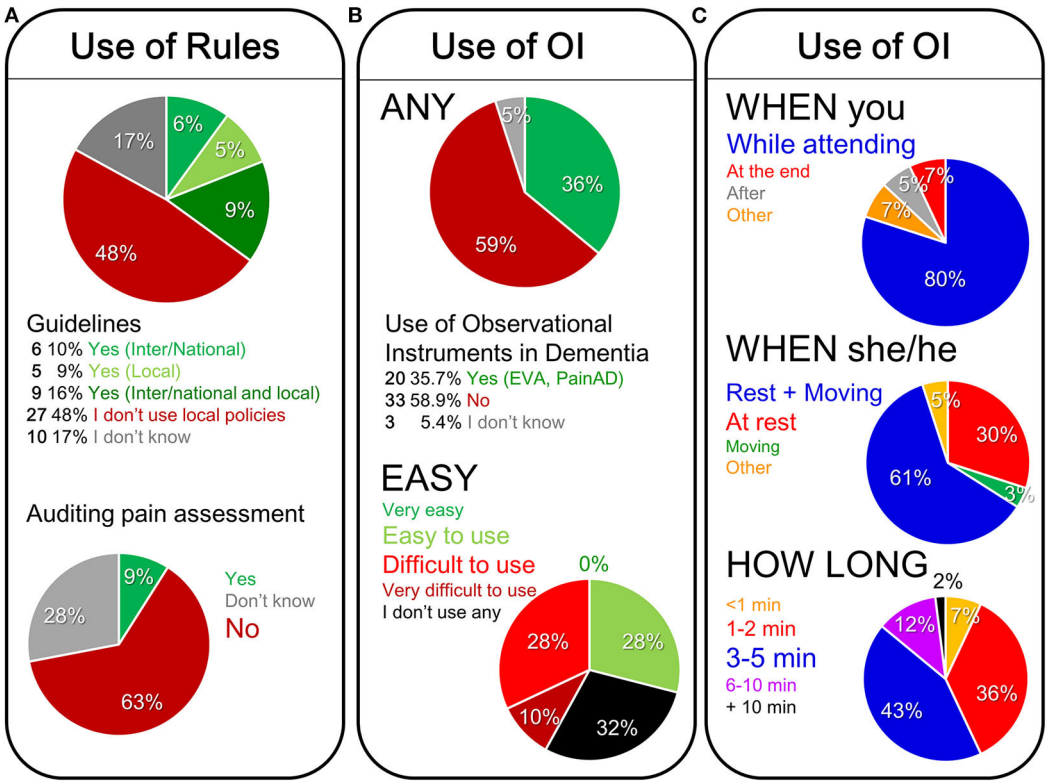

FIGURE 4 | On the use of rules and audition of pain assessment **(A)**, the use of observational instruments **(B)**, and the different conditioning factors on the use of observational instruments for pain assessment in people with dementia **(C)**.

persons are more likely not to have their pain assessed and therefore undertreated (27). Untreated pain is not only distressing for the individual, but causes other problems, including reduced quality of life, interrupted or poor sleep patterns, impaired social interactions, and reduced appetite. Severe pain is less likely to cause wandering but more likely to display aggressive and agitated behaviors (28). Conversely, treating pain in patients has been reported to have concomitant relief of agitation (29). The high number of physical assaults on staff working in dementia wards (30) may be related to unidentified and unmanaged pain and often results in antipsychotic medication rather than person-centered care. In such a case, a worrisome clinical situation arises due to the increased mortality risk that has been associated with antipsychotics in the elder but mostly those with dementia (15, 16). Therefore, "Pain in Cognitive Impairment, especially Dementia" is one of the health topics with clear snail effect (poor and slow diagnosis in frail elderly patients) (31) that raises growing concern among professionals since the pain in these patients is known to be under-detected and under-treated (13, 32). Despite this evidence, statements such as "pain is a normal part of the aging process," "the older person who has dementia cannot feel pain," or "if an older person does not verbalize pain, it does not exist" are often displayed amongst health care professionals and result in poor pain management for these elderly groups (33, 34). Also, the belief that older adults should not be prescribed strong opioids results in avoidance of their use (35, 36).

In the present work, we estimated a low implementation and use of pain scoring systems in patients with cognitive impairment/dementia, probably to the poor reliability given to them by Spanish professionals. The survey reflected that the lack of guidelines is a major problem. Similarly, the ratio of organizations reporting auditions of pain assessment in older people with dementia to assess care quality was low. The low response rate (taking part in the study) and an important percentage of uncertain responses (I don't know) were also notorious. Most research works never achieve a 100% response rate, and reasons for low rates include refusal, ineligibility, inability to respond, and contact not been possible (37). Limitations associated with poor response are referred to as each non-response being liable to sample bias. Here, the participants found observational tools difficult or very difficult to use, an important number do not use them, and many complained they have low reliability in the advanced stages of the disease. Therefore, the current study confirmed many findings from the original E.U. survey (22). The majority of participants did not use national/international standards, guidelines, or local policies or were unsure if their institutions had any pain management guidelines (E.U. survey, 42, and 17%, respectively). In many other

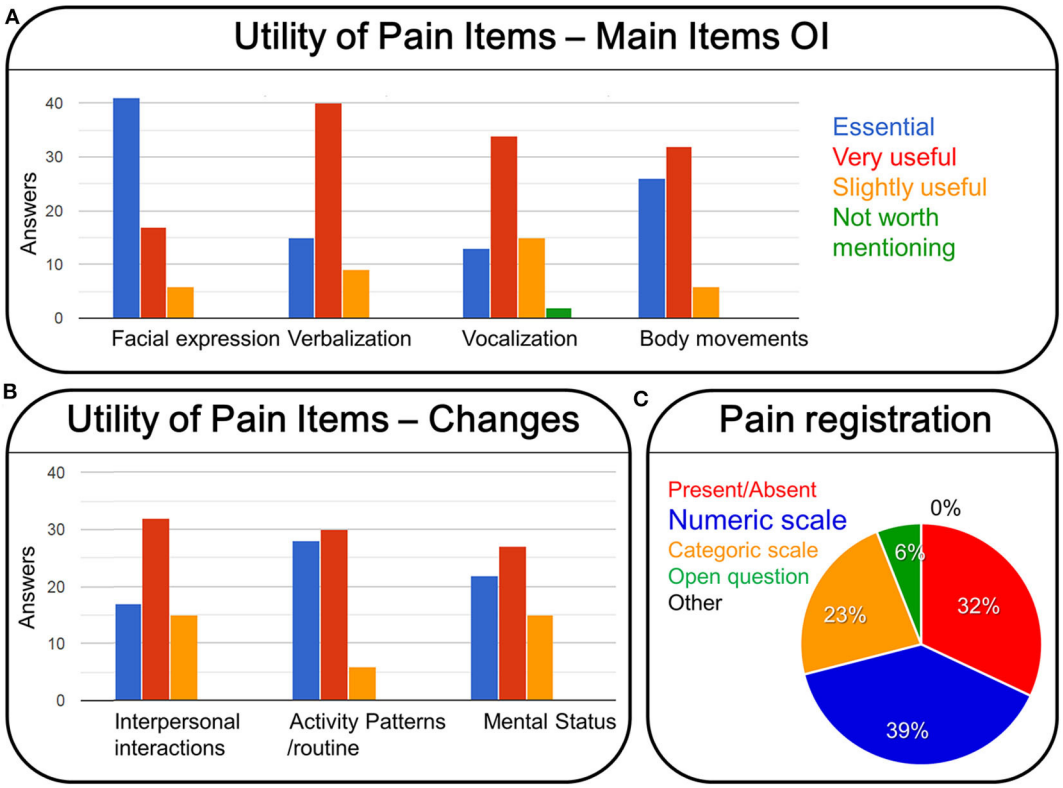

FIGURE 5 | Utility of pain items of observational scales for pain assessment in people with dementia (**A**, main items: facial expression, verbalization, vocalization, body movements; **B**, changes in interpersonal interactions, activity patterns/routine, mental status), and the professional preference about how to register pain **(C)**.

countries globally, pain is not routinely assessed, even though we have the tools available to help us do this.

An important number of observational scales have been developed so far to assess pain in persons with dementia, and their use seems to respond to experience-based confidence in them (20). The EU-COST-Action "Pain in impaired cognition, especially dementia" selected items out of existing observational scales, critically re-assessed their suitability to detect pain in dementia. The EU-consortium created an improved "best-of" meta-tool built on the knowledge and expertise implemented in these scales (38). In both the current study and the E.U. survey, approximately one-third reported using observational pain assessment tools for older people with dementia (36 vs. 34%). Furthermore, in both studies, most of the respondents complete the observational assessment while providing care for the patient and over a period of rest and movement, with a preference for short assessments of 3–5 min and numeric or easy tick scales, as opposed to selecting categories or open-ended questions. In the original survey, participants from the U.K. unsurprisingly demonstrated a preference for the Abbey scale, which is simple and easy to apply. In contrast, other countries in the previous study opted for the Doloplus scale. Interestingly, the Spanish survey participants did not choose the Abbey pain scale as a preferred tool.

While there were similarities in the preference for verbal, vocal, and body movements as being essential elements of a behavioral pain assessment, the Spanish participants reported facial expression as the most important and essential pain indicator (41%), the original survey found facial expressions were regarded as being less important in the clinical setting (18% rated as essential). Facial expressions were reported as not easily observed when providing care, such as washing or moving a patient. Probably, the relevance of facial expressions to infer pain depends on cultural aspects on the expressivity (how one expresses) and detection and codification (how one detects from others) of non-verbal communication, known to be higher in Latin countries. In fact, checklists of non-verbal pain indicators (39) do a specific analysis of the facial expression coding system (FACS). Most studies on the use of FACS on pain in adults and elderly health conditions, or cognitive deficits and/or chronic pain, show that it is a reliable and objective tool in the detection and quantification of pain in all patients (40, 41).

Regarding the professional concerns on problems experienced in Spain and solutions for its improvement (23), pain management in patients with severe cognitive impairment was mostly the one raising more concern. The poor standardization of practices, poor or lack of guidelines or recommendations for the complex and heterogeneous features of the last stages of dementia were also considered as limiting point for their professional activity. Here, the detailed analysis of the answers unveils the specific aspects behind these reasons. First, the main functional limitation was the poor or absent verbal

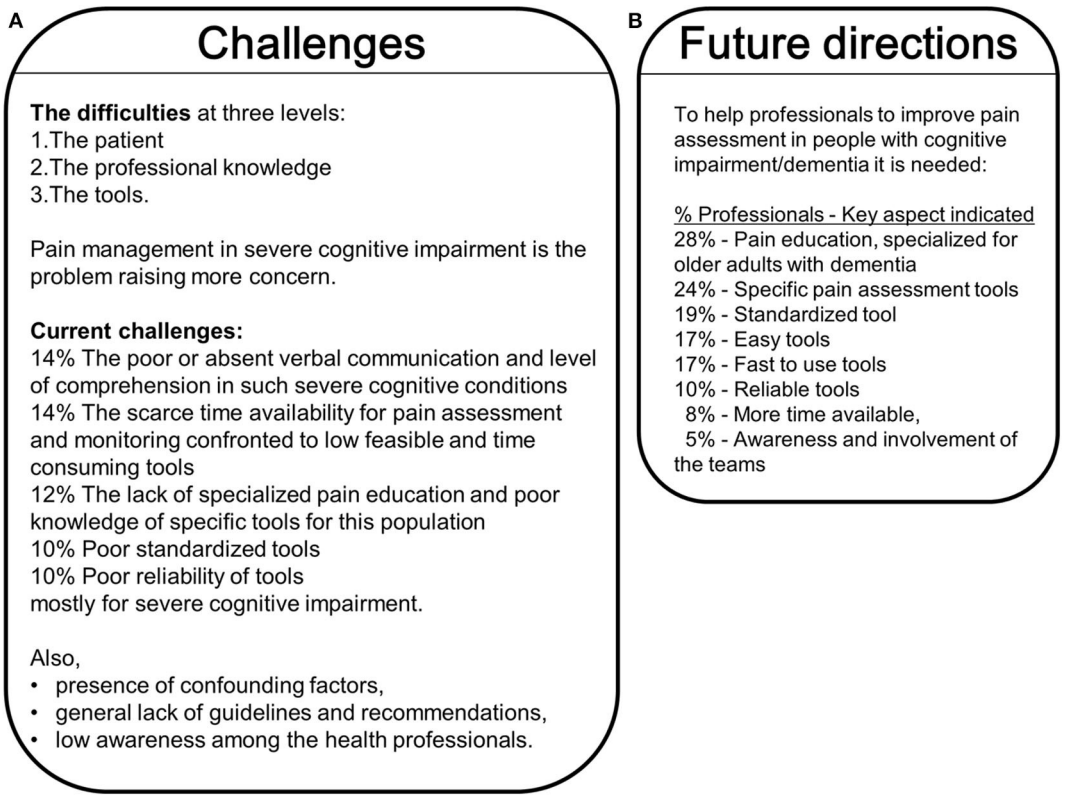

FIGURE 6 | Challenges **(A)** and Future directions **(B)** in the pain assessment and management in the cognitively impaired/dementia population.

communication of the patient but also of his/her comprehension. Second, the low feasible and time-consuming tools in a professional activity with a short time to assess the patients were seen as worsening pain assessment challenges, mostly in these patients with severe cognitive impairment. Third, the professionals' complain about knowledge and education indicated that the poor knowledge of specific tools for pain assessment in these populations and the lack of specialized pain education were important regrets. Standardization and reliability of tools, mostly for severe cognitive impairment, were also mentioned by many professionals, as well as confounding factors, and low awareness among health professionals. Most of these challenges pointed out by Spanish professionals agreed with aspects reported from the original survey (22). There, concerns referred to uncertainty about the observation; lack of information; lack of objectivity; lack of education, knowledge and expertise; lack of time; lack of interest and awareness; and lack of available pain tools. In the current survey, the key challenges were similar and focused on difficulties in communicating with the patient; short time available for pain assessment and monitoring; lack of specialized pain education and poor knowledge of tools suitable for this population; poor standardization and reliability of tools; and general lack of guidance and recommendations.

Inadequate pain education from both studies came out as a key problem relating to pain assessment in dementia patients. In Spain, professionals reported feeling very (32%) or slightly (26%) dissatisfied with their pain assessment knowledge. Health professionals who did have multidisciplinary staff meetings regarding pain assessment and specialized pain training reported higher satisfaction with their knowledge and pain management. This study's findings indicate that training needs are still not being met, despite the clinician's best efforts to learn.

It is evident from the comparable findings that health professionals across the E.U. struggle with the same challenges in pain assessment in people with dementia. The recommendations made by participants from Spain closely align with those made by authors of the E.U. survey; these include improved pain education for understanding pain assessment and management in people with dementia; improved pain assessment tools that are fast and easy to use and interpret; and more time allowed for pain assessment. Efforts to establish more feasible instruments and policies together with education are key targets of our future directions in Spain as they are for other countries.

Among the limitations of the study, the first to be mentioned is the number of participants, lower than in the center-north Europe survey, mostly due to the lack of submissions received from an important geographical area. The length and the number of sensitive questions regarding the use of guidelines probably was a strong limitation to receive submissions. As mentioned before, despite the chance to stop completing the survey at any moment, all the submissions were completed surveys. The sex bias of participants, mostly females, was observed but probably less than expected for the gender bias in healthcare professions. Finally, since there are different types and degrees of dementia,

a survey requesting answers for each one of them would be more specific.

The Spanish survey was in clear agreement in the results obtained in center-north Europe, except for considering facial expression as a key aspect of observational tools, probably due to cultural bias. This convergence in the professional criteria through south-center-north Europe is relevant to note because, in Spain, the distribution of the survey to different health professional profiles, not always confident with the approach to patients with severe dementia, could have been a source of bias in the findings and interpretation of the results.

The results of the present work and the center-north Europe survey (22) were considered as one of the sources of knowledge for the PAIC-15 scale, a new internationally agreed-on meta-tool for Pain Assessment in Impaired Cognition, composed by a list of 15 observational items that have demonstrated psychometric quality and clinical usefulness both in their former scales and in their international evaluation (38). Also, these surveys' professional opinions were taken into consideration in the scientific development and discussion of our most recent work on observational pain assessment in older persons with dementia (42).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LG-L and PS: WG2 COST-Action TD1005 development of the concept and study design. LG-L, MB, AM-S, and VT-L: study conduct, survey and data collection from the different geographical areas. LG-L: data analysis and illustrations. LG-L, RD, and PS: drafting manuscript. AB and MB: critical discussion. All authors participated in the scientific discussions and approved final version of manuscript.

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Opioids in Post-stroke Pain: A Systematic Review and Meta-Analysis

Damiana Scuteri[1], Elisa Mantovani[2], Stefano Tamburin[2], Giorgio Sandrini[3], Maria Tiziana Corasaniti[4,5], Giacinto Bagetta[1] and Paolo Tonin[6]*

[1]Pharmacotechnology Documentation and Transfer Unit, Section of Preclinical and Translational Pharmacology, Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende, Italy, [2]Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy, [3]Department of Brain and Behavioral Sciences, University of Pavia, IRCCS C. Mondino Foundation Neurologic Institute, Pavia, Italy, [4]Department of Health Sciences, University "Magna Graecia" of Catanzaro, Catanzaro, Italy, [5]School of Hospital Pharmacy, University "Magna Graecia" of Catanzaro, Catanzaro, Italy, [6]Regional Center for Serious Brain Injuries, S. Anna Institute, Crotone, Italy

Background: Post-stroke pain is one of the most common *sequelae* of stroke, which stands among the leading causes of death and adult-acquired disability worldwide. The role and clinical efficacy of opioids in post-stroke pain syndromes is still debated.

Objectives: Due to the important gap in knowledge on the management of post-stroke pain, this systematic review aimed at assessing the efficacy of opioids in post-stroke pain syndromes.

Methods: A literature search was conducted on databases relevant for medical scientific literature, i.e. PubMed/MEDLINE, Scopus, Web of Science and Cochrane Library databases from databases inception until August 31st, 2020 for clinical trials assessing the effects of opioids and opioid antagonists on pain reduction and pain related symptoms in patients with post-stroke pain syndromes. Studies assessing the effects of other medications (e.g., tricyclic antidepressant, pregabalin) or non - pharmacological management strategies (e.g., neurostimulation techniques) were excluded. The selected studies have been subjected to examination of the risk of bias.

Results: The literature search retrieved 83,435 results. After duplicates removal, 34,285 articles were title and abstract screened. 25 full texts were assessed and 8 articles were identified to be eligible for inclusion in the qualitative summary and narrative analysis, of which three were placebo-controlled and two were dose-response. Among placebo-controlled studies, two evaluated the analgesic effect of morphine and one assessed the effects of the opioid antagonist naloxone on patients with central post-stroke pain. With regard to dose-response studies, both were on patients with central post-stroke pain, one assessing the efficacy of levorphanol, and the other on naloxone. Seven out of eight included studies showed an overall slight analgesic effect of opioids, with less consistent effects on other pain-related symptoms (e.g., mood, quality of life). The randomized controlled trials were subjected to meta-analysis and rating of the quality of evidence for the two outcomes considered according to GRADE (Grading of Recommendations, Assessment, Development and Evaluations) system. The overall results are inconclusive because of the small number of studies and of patients.

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****Correspondence:***
Giacinto Bagetta
g.bagetta@unical.it

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Conclusions: The limited number of the included studies and their heterogeneity in terms of study design do not support the efficacy of opioids in post-stroke pain and in pain-related outcomes. Large double-blind randomized clinical trials with objective assessment of pain and related symptoms are needed to further investigate this topic.

Keywords: post-stroke pain, stroke, pain, central pain, opioids, rehabilitation, systematic review, meta-analysis

INTRODUCTION

Post-stroke Pain

Stroke stands among the leading causes of death and adult-acquired disability with 13.7 million new strokes every year worldwide (Collaborators, 2019). Post-stroke pain is one of the most poorly understood complications, arising either in the acute, but mainly in the subacute or chronic stages (i.e., often within 6 months) of stroke (Merskey, 1994) . The prevalence of post-stroke pain varies largely depending on the definition of pain; the musculoskeletal pain appears to be the most common being reported in up to 72% of stroke patients (Harrison and Field, 2015). While post-stroke pain syndromes in general are estimated to affect up to 30–40% of stroke survivors (Paolucci et al., 2016), central post-stroke pain (CPSP) is more rare: definite CPSP was found in 3.5%, definite/probable in 5.8% and CPSP-like pain or dysesthesia in 6.7% of patients in a specific population-based study of post-stroke pain (Klit et al., 2011). Pain after stroke can remarkably reduce the quality of life, causing depression, anxiety and sleep disorders making rehabilitation more difficult.

Post-stroke Pain Syndromes

Pain after stroke is often under-reported, being diagnosed only if actively searched by the clinician (Harrison and Field, 2015). There are multiple types of post-stroke pain syndromes that can also occur in combination, with both neuropathic and nociceptive features. The most common types of pain after stroke include CPSP, pain secondary to spasticity, shoulder pain, complex regional pain syndrome (i.e., CRPS), and headache (O'Donnell et al., 2013). Dysesthesia and allodynia often occur and the symptoms generally develop within the area corresponding to the lesion with frequent involvement of face, hand and foot, but sometimes also of thigh and shoulder (Kim, 2014). In particular, CPSP is often characterized by dysesthesia, constant or intermittent pain and hyperalgesia/allodynia (Harrison and Field, 2015). CPRS is of type I when nerve lesion is not identifiable, while of type II when there is a definite nerve lesion.

Treatment and Limitations

Treatment of post-stroke pain is made challenging by the lack of universally accepted guidelines (Kim, 2014), due to the paucity of high quality evidence from controlled clinical trials guiding pharmacological management and, expecially, for non neuropathic syndromes despite their high frequency (Hansson, 2004). In neuropathic pain, tricyclic antidepressants (e.g., amytriptiline), serotonin and norepinephrine reuptake inhibitors (e.g., duloxetine) and calcium channel α2δ ligands (e.g., gabapentin or pregabalin) are recommended as first-line agents, but data supporting their use is based on studies in peripheral neuropathic pain, while the evidence in central neuropathic pain is very limited (Mulla et al., 2015). A single study suggested lamotrigine to have a moderate effect on CPSP (Klit et al., 2009). Botulinum toxin injections represent the gold standard for the treatment of post-stroke spasticity and related pain (Hillis, 2020). Given their potential for misuse and other adverse effects (McNicol et al., 2013), opioids stand among the third-line therapy and evidence on their effectiveness for post-stroke pain syndromes is even more limited.

Aim of the Research

The aim of manuscript is to conduct a systematic review and meta-analysis of evidence on the efficacy of opioid and opioid antagonist medications, important and useful under the recommended conditions (Morrone et al., 2017), for reducing post strokepain and improving pain-related symptoms. Agonists and antagonists at opioid receptors were included in the search. In the brain area subjected to stroke, altered perfusion (Strahlendorf et al., 1980) and changes in opioid neurotransmission (Baskin and Hosobuchi, 1981; Willoch et al., 2004) were suggested to be positively affected by naloxone; incidentally, opioids can reduce blood flow during cerebral ischemia, through inhibition of the release of noradrenaline in the *locus coeruleus* (Budd, 1985). Moreover, naloxone and kappa opioid receptor antagonists were tested in acute ischemic stroke showing in some cases benefit and improvement of neurological conditions (Fallis et al., 1984; Jabaily and Davis, 1984; Perey et al., 1984; Adams et al., 1986; Czlonkowska and Cyrta, 1988; Federico et al., 1991; Czlonkowska et al., 1992; Clark et al., 1996; Lyden, 1996; Clark et al., 2000). Levorphanol, an opioid agonist with high affinity for all the mu, delta and kappa opioid receptors, reported to interact with both N-methyl-D-aspartate (NMDA) receptors and serotonin and norepinephrine uptake (Codd et al., 1995), was included because of its favourable pharmacodynamic and pharmacokinetic characteristics and it showed efficacy in neuropathic pain (Le Rouzic et al., 2019). Oliceridine, a novel mu opioid agonist, was included because it can confer analgesia with less respiratory depression (Dahan et al., 2020).

METHODS

This work was conducted according to the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) recommendations (Liberati et al., 2009; Moher et al., 2009).

The systematic review focused on the following question: are opioids effective in reducing pain after stroke and improving

pain-related symptoms? Detailed PICOS (i.e., participants, interventions, comparisons, outcomes, study design) framework is shown below:

Participants: patients with pain after stroke; - Intervention: opioid and opioid antagonist medications; - Comparison: placebo or usual/other treatment; - Outcomes: 1) improvement of assessed pain intensity and 2) of pain-related outcomes (e.g., mood, quality of life); - Design of the studies: clinical trials.

The efficacy and safety of opioids on intractable post-stroke pain is a fundamental gap of knowledge due to the lack of studies. Therefore, our systematic review and meta-analysis addresses this broad question, providing an overview of the existing evidence also originating from studies with different design and prompting further future research (Peters et al., 2015). This research aims at highlighting whether opioids and their antagonists are used in post-stroke pain, including medications with different mechanisms of action, and if they are safe and efficacious on the primary outcome of pain reduction and on secondary related outcomes like physical functioning.

Eligibility Criteria

Studies eligible to be included in this systematic review and meta-analysis were required to meet the following criteria:

clinical trials assessing the effects of opioids on pain in post-stroke patients. No restrictions were placed on the publication date, study duration or follow-up; - patients of any age or ethnicity with post-stroke pain; - interventions include opioids.

Studies meeting the following criteria were excluded from the review:

in vitro and *in vivo* animal studies, narrative or systematic reviews and meta-analysis, abstracts and congress communications, proceedings, editorials and book chapters; - clinical trials assessing the effects of other pharmacological treatments (e.g., tricyclic antidepressant, pregabalin) or non - pharmacological management strategies (e.g., neurostimulation techniques); - studies not published in English.

Primary outcomes of interest were changes in objective measures of pain intensity (e.g., pain visual analog scale VAS) and secondary outcomes of interest were changes in pain-related outcomes (e.g., quality of life and physical functioning).

Search Strategy

The literature search was conducted on PubMed/MEDLINE, Scopus, Web of Science and Cochrane Library databases for peer-reviewed studies on opioid medications for the treatment of post-stroke syndromes and published from databases inception until August 31st, 2020 (date of last search). The search strings consisted in a combination of the following keywords: "stroke," "post-stroke pain," "pain after stroke," "central post-stroke pain," "CPSP," "shoulder post-stroke," "thalamic pain syndrome," "central pain syndrome," "shoulder hand syndrome," "complex regional pain"; "Dejerine Roussy," "facial pain," "headache," "facial neuralgia," "trigeminal autonomic cephalalgia," "temporomandibular joint disorders," "allodynia," "pain secondary to spasticity," musculoskeletal pain," "myofascial pain," "neuropathic pain," "opioids," "methadone," "tramadol," "codeine," "morphine," "buprenorphine," "oxycodone," "fentanyl," "tapentadol," "loperamide," "oxymorphone," "hydrocodone," "levorphanol," "sufentanil," "remifentanil," "R-dihydroetorphine," "Morphine-6-glucuronide," "oliceridine," "naloxone," "naltrexone."

Study Selection

Two authors independently screened titles and abstracts of the studies in agreement to the previously established inclusion and exclusion criteria. The reference lists of relevant papers were inspected for additional studies potentially missed in the database search. Any disagreement was planned to be solved by consensus or by consulting a third Author.

Data Collection Procedure

Two authors independently extracted the following data, according to the PICOS framework discussed above: study design, sample size, subtype of post-stroke pain syndrome, interventions, route of drugs administration, comparators, outcomes of interest (primary and secondary), drop-out rates, adverse effects.

Data Analysis

A systematic and descriptive analysis of the results was provided with information presented in the text and tables. The narrative synthesis has been carried out according to the Cochrane Consumers and Communication Review Group guidelines (Ryan, 2013). Risk of bias and quality of the studies have been assessed, considering study limitations including lack of allocation concealment, lack of blinding, selective outcome reporting bias, inadequate sample or lack of sample size calculation. The revised Cochrane risk of bias tool for randomized trials RoB2 (Sterne et al., 2019) has been used. only the randomized clinical trials included were subjected to meta-analysis to assess imprecision. Indeed, the quality of the body of evidence for both outcomes was rated through the GRADE (Grading of Recommendations, Assessment, Development and Evaluations) system providing the evidence profile including the quality assessment and the summary of findings (Guyatt et al., 2011). Absolute and relative risk with 95% confidence intervals (CI) were calculated using the Cochrane Review Manager 5.3 (RevMan5.3; Copenhagen: The Nordic Cochrane Center, The Cochrane Collaboration). The random effect model (DerSimonian and Kacker, 2007) was used to manage eventual heterogeneity of the studies and to assess intra- and inter-study variation. In particular, for the assessment of inconsistency in results, since the retrieved studies number is small, the Higgins I^2 value was calculated to assess the heterogeneity of the studies (Higgins and Thompson, 2002). Relative risk below one favors the intervention (opioids)

rather than the control/other treatment. Subgroup analysis, sensitivity testing and meta-regression have been performed to evaluate the impact and the causes of heterogeneity and publication bias has been assessed through Egger's linear regression test to measure funnel plot asymmetry, adjusted through "trim and fill" method (Egger et al., 1997; Duval and Tweedie, 2000; Sterne and Egger, 2001).

RESULTS

Identification and Selection of the Studies

The literature search retrieved a total of 83,435 results. The 83,435 references obtained have been searched for duplicates, leaving 34,285 articles to screen. After titles and abstract screening, not original articles like reviews, book chapters and conference proceedings have been eliminated leaving 24,950 titles and abstracts to screen. After elimination of *in vivo* and *in vitro* studies 2,736 have been screened to exclude observational and retrospective studies, thus leading to 2,531 clinical studies, among which 25 were obtained for full-text reading. One of these trials (Fallis et al., 1984) was not available in full text and one significant paper (Yamamoto et al., 1991) was further identified by the inspection of the reference lists of the relevant records. Eight studies met the inclusion criteria and were therefore included in qualitative synthesis. The four randomized controlled trials (RCTs) (Bainton et al., 1992; Attal et al., 2002; Maier et al., 2002; Rowbotham et al., 2003) were subjected to meta-analysis. The selection process is illustrated in the PRISMA flow diagram (**Figure 1**).

Qualitative Summary and Narrative Analysis

The 8 included articles (Budd, 1985; Yamamoto et al., 1991; Bainton et al., 1992; Yamamoto et al., 1997; Attal et al., 2002; Maier et al., 2002; Rowbotham et al., 2003; Saitoh et al., 2003) were clinical trials meeting the previously mentioned inclusion criteria. Studies were grouped according to the intervention (i.e., type of opioid medication), following the Cochrane Consumers and Communication Review Group guidelines (Ryan, 2013). Details of the included studies are summarized in **Tables 1** and **2**.

Morphine

Two studies assessed the analgesic effect of morphine on CPSP (Attal et al., 2002; Maier et al., 2002). Attal et al. (2002) performed a double-blind, placebo-controlled, crossover study with the two-fold aim to evaluate the efficacy of intravenous morphine on spontaneous and evoked pain and the long-term benefit of oral morphine on neuropathic pain caused by spinal cord injury or stroke. They reported that the analgesic effect of intravenous morphine regarded only some components of evoked pain (i.e., the intensity of brush-induced allodynia) and that the effects of morphine on ongoing pain were not significantly different from those of the placebo and some patients were reported to receive other pharmacological treatment for pain. Regarding the long-term benefit of oral morphine, only three patients were reported to be still treated after 1 year with persistent pain relief, while the others dropped out before three months due to side effects. It is however unclear whether the patients still on oral morphine treatment at 1 year were those belonging to the group of neuropathic pain caused by stroke or by spinal cord injury. Maier et al. (2002) conducted a prospective, randomized, double-blind, placebo-controlled, crossover study on forty-nine patients with either neuropathic (of which only two had CPSP) or nociceptive pain syndromes, assessing the efficacy and effectiveness of 1 week of oral morphine administration. In fact, the MONTAS study assessed the efficacy of morphine on chronic non-tumor associated pain syndromes (Maier et al., 2002). An interdisciplinary consensus protocol on compulsory and optional treatments for pain, excluding strong opioids was followed before inclusion. The two patients with CPSP were classified as partial responders according to the reduction from 7.8 to 5.6 of mean pain intensity measured with an 11 points Numerical Rating Scale (NRS) and to the overall tolerability of adverse effects connected with opioid medications. Pain reduction was reported to correlate with improvement of physical function. Moreover, the Authors found a reduction of pain disability, depression score, mood and exercise endurance, secondary pain-related outcomes. The pharmacological background of intractable CPSP was characterized in three studies through morphine tests: two by Yamamoto and coworkers (Yamamoto et al., 1991; Yamamoto et al., 1997) and one by Saitoh and collaborators (Saitoh et al., 2003). The first study evaluated deafferentation pain using the morphine/thiamylal test enrolling twenty-five patients suffering from intractable deafferentation pain (thalamic/suprathalamic lesions n = 16; brainstem lesions n = 2; spinal cord lesions n = 2; peripheral nerve lesions n = 5) (Yamamoto et al., 1991). The morphine test consists in intravenously administering 3 mg morphine hydrochloride every 5 min up to reach 18 mg, followed by injection of naloxone to reverse thus confirming the effect of morphine, and assessing pain through a visual analog scale at 5 min intervals (Yamamoto et al., 1991). All the patients included were resistant to pharmacological therapy. According to the results, only two patients with thalamic or suprathalamic lesions were responding to morphine and thiamylal (Yamamoto et al., 1991). In the second study by Yamamoto et al. (1997) thirty-nine patients with intractable hemibody CPSP associated with dysesthesias and allodynia (twenty-five affected by a small thalamic infarct or hemorrhage and fourteen affected by infarct or hemorrhage in the posterior limb of the internal capsule or subcortical parietal area sparing the thalamus) were subjected to the morphine and thiamylal tests and only twenty-three recent cases were subjected to the ketamine test. All the patients had been received treatment with tricyclic and heterocyclic antidepressants, benzodiazepines and non-narcotic analgesics without satisfactory pain reduction. During this study, eight patients with CPSP were sensitive to morphine experiencing transient satisfactory pain reduction (Yamamoto et al., 1997). In the study by Saitoh and colleagues (Saitoh et al., 2003) nineteen patients with central and peripheral deafferentation pain (seven who had thalamic hemorrhage, one putaminal hemorrhage, one pontine hemorrhage, six brachial plexus injury, two phantom limb pain, one spinal cord injury and one pontine injury) of

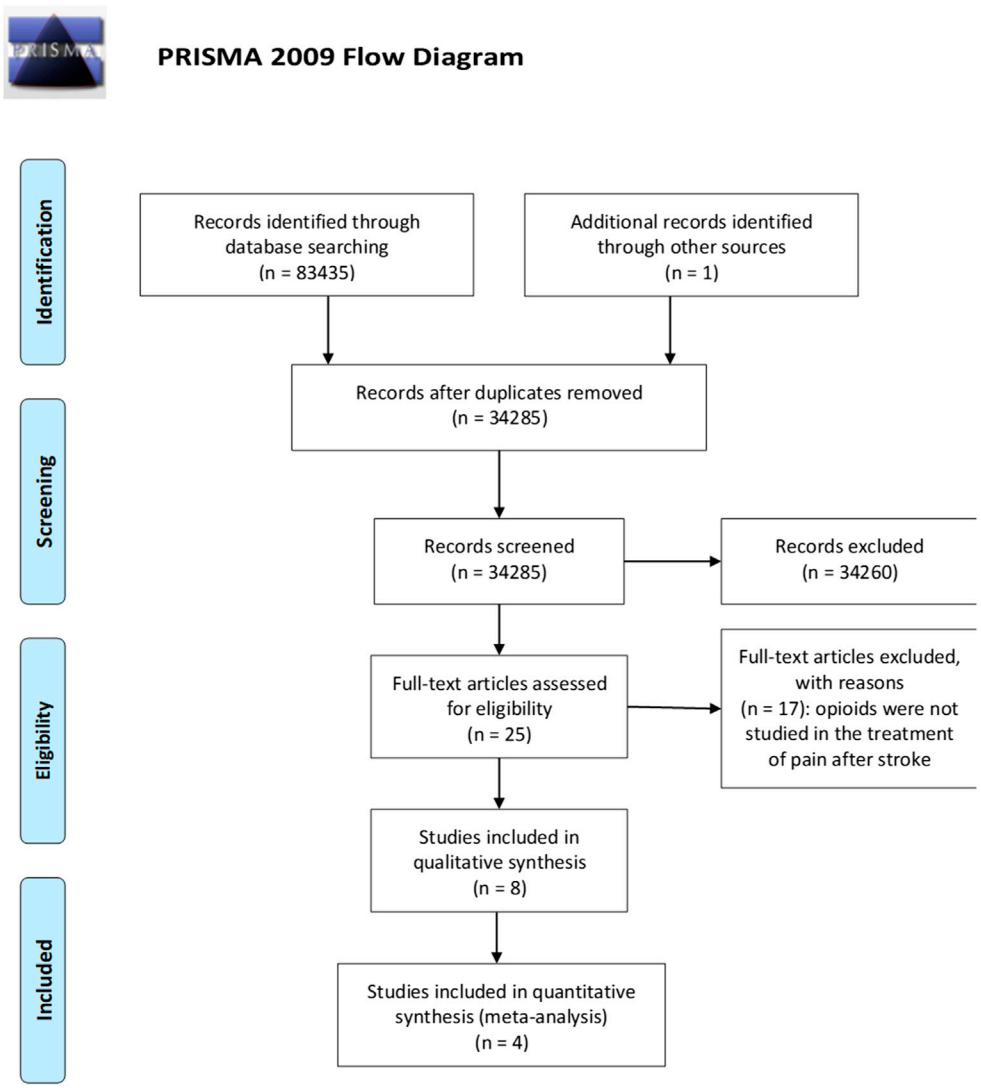

FIGURE 1 | Process of selection of the studies eligible for the qualitative analysis according to the PRISMA guidelines (Moher et al., 2009).

which eighteen underwent drug challenge test and pain was assessed through a visual analog scale and the McGill Pain Questionnaire. All the patients were treated with various medications including NSAIDs, anticonvulsants and antidepressants also used in combination, without sufficient reduction of pain. Among these patients, five were sensitive to morphine (Saitoh et al., 2003).

Levorphanol

One study (Rowbotham et al., 2003) evaluated the efficacy of low and high doses of the opioid agonist levorphanol on eighty-one patients with neuropathic pain of different aetiology (patients with CPSP were ten). All patients had not achieved pain relief with previous non opioid medications and a trend towards previous use of low dose opioid was reported in the low-strength group. Compared to low ones, high doses of levorphanol resulted in higher rates of reduction in the intensity of neuropathic pain, considering the whole patient sample; however, high doses of levorphanol also resulted in more severe side effects that led to higher drop-out rates. Despite the additional outcomes of affective distress and interference with functioning were reduced, no difference between groups were observed. Moreover, pain relief was less frequent for patients suffering from CPSP. Pain effect on physical functioning was evaluated only in this study (Rowbotham et al., 2003).

Naloxone

Two studies assessed the effect of the opioid antagonist naloxone on CPSP (Budd, 1985; Bainton et al., 1992). Budd (1985) performed a single group study on thirteen patients with pain due to thalamic syndrome resistant to prior analgesic or other therapies and reported analgesia, assessed by direct questioning, for seven patients after twenty intravenous administrations of naloxone. The duration of the effects

TABLE 1 | Summary of the characteristics of study design of the trials included in qualitative analysis.

Study	Attal et al. (2002)	Bainton et al. (1992)	Budd (1985)	Maier et al. (2002)	Rowbotham et al. (2003)	Yamamoto et al. (1991)	Yamamoto et al. (1997)	Saitoh et al. (2003)
Study design	Randomized, double-blind, placebo-controlled and crossover	Randomized, double-blind, placebo-controlled and crossover	Single-arm trial	Multicenter prospective, randomized, double-blind placebo-controlled and crossover	Randomized, double-blind, dose-response	Single-arm trial: drug challenge test	Single-arm trial: drug challenge test	Single-arm trial: drug challenge test
Patient sample and pain condition	Patients with CPSP (N = 6) or pain due to spinal cord injury (N = 9)	Patients with CPSP (N = 20)	Patients with CPSP (N = 13)	49 patients with neuropathic or nociceptive pain syndromes (CPSP = 2)	81 patients with chronic neuropathic pain of different etiology (CPSP = 10)	Twenty-five patients suffering from intractable deafferentation pain (thalamic/suprathalamic lesions n = 16; brainstem lesions n = 2; spinal cord lesions n = 2; peripheral nerve lesions n = 5)	Thirty-nine patients with intractable hemibody CPSP associated with dysesthesias and allodynia (twenty-five affected by a small thalamic infarct or hemorrhage and fourteen affected by infarct or hemorrhage in the posterior limb of the internal capsule or subcortical parietal area sparing the thalamus)	Nineteen patients with central and peripheral deafferentation pain (seven who had thalamic hemorrhage, one putaminal hemorrhage, one pontine hemorrhage, six brachial plexus injury, two phantom limb pain, one spinal cord injury and one pontine injury)
Intervention	First phase: intravenous infusion of morphine (9–30 mg; mean dosage, 16 mg) for a 20 -minute period; infusion of saline solution was conducted on a separate session after 2 weeks Second phase: within one week after the second infusion, all patients began to take sustained release oral morphine (starting from 20 mg/d during four weeks up to the maximum tolerated dosage)	First intravenous injection of naloxone (8 mg in a 20 ml manufacturer's vehicle) or of placebo (20 ml of saline solution).The second injection took place 2 or 3 weeks later and contained either naloxone or placebo, depending on previously injected compound	Twenty treatments with intravenous naloxone (from 4.0 to 8.0 mg)	Sustained-release morphine in the first week and placebo in the second week (or reverse order)	Low-strength group: eight weeks levorphanol treatment (max. daily dosage 3.15 mg) High-strength group: eight weeks levorphanol (max. daily dosage 15.75 mg)	Drug challenge tests including morphine test to predict the efficacy of brain stimulation therapy	Drug challenge tests including morphine test to predict the efficacy of brain stimulation therapy	Drug challenge tests including morphine test to predict the efficacy of brain stimulation therapy
Route of drug administration	First phase: intravenous Second phase: oral	Intravenous	Intravenous	Oral	Oral	Intravenous	Intravenous	Intravenous
Comparator	Saline (0.9% NaCl)	Saline (0.9% NaCl)	NA	NS	Low-strength levorphanol	Saline (0.9% NaCl)	Saline (0.9% NaCl)	Saline (0.9% NaCl)

CPSP, Central Post-Stroke Pain; DS, Depression Scale; MPI, Multidimensional Pain Inventory; NA, not applicable; NaCl, sodium chloride; NRS, Numerical Rating Scale; NS, not specified; OAES, Opiate Agonist Effects Scale; OWS, Opiate Withdrawal Scale; PMS, Profile of Mood States; SC-S, Symptom Complaint Score; SDMT, Symbol Digit Modalities Test; VAS, Visual Analogue Scale; VRS, Visual Rating Scale.

TABLE 2 | Summary of the findings of the studies included in qualitative analysis.

Study	Attal et al. (2002)	Bainton et al. (1992)	Budd (1985)	Maier et al. (2002)	Rowbotham et al. (2003)	Yamamoto et al. (1991)	Yamamoto et al. (1997)	Saitoh et al. (2003)
Primary outcomes	First phase: spontaneous pain = ongoing pain intensity (VAS); evoked pain = intensity of allodynia (VAS); intensity of mechanical pain (VAS); intensity of thermal pain (VAS) Second phase: mean pain intensity (VAS)	Pain intensity (VAS and 5 – word pain score) assessed immediately after the injection	Changes in pain state (direct questioning)	Pain intensity (NRS) Pain tolerability (VRS) Rate and intensity of adverse effects (VRS)	Daily pain intensity (VAS) Pain relief (NRS)	Pain assessed through a visual analog scale	Pain assessed through a visual analog scale	Pain assessed through a visual analog scale and the McGill Pain Questionnaire
Secondary outcomes	Global assessment of pain relief (complete, a lot, moderate, slight, none, or worse pain) Reports of side effects (direct questioning)	Long-term pain intensity reduction (VAS)	Pain relief duration	Sleep quality (VRS) Physical fitness and endurance (NRS) Pain disability index (NRS) Mental state and mood (NRS) Depression (DS) Intensity of symptoms (SC-S)	Mood disturbances (PMS) Quality of life (MPI) Cognitive functioning (SDMT) Symptoms related to agonist and antagonist activity (OAES; OWS) Number of capsules/day Blood levorphanol levels	NA	NA	NA
Results	[a]First phase: morphine significantly reduced dynamic mechanical allodynia (in 9 patients reduction of 50% of pain intensity - VAS) respect to placebo; no significant differences on ongoing pain intensity between morphine and placebo [a]Second phase: 3 patients out of 15 still took oral morphine after one year follow-up, reporting a 50–70% reduction of mean pain intensity measured with VAS	Inconsistent effects of naloxone compared to placebo on pain intensity reduction: mean ±SE of VAS for naloxone (– 9.35 ± 4.86) vs saline (– 10.05 ± 4.99) Pain relief obtained either with naloxone or placebo was not maintained beyond one day after the injection	7 patients experienced analgesia within 5 min of the completion of naloxone administration lasting from 4 days to 2 and a half years	2 CPSP patients were classified as partial responders ([a]mean pain intensity from 7.8 to 5.6 after morphine; tolerable side effects) [a]Pain intensity reduction correlated with improvement of physical function [a]Other secondary outcomes measures did not show significant improvement after morphine treatment compared to placebo	[a]Pain reduction from baseline (high-strength 23 mm vs low-strength 14 mm VAS) [a]66% patients under high-strength treatment reported pain relief [a]No significant changes in total mood disturbance in either treatment group [a]No significant changes in quality of life measures in either treatment group [a]No significant changes in cognitive functioning in either treatment group [a]Fewer capsules each day for the high-strength group compared to low-strength (11.9 ± 5.5 vs. 18.3 ± 4.3) [a]Mean blood levorphanol level closely mirrored the ratio of the actual levels of levorphanol intake in either treatment group	Only 2 patients with thalamic or suprathalamic lesions were responding to morphine	8 patients with CPSP were sensitive to morphine	5 patients resulted responding to morphine
Drop – out rates	[a]First phase: None [a]Second phase: 60% of patients dropped out because of insufficient pain relief and/or side effects	NA	NA	[a]Only 1 patient dropped the trial	7 out of 10 patients with CPSP dropped	NA	NA	NA
Adverse effects	[a]Nausea, somnolence, headache (mild, rapidly reversible) mainly for morphine administration (60% patients) [a]Somnolence after placebo (40% patients)	Slight side effects (i.e., rise in pulse rate, sweating, tremor, salivation, pain, nausea, faintness) either after naloxone	Slight transitory changes in heart rate (increase of 10–40 beats/min)	[a]Severe side effects (constipation, vomiting, nausea, sedation and micturition disturbances) occurred in 58% of patients under morphine and in 22% of patients under placebo, independently of dose	[a]Physical or psychological adverse events, treatment failure, lack of adherence	NS	Two patients reported an increase in pain with transient abnormal sensations and anxiety in the ketamine test	NS

CPSP = Central Post-Stroke Pain; DS = Depression Scale; MPI = Multidimensional Pain Inventory; NA = not applicable; NaCl = sodium chloride; NRS = Numerical Rating Scale; NS = not specified; OAES = Opiate Agonist Effects Scale; OWS = Opiate Withdrawal Scale; PMS = Profile of Mood States; SC-S = Symptom Complaint Score; SDMT = Symbol Digit Modalities Test; VAS = Visual Analogue Scale; VRS = Visual Rating Scale.

[a]Considering the whole sample (no separation between patients with CPSP and those with other types of pain).

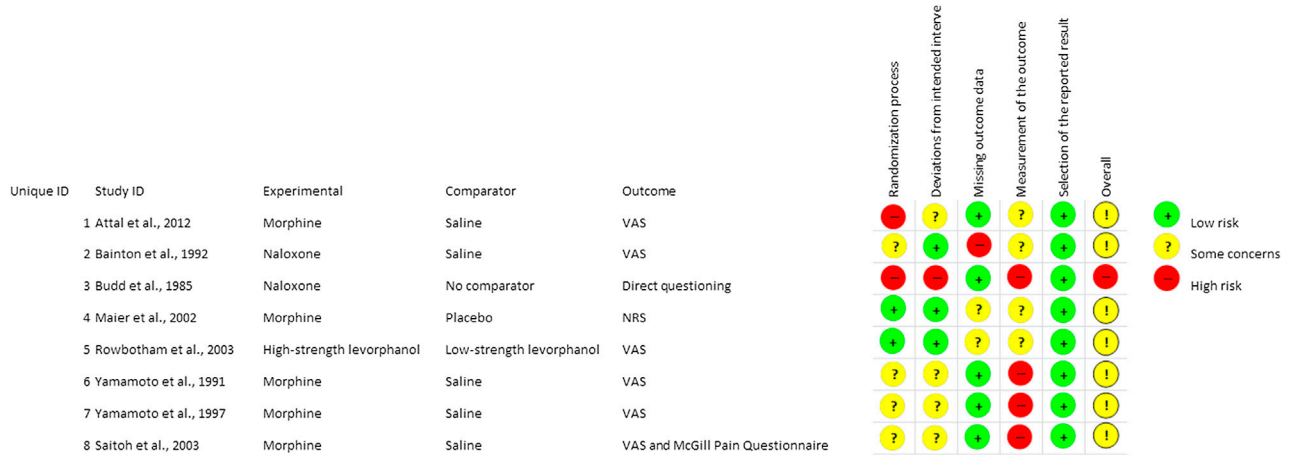

FIGURE 2 | Summary of risk of bias assessment for the studies included in qualitative analysis from low to some concerns and high. The mark (+) indicates low risk of bias and the mark (–) indicates high risk of bias.

varied from 4 days to two and a half years. On the other hand, the placebo-controlled study by Bainton et al. (1992) failed to demonstrate the efficacy of intravenous administration of naloxone in alleviating CPSP.

Risk of Bias Assessment

Four of the studies are randomized clinical trials (Bainton et al., 1992; Attal et al., 2002; Maier et al., 2002; Rowbotham et al., 2003), one is a single arm trial (Budd, 1985) and three (Yamamoto et al., 1991; Yamamoto et al., 1997; Saitoh et al., 2003) are drug challenge tests. Therefore, the included studies are very heterogeneous in terms of study design. Moreover, four studies (Budd, 1985; Yamamoto et al., 1991; Yamamoto et al., 1997; Saitoh et al., 2003) included one single group without control. In the study by Rowbotham et al. (2003) the groups compared are high and low strength. The lack of a control arm can rise some concerns in terms of bias as for concealment. The population enrolled is heterogeneous across the eight studies and the number of patients is small for all the trials except for Maier et al. (2002) and for Rowbotham et al. (2003); however, the CPSP patients are only two and ten, respectively. Moreover, in the MONTAS study, with crossover design, number needed to treat and number needed to harm are reported to have been calculated only with reference to first week since the results of the second week could feel the effect of opioid withdrawal symptoms. Compliance to treatment has been assessed by pill counts and repeated urine screening, revealing only minor protocol violations. Interestingly, double masking was applied to three trials (Bainton et al., 1992; Attal et al., 2002; Rowbotham et al., 2003), but for the study by Attal et al. (2002) it was reported that seven patients and the examiner (in ten cases) had identified the active treatment, thus impairing blindness. In the study of Maier et al. (2002), a random generator was used for patients randomization and the medication package was blinded. The summary of risk of bias assessment according to intention-to-treat analysis is reported in **Figure 2**.

Meta-Analysis and GRADE Evidence Profile (EP)

The quality of evidence of the two selected outcomes, i.e. analgesic efficacy of opioids in post-stroke pain and effectiveness on pain-related domains, was rated through the GRADE system (Guyatt et al., 2008; Guyatt et al., 2011). The quality assessment was based on: Limitations; Inconsistency; Indirectness; Imprecision and Publication bias. For each outcome, the four retrieved randomized clinical trials (Bainton et al., 1992; Attal et al., 2002; Maier et al., 2002; Rowbotham et al., 2003) were subjected to meta-analysis (**Figure 3**), for the assessment of absolute and relative risk and width in the CIs to calculate imprecision, with funnel plot for the evaluation of publication bias (**Figure 4**). The GRADE assessment reveals very low quality of evidence for the outcome of pain reduction and low quality of evidence for pain-related outcomes. This meta-analysis follows the Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT) recommendations (Turk et al., 2003). The core outcome domains for clinical trials of chronic pain treatment efficacy and effectiveness have been identified as pain; physical functioning; emotional functioning; participant ratings of global improvement; symptoms and adverse events; participant disposition (including adherence to the treatment regimen and reasons for premature withdrawal from the trial) (Turk et al., 2003). A meaningful decrease in chronic pain representing a clinically important difference in pain intensity is determined as change of approximately 2.0 points of Numerical Rating Scale (NRS) or 30–36% (Dworkin et al., 2008). Therefore, administration of opioids (agonists or antagonists) is not associated to meaningful pain relief (Relative Risk RR 1.05; 95% CI 0.57–1.92; I^2 = 0%; p = 0.53; **Figure 3**) and data are influenced by the paucity and the design of the studies. Though in agreement with I^2, heterogeneity allows comparison of these RCTs, RR is not estimable for the study by Rowbotham et al. (2003), since there is not a real control arm, but a high- and a low-strength arm. This occurs also for the pain-related outcome, thus influencing the RR calculation (RR 1.00; 95% CI 0.49–2.05;

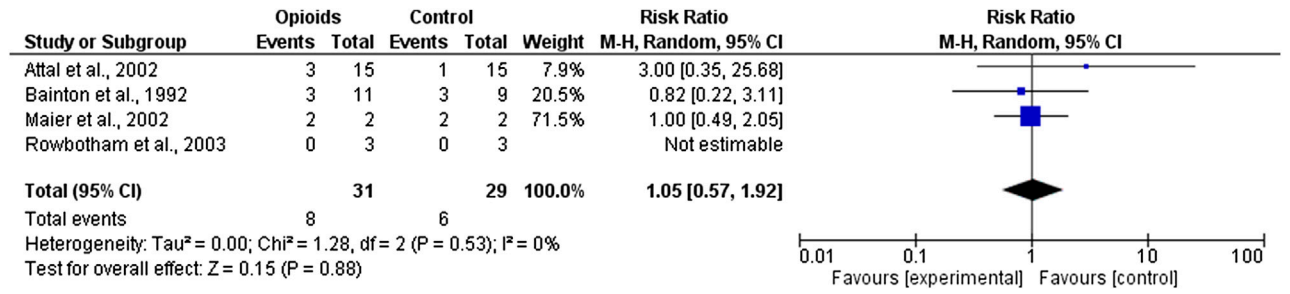

FIGURE 3 | Forest plot for efficacy of opioid agonists and antagonists in pain reduction in trials included in the meta-analysis.

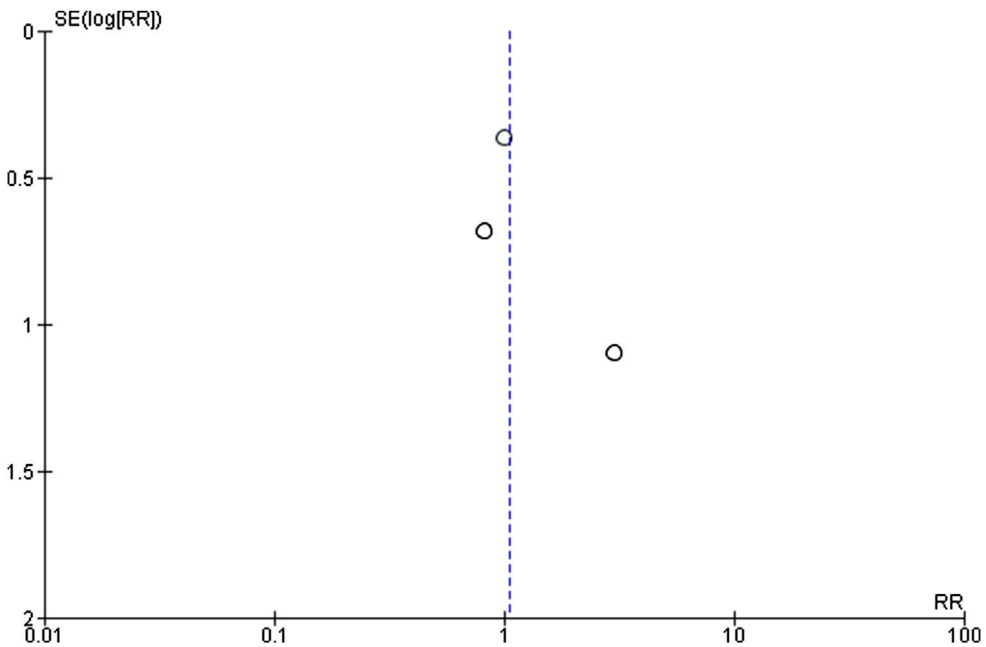

FIGURE 4 | Funnel plot for efficacy of opioid agonists and antagonists in pain reduction in trials included in the meta-analysis.

heterogeneity not applicable; **Figure 4**), since only two RCTs evaluate this outcome. In this study (Rowbotham et al., 2003) no significant changes in total mood disturbance and in quality of life were reported in either treatment group. In the study by Maier et al. (2002), the improvement of pain-related outcomes exerted by morphine administration reached statistical significance ($p \leq 0.05$) only for pain disability and sleep quality. Therefore, RR is based only on the study by Maier et al. (2002), for pain-related outcome, thus forest and funnel plots are not reported; evidence coming from a single trial is uncertain. According to the forest plot in **Figure 3** the results do not favor the experimental treatment (opioid agonist or antagonist) rather than the placebo for the outcome of pain reduction.

The GRADE assessment is based on rating of the following four domains:

(1) Limitations: lack of allocation concealment and/or of blinding, loss to follow-up, failure to adhere to an intention to treat analysis and failure to report outcomes. This key outcome was downgraded for failure of concealment and blinding (Attal et al., 2002), lack of control arm and large loss to follow-up (Rowbotham et al., 2003) and minor protocol violations (Maier et al., 2002) for pain reduction; for the same reasons, this outcome was downgraded for pain-related outcomes.

(2) Inconsistency: variability in results across studies can be due to true differences in treatment effect. The rate of this domain was downgraded since efficacy is partial across the studies for pain reduction, and for pain-related outcomes since results are opposite in the two RCTs.

(3) Indirectness: differences between the population, intervention, comparator and outcome of interest and those included in the relevant studies. This key outcome was downgraded both for pain reduction and for pain-related outcomes since these studies are conceived for populations including mixed types of pain and not only post-stroke pain as defined in the PICOS.

Table 3 | GRADE rating of overall quality of evidence: quality assessment and summary of findings of the body of evidence for pain reduction and pain-related outcomes.

Quality assessment						Summary of Findings		
No of studies (Design)	Study limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Relative effect (95% CI) [b]	Risk difference (95% CI)	Quality
Analgesic efficacy[a]: 4 (RCT)	Serious limitations (−1)	Serious inconsistency (−1)	Serious indirectness (−1)	Imprecision[c]	Likely	RR 1.05 (0.57–1.92)	RD 0.06 (−0.12 to 0.24)	Very low ⊕OOO
Effectiveness on pain-related domains[a]: 2 (RCT)	Serious limitations (−1)	Serious inconsistency (−1)	Serious indirectness (−1)	Imprecision[c]	Likely	RR 1.00 (0.49–2.05)	RD 0.00 (−0.60 to 0.60)	Very low ⊕OOO

RCT, Randomized clinical trial; CI, Confidence Interval.
[a]Please refer to PICOS outcomes in the Methods paragraph.
[b]Relative risks (95% CI) are based on random effect models.
[c]The retrieved RCTs include relatively few patients.

(4) Imprecision: wide CIs. The retrieved RCTs include relatively few patients and thus have wide CIs.
(5) Publication bias: studies showing no significant results are often unpublished. Due to the asymmetry of the funnel plot and to the paucity of studies, there is strong suspicion of publication bias. Therefore, the rating of this domain is defined as "likely".

The EP with quality assessment and Summary of Findings (SoF) is reported in **Table 3**.

DISCUSSION

Post-stroke pain is a complex condition representing both an underdiagnosed and an undertreated chronic consequence of cerebrovascular events. Pain after stroke encompasses neuropathic and nociceptive features and can be either spontaneous or evoked, constant or intermittent (Klit et al., 2009). It consists in a variety of pain syndromes going from CPSP, CRPS, pain secondary to spasticity and musculoskeletal pain, which may occur with variable prevalence and that can be present simultaneously being different and difficult to characterize and adequately treated in each individual patient (Delpont et al., 2018). Post-stroke pain management is complex, considering its multifaceted nature and the existence of multiple pharmacological and non-pharmacological therapeutic approaches depending on the pain subtype. In fact, according to the underlying pathophysiology a different management is needed (Harrison and Field, 2015): local neuromuscular blockade for pain secondary to spasticity, mechanical stabilization and rehabilitation with shoulder strapping in musculoskeletal pain, while first line for neuropathic pain consists in α2δ-1 ligands and antidepressants and in case of severe resistant pain opioids can represent an adjuvant treatment. Data and evidence for second and third-line therapies are even more scant. Also opioid antagonists could exert some efficacy in pain after stroke since anomalous perfusion (Strahlendorf et al., 1980) or increased levels of endogenous opioids (Baskin and Hosobuchi, 1981; Willoch et al., 2004) can affect the area subjected to stroke. Indeed, naloxone is studied in acute ischemic stroke. Other opioids exert peculiar actions like levorphanol that can interact with both NMDA receptors and serotonin and norepinephrine uptake (Codd et al., 1995). This systematic review and meta-analysis evaluated clinical trials investigating the effect of opioids and opioid antagonists on pain after stroke and its functional consequences. A small number of studies (i.e., 8 results) met the inclusion criteria and was therefore included in the qualitative analysis and narrative synthesis (Budd, 1985; Yamamoto et al., 1991; Bainton et al., 1992; Yamamoto et al., 1997; Attal et al., 2002; Maier et al., 2002; Rowbotham et al., 2003; Saitoh et al., 2003). Despite the differences among study design (i.e., placebo-controlled, single arm, dose-response) and mechanism of the various opioids investigated (i.e., morphine, naloxone, levorphanol), nearly all the included studies (except one) showed an overall only slight analgesic effect of opioid medications on CPSP, with various primary outcomes (VAS/

NRS, pain relief, pain tolerability) and less consistent effects on other pain-related symptoms. All the patients included in these studies suffered from intractable pain resistant to previous analgesic treatment. Because of the small number of studies and patients, and the different study design across them, no robust evidence can be drawn (Sharpe, 1997). Due to their design, the three drug challenge tests and the study of Budd (1985) are single group without control and lacking information concerned with masking of patients, deliverers and assessors, thus rising some concern in terms of concealment. Double masking was applied to three trials (Bainton et al., 1992; Attal et al., 2002; Rowbotham et al., 2003) and, in the MONTAS study, patients were randomized using a random generator and received the same blinded medication package. However, in the study by Attal et al. (2002), blindness was put at risk since seven patients and the examiner (in ten cases) identified the active treatment. Moreover, studies differed in terms of pain scales used and they are very heterogeneous in terms of population enrolled, impairing directness. Trials are adequately designed but not specifically for post-stroke pain, e.g. the number of patients is small for all the trials except for the MONTAS study and the study by Rowbotham et al. (2003), whereas the CPSP patients are only two and ten, respectively. Moreover, sample power calculation is not reported. Overall, in this population, any estimate of effect for the first PICOS outcome is very uncertain and results are inconclusive due to the small number of studies and of patients: in fact, each of the four important GRADE criteria ranges from moderate/low to very low quality of evidence, downgrading to very low the overall quality of evidence of efficacy of opioids (Atkins et al., 2004; Guyatt et al., 2008; Guyatt et al., 2011). The IMMPACT recommendations support the importance of physical functioning as core outcome for pain (Turk et al., 2003), an issue of the utmost importance in these patients. However, only the study by Rowbotham et al. (2003) included physical functioning as an outcome and the MONTAS study highlighted an improvement of pain-associated sensory and affective variables and disability. Therefore, there is low quality of evidence for the second PICOS outcome.

The poor/unclear response of CPSP to opioids is in keeping with reduced binding to opioids in pain circuitry in CPSP (Willoch et al., 2004). Indeed, CPSP patients show decreased brain opioid receptors binding in posterior midbrain, medial thalamus and the insular, temporal and prefrontal cortices contralateral to pain, being this pattern different from the opioid receptors binding occurring in peripheral neuropathic pain, thus supporting different response of central vs. peripheral neuropathic pain to opioids (Maarrawi et al., 2007). Of interest, despite the wide search criteria we used, all the included studies pertained CPSP, and we found no evidence on other types of post-stroke pain. Indeed, all the included studies were quite old and dating prior to the 2009 CPSP redefinition (Klit et al., 2009), which made the differential diagnosis between CPSP and other types of post-stroke pain clearer and more reliable. Pain assessment represents an important issue in non-communicative patients, who can have difficulties to describe their pain, contributing to behavioral disturbances in some neurological conditions (Scuteri et al., 2017; Scuteri et al., 2018; Scuteri et al., 2019a; Scuteri et al., 2019b). Indeed, in the real-life clinical setting, the use of opioids in patients with post-stroke pain, who are not able to communicate is frequent, but the response to treatment is unclear (Schuster et al., 2020). Post-stroke pain can occur also in patients with neurodegenerative disorders (Scherder and Plooij, 2012) and clinical trials to assess the efficacy and safety of opioids are needed, being the treatment of pain often inappropriate in this population (Scuteri et al., 2017; Scuteri et al., 2018; Scuteri et al., 2020a; Scuteri et al., 2020b). Future double-blind randomized clinical trials designed specifically for post-stroke pain, methodology and statistical power are needed to assess the efficacy and safety of opioids in post-stroke pain and to understand the impact of pain treatment on physical function. In fact, being post-stroke pain often severe, it may be resistant to first line treatments, as it occurs in all of the studies included in the analysis; the latter condition makes treatment with opioids sometimes necessary. In these eight studies morphine induced nausea, somnolence, headache and severe side effects (in 58% of patients of the study by Maier et al. (2002), consisting in constipation, vomiting, nausea, sedation and micturition disturbances), also psychological effects were reported with levorphanol and naloxone caused slight increase in pulse rate, sweating, tremor, salivation, pain, nausea and faintness. In the light of the most rigorous analysis of the literature, it is conceivable that opioid use within a time frame of no longer than 12 weeks is not linked to respiratory depression and to the potential for abuse with overdose death (Dowell et al., 2016). However, further evidence is necessary for the best clinical use of these effective analgesics, also limiting the most serious consequences of inappropriate opioids prescription.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article.

AUTHOR CONTRIBUTIONS

DS, GB and PT conceived the study. All Authors have participated in the manuscript preparation and have read and approved the final manuscript.

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REVIEW
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Analgesic Effect of Acetaminophen: A Review of Known and Novel Mechanisms of Action

Nobuko Ohashi[1] *and Tatsuro Kohno*[2]*

[1]Division of Anesthesiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan, [2]Department of Anesthesiology and Intensive Care Medicine, International University of Health and Welfare School of Medicine, Narita, Japan

Acetaminophen is one of the most commonly used analgesic agents for treating acute and chronic pain. However, its metabolism is complex, and its analgesic mechanisms have not been completely understood. Previously, it was believed that acetaminophen induces analgesia by inhibiting cyclooxygenase enzymes; however, it has been considered recently that the main analgesic mechanism of acetaminophen is its metabolization to *N*-acylphenolamine (AM404), which then acts on the transient receptor potential vanilloid 1 (TRPV1) and cannabinoid 1 receptors in the brain. We also recently revealed that the acetaminophen metabolite AM404 directly induces analgesia via TRPV1 receptors on terminals of C-fibers in the spinal dorsal horn. It is known that, similar to the brain, the spinal dorsal horn is critical to pain pathways and modulates nociceptive transmission. Therefore, acetaminophen induces analgesia by acting not only on the brain but also the spinal cord. In addition, acetaminophen is not considered to possess any anti-inflammatory activity because of its weak inhibition of cyclooxygenase (COX). However, we also revealed that AM404 induces analgesia via TRPV1 receptors on the spinal dorsal horn in an inflammatory pain rat model, and these analgesic effects were stronger in the model than in naïve rats. The purpose of this review was to summarize the previous and new issues related to the analgesic mechanisms of acetaminophen. We believe that it will allow clinicians to consider new pain management techniques involving acetaminophen.

Keywords: acetaminophen, *N*-acylphenolamine, analgesia, brain, spinal dorsal horn

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Damiana Scuteri,
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Danilo De Gregorio,
McGill University, Canada

***Correspondence:**
Tatsuro Kohno
kohno-t@umin.net

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INTRODUCTION

Acetaminophen is one of the most commonly used analgesic agents for alleviating acute and chronic pain. Due to its safety, acetaminophen is prescribed for patients in whom non-steroidal anti-inflammatory drugs (NSAIDs) are contraindicated, such as those with gastric ulcers and bronchial asthma, pregnant women, nursing mothers, and children (Leung, 2012; Roberts et al., 2016). It has also been placed on all three steps of pain treatment intensity of the WHO analgesic ladder for the treatment of cancer pain. However, its metabolism is complex, and its analgesic mechanisms have not been completely understood. Previously, it was thought that acetaminophen induces analgesia by inhibiting the enzyme cyclooxygenase (COX), but now it is believed that acetaminophen is metabolized to *p*-aminophenol, which crosses the blood-brain barrier and gets metabolized by fatty acid amide hydrolase to yield *N*-acylphenolamine (AM404). AM404 acts on the transient receptor potential vanilloid 1 (TRPV1) and cannabinoid 1 (CB1) receptors in the midbrain and medulla (Roberts et al., 2002; Jennings et al., 2003; Mallet et al., 2010), which are co-localized

mediators of pain modulation (De Petrocellis et al., 2000; Palazzo et al., 2002; Maione et al., 2006). Therefore, acetaminophen induces analgesia via direct action on the brain (Bannwarth et al., 1992; Gelgor et al., 1992; de Lange et al., 1994; Hogestatt et al., 2005), and these receptor sites on the brain are the main mediators of acetaminophen-induced analgesia. However, our group recently revealed a new analgesic mechanism of acetaminophen, using behavioral measures, *in vivo* and *in vitro* whole-cell patch-clamp recordings with rats, wherein the acetaminophen metabolite AM404 directly induces analgesia via TRPV1 receptors on the spinal dorsal horn (Ohashi et al., 2017). Similar to the brain, the spinal cord, especially substantia gelatinosa (SG, lamina II of Rexed), is also critical to pain pathways, and modulates nociceptive transmission via primary afferent Aδ- and C-fibers (Kohno et al., 1999; Ohashi et al., 2017). Furthermore, TRPV1 receptors are abundant in the spinal cord dorsal horn (Yang et al., 1998; Yang et al., 1999; Yang et al., 2000). Therefore, our results describing the new analgesic mechanism underlying the action of acetaminophen on the spinal dorsal horn, are reasonable compared to previous reports (Ohashi et al., 2017).

Acetaminophen does not possess any anti-inflammatory activity, because it is a very weak inhibitor of COX and does not inhibit neutrophil activation (Hanel and Lands, 1982). Therefore, even though it has always been discussed together with NSAIDs in terms of pharmacological mechanism, acetaminophen is not regarded as an NSAID and is not appropriate for treating inflammatory pain conditions. However, we also revealed that acetaminophen metabolite AM404 induces analgesia via TRPV1 receptors on the spinal dorsal horn in a rat model of inflammatory pain, and these analgesic effects were stronger in the inflammatory pain model than in naïve rats (Ohashi et al., 2017).

The purpose of this review was to summarize the previous and new issues related to the analgesic mechanisms of acetaminophen and discuss our understanding that acetaminophen metabolite AM404 also acts on the spinal dorsal horn and induces analgesia in inflammatory pain conditions. This review will allow clinicians to consider new pain management techniques using acetaminophen.

ANALGESIC MECHANISMS OF ACETAMINOPHEN

Inhibition of Cyclooxygenase Activity

It has been thought that acetaminophen induces analgesia by blocking prostaglandin synthesis from arachidonic acid by inhibiting the enzymes, COX-1 and -2. However, unlike NSAIDs, acetaminophen interferes with the peroxidase activity of COX isoenzymes, predominantly COX-2, with little clinical effect and depends to a great extent on the state of environmental oxidation (Graham et al., 2013; Aminoshariae and Khan, 2015). It has also been reported that the third COX isoenzyme, COX-3, which is an exon splice variant of COX-1, is especially sensitive to acetaminophen (Chandrasekharan et al., 2002). However, it soon appeared that COX-3 is not found in humans, and further studies suggest that acetaminophen has no clinically significant effects on the COX-1 exon splice variants found in humans so far (Graham and Scott, 2005). It is now considered that the inhibition of COX activity is not the main analgesic mechanism of acetaminophen (**Table 1**; **Figure 1**).

Activating the Transient Receptor Potential Vanilloid 1 and Cannabinoid 1 Receptors

Acetaminophen is first metabolized to *p*-aminophenol, which easily crosses the blood-brain barrier and is converted to AM404 by fatty acid amide hydrolase (Hogestatt et al., 2005). Acetaminophen is also metabolized to other compounds through another pathway, such as *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which also appears to produce analgesia by activating transient receptor potential ankyrin 1 receptors (Andersson et al., 2011; Gentry et al., 2015). However, AM404 is widely known to be the most important mediator of acetaminophen metabolite-induced analgesia. Although AM404 was thought to be just an anandamide analog which acts on CB1 receptors (Beltramo et al., 1997), it was recently shown that AM404 also acts on TRPV1 receptors (Zygmunt et al., 2000; Hogestatt et al., 2005; Barrière et al., 2013). In particular, it is known that TRPV1 receptors in the brain are important for pain modulation. Two examples involving TRPV1 receptors are cannabidiol, the primary nonaddictive component of cannabis, which induces analgesia through TRPV1 receptor activation in the dorsal raphe nucleus (De Gregorio et al., 2019), and dipyrone, an antipyretic and non-opioid analgesic drug which causes analgesia by acting on TRPV1 and CB1 receptors in rostral ventromedial medulla (Maione et al., 2015). Therefore, it is now considered that AM404 acts on TRPV1 receptor in the brain and induces analgesia. For example, by activating TRPV1 receptor, AM404 produced outward currents that were measured using whole-cell patch-clamp recordings and acted as a partial agonist in trigeminal neurons (Roberts et al., 2002; Jennings et al., 2003). Moreover, intracerebroventricular injection of AM404 produced analgesia in the formalin test (Mallet et al., 2010). Therefore, these receptors in the brain are widely considered to be the main mediators of acetaminophen-induced analgesia. They are also the reason why acetaminophen exhibits a "central" effect for long periods.

Similar to the brain, it is also known that the spinal cord, especially SG neurons, is critical to pain pathways, and modulates nociceptive transmission via primary afferent Aδ- and C-fibers (Kohno et al., 1999; Ohashi et al., 2015). Furthermore, it is also known that TRPV1 and CB1 receptors are abundant in the spinal cord dorsal horn (Yang et al., 1998; Yang et al., 1999; Yang et al., 2000). Therefore, there is a possibility that, in addition to its actions in the brain, acetaminophen and/or its metabolite AM404 also induce analgesia via direct activation of TRPV1 and/or CB1 receptors in the spinal cord dorsal horn. In fact, a few previous studies have shown that AM404 decreases neuronal *c-fos*-positive immunoreactivity induced by non-noxious stimulation of the spinal cord in a rat model of neuropathic or inflammatory pain, and these responses are inhibited by TRPV1 or CB1 receptor

TABLE 1 | Analgesic mechanism of acetaminophen.

Medicine	Target site	Effect/mechanism	References
Acetaminophen	COX-1, COX-2	Inhibitory	Aminoshariae and Khan (2015)
Acetaminophen	COX-2	Inhibitory	Graham et al. (2013)
Acetaminophen	COX-3	Inhibitory	Chandrasekharan et al. (2002)
Acetaminophen	COX-3	No clinically relevant effects	Graham and Scott (2005)
NAPQI	TRPA1	Activating	Andersson et al. (2011)
Acetaminophen	TRPA1	Activating	Gentry et al. (2015)
AM404	Anandamide transport inhibitor, CB1 receptor	Re-uptake inhibitor, Activating	Beltramo et al. (1997)
AM404	TRPV1 receptor	Activating	Zygmunt et al. (2000), Hogestatt et al. (2005), Roberts et al. (2002), Jennings et al. (2003), Mallet et al. (2010), Barrière et al. (2013),
AM404	CB1 receptor, TRPV1 receptor	Activating	Rodella et al. (2005), Borsani et al. (2007)
AM404	CB1 receptor, TRPV1 receptor	Not activating, Activating	Ohashi et al. (2017)
AM404	CB1 receptor < TRPV1 receptor	Activating	Szallasi and Di Marzo (2000)
Acetaminophen	Opioids	Activating	Raffa et al. (2000), Raffa et al. (2004)
Acetaminophen	Serotonin	Increases content	Pini et al. (1996)
Acetaminophen	5-HT_3 receptor	Activating	Alloui et al. (2002), Pickering et al. (2006), Pickering et al. (2008)

NAPQI, N-*acetyl*-p-*benzoquinoneimine; AM404,* N-*acylphenolamine; COX, cyclooxygenase; TRPA1, transient receptor potential ankyrin 1; CB1, cannabinoid 1; TRPV1, transient receptor potential vanilloid 1.*

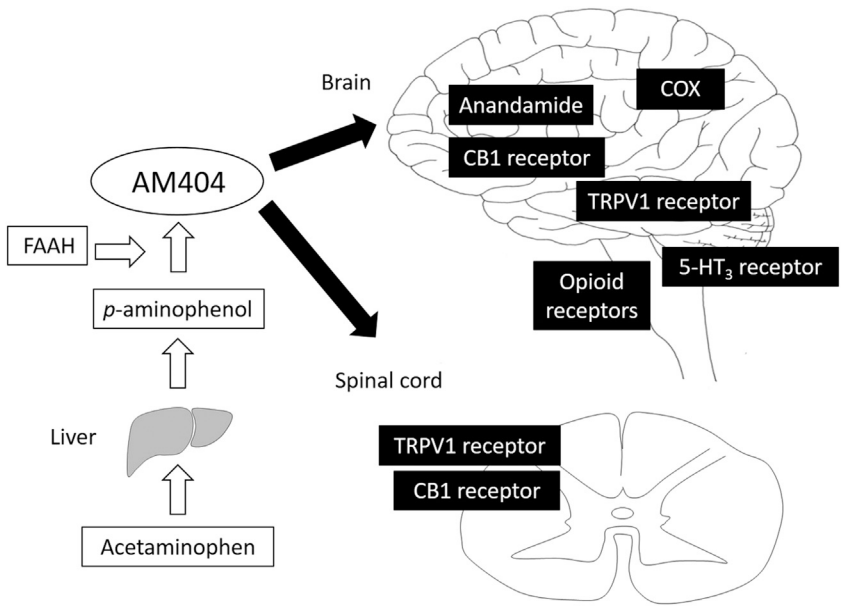

FIGURE 1 | Analgesic mechanism of acetaminophen. Acetaminophen is metabolized to *p*-aminophenol, which easily crosses the blood-brain barrier and is converted to AM404 by FAAH. AM404 mainly acts on both the brain and spinal cord via COX, anandamide, CB1, TRPV1, opioid, and 5-HT3 receptors. AM404, N-acylphenolamine; FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; CB1, cannabinoid 1; TRPV1, transient receptor potential vanilloid 1.

antagonists (Rodella et al., 2005; Borsani et al., 2007). Nevertheless, the precise analgesic mechanisms of acetaminophen in the spinal cord via its AM404 metabolite are still unknown, because previous studies have not examined the synaptic transmission at the cellular level. Therefore, it was believed that acetaminophen does not act on the spinal cord. However, our group recently revealed a new analgesic mechanism of acetaminophen, using behavioral measures, and *in vivo* and *in vitro* whole-cell patch-clamp recordings with naïve rats (Ohashi et al., 2017). We first demonstrated with behavioral experiments that intraperitoneal injections of acetaminophen and intrathecal injections of AM404 induce analgesia to thermal stimulation. We next conducted *in vivo* and *in vitro* whole-cell patch-clamp recordings of SG neurons in the spinal cord dorsal horn and recorded the excitatory post-synaptic currents (EPSCs). With *in vivo* patch-clamp recording, the areas under the curve, which is surrounded by the baseline and border of the EPSCs, were significantly reduced after intravenous injection of

acetaminophen following peripheral pinch stimuli. However, with *in vitro* patch clamp recording, direct application of acetaminophen to the spinal cord did not change miniature EPSCs (mEPSCs), but AM404 did. These results suggest that systemic administration of acetaminophen metabolizes to AM404, which directly acts on spinal cord dorsal horn and induces analgesia. We also examined the effects of AM404 on EPSCs evoked from primary afferent neurons by stimulating the dorsal root and demonstrated that AM404 reduces the amplitudes of monosynaptic EPSCs evoked by stimulating C-fibers, but not Aδ-fibers. These responses were inhibited by the TRPV1 receptor antagonist, but not CB1 receptor antagonist. Therefore, we found that acetaminophen was metabolized to AM404, which induces analgesia by directly inhibiting the excitatory synaptic transmission via TRPV1 receptors expressed on terminals of C-fibers in the spinal dorsal horn. Contrary to previous studies on the brain, we failed to find the analgesic effect of acetaminophen/AM404 on the CB1 receptor on spinal dorsal horn neurons. We believe that the main reason for the differences between our results and that of previous reports was the concentration of AM404 (30 μM) in our study, which is equivalent to the clinically recommended dosage of acetaminophen (20 mg/kg). Szallasi *et al.* compared the affinities of AM404 for brain TRPV1 and CB1 receptors and reported that the concentration of AM404 required to activate TRPV1 receptors is much lower than that required for CB1 receptors (Szallasi and Di Marzo, 2000). Therefore, there is a possibility that the concentration of AM404 in our study was insufficient to activate CB1 receptors in dorsal horn neurons and higher doses of AM404 may also act on the CB1 receptor in the spinal dorsal cord. We believe that our new analgesic mechanism of acetaminophen will contribute to the development of new techniques for clinical pain management using acetaminophen.

Other Mechanisms

Another possible reason for the analgesic action of acetaminophen could be the action of endogenous neurotransmitter systems including opioid and serotonergic systems. Previous studies have reported that the analgesic effect of acetaminophen involves the recruitment of endogenous opioid pathways that lead to analgesic spinal-supraspinal self-synergy (Raffa et al., 2000), and the analgesic effects induced by intrathecal injection or intracerebroventricular injection of acetaminophen were attenuated by *mu*-, *delta*-, and *kappa*-opioid receptor antagonists (Raffa et al., 2004). This analgesic self-synergy is significantly attenuated by the administration of naloxone, an opioid receptor antagonist, at the spinal level (Raffa et al., 2000). Similarly, another study reported that depletion of brain serotonin prevented the analgesic effect of acetaminophen in the hot-plate test and in the first phase of the formalin response. Furthermore, acetaminophen significantly increased the serotonin content in the pontine and cortical areas (Pini et al., 1996). It is also reported that the serotonin receptor has several subtypes, and acetaminophen-induced analgesia was inhibited by intrathecal or intravenous injection of tropisetron, a 5 hydroxytryptamine$_3$ (5-HT$_3$) receptor antagonist (Alloui et al., 2002; Pickering et al., 2006; Pickering et al., 2008). These findings implied that acetaminophen may be involved in endogenous opioid or descending serotonergic pathways as contributors to the analgesic action of acetaminophen.

Analgesic Effect of Acetaminophen for Inflammatory Pain

For many decades, acetaminophen was not considered to possess any anti-inflammatory activity and was, therefore, not appropriate for treating allodynia or hyperalgesia in inflammatory pain conditions. A study has reported that acetaminophen is a very weak inhibitor of COX, which does not inhibit neutrophil activation (Hanel and Lands, 1982). For example, at the therapeutic concentration, acetaminophen inhibits COX activity when the levels of arachidonic acid and peroxide are low but has little effect when the levels of arachidonic acid or peroxide are high as seen in severe inflammatory conditions such as rheumatoid arthritis (Hanel and Lands, 1982). However, our group also revealed that acetaminophen metabolite AM404 induces analgesia in rats of the inflammatory pain model (Ohashi et al., 2017). Similar to the results in naïve rats, our behavioral studies in an inflammatory pain rat model suggest that acetaminophen and AM404 induce analgesia to thermal stimulation. Moreover, both *in vivo* and *in vitro* whole-cell patch-clamp recordings have shown that acetaminophen metabolite AM404 directly inhibits excitatory synaptic transmission via TRPV1 receptors expressed on terminals of C-fibers in the spinal dorsal horn. Moreover, analgesic effects induced by acetaminophen and AM404 in the rats used for the inflammatory pain model were stronger than those in naïve rats (Ohashi et al., 2017). It is known that there is an increased proportion of TRPV1-protein-positive neurons during inflammation in dorsal root ganglion and unmyelinated axons of the digital nerves (Carlton and Coggeshall, 2001). Therefore, increased TRPV1 activity in the rats used for the inflammatory pain model suggests strong analgesic effects following acetaminophen and AM404 administration. Therefore, our findings are consistent with previous research, and we believe that our results will allow clinicians to consider new pain management techniques involving acetaminophen.

PHARMACOKINETICS AND SIDE EFFECTS

When the appropriate dosage of acetaminophen is used, serious side effects seldom occur; however, some case studies have reported liver toxicity caused by acetaminophen. Usually, acetaminophen is administered orally or intravenously. The maximum single-dose of acetaminophen for the treatment of pain or fever is 1,000 mg every 4 h as needed, up to a recommended maximum daily dose of 4 g. These therapeutic concentrations range from 5 to 20 mg/ml. Acetaminophen has a very high oral bioavailability of 60–88% (Bertolini et al., 2006), and after oral administration of 1,000 mg acetaminophen, the plasma maximum concentration (C_{max}) is 12.3 μg/ml, area under the curve over 6 h (AUC_{0-6}) is 29.4 μg/h/ml, and AUC

extrapolated to infinity ($AUC_{0-\infty}$) is 44.4 μg/h/ml. The time to maximal concentration (T_{max}) is 1.0 h, and the elimination half-life ($t_{1/2}$) is 2.53 h. In contrast, after intravenous administration of 1,000 mg acetaminophen, the plasma C_{max} is 21.6 μg/ml, AUC_{0-6} is 42.5 μg/h/ml, and $AUC_{0-\infty}$ is 50.0 μg/h/ml. The T_{max} is 0.25 h, and the $t_{1/2}$ is 2.17 h (Singla et al., 2012). These findings suggest that intravenous administration of acetaminophen shows earlier and higher peak plasma levels than oral administration; however, there is no difference in AUC and $t_{1/2}$ between the intravenous and oral administration.

Once acetaminophen metabolizes in the liver by conjugation with glucuronic acid (40–67%), sulfuric acid (20–46%), and *p*-aminophenol, it easily crosses the blood-brain barrier and is converted to AM404 (Gazzard et al., 1973; Duggin and Mudge, 1975). Furthermore, about 5% of acetaminophen is subjected to *N*-hydroxylation in the liver with the involvement of cytochrome P450 enzymes (especially CYP2E1) to form the toxic metabolite, NAPQI (Bertolini et al., 2006). Normally, NAPQI is detoxified into harmless metabolites via conjugation of the sulfhydryl groups of glutathione by glutathione S-transferase into mercapturic acid, which is eliminated in the urine (Mitchell et al., 1974; Potter et al., 1974; Benson et al., 2005; Bertolini et al., 2006). However, glutathione can become depleted after overuse of acetaminophen or in cases of weakened hepatic function (caused by slimming, malnutrition, hepatitis C virus, or alcohol overuse), which causes accumulation of NAPQI. When this happens, NAPQI interacts covalently with liver cell components resulting in hepatic damage. To detoxify the liver toxicity caused by NAPQI, *N*-acetylcysteine must be ingested as soon as possible.

Usually, acetaminophen is administered by oral, transanal, and intravenous routes, and NAPQI is produced by acetaminophen during the metabolic pathways. However, we think that if we administer AM404 instead of acetaminophen using intrathecal or intracerebroventricular injection, we could observe a stronger analgesic effect with reduced side effects at a smaller dosage. Therefore, further clinical studies on the effectiveness and safety of acetaminophen will be needed.

CONCLUSION

Acetaminophen acts not only on the brain but also the spinal cord and induces analgesia. Moreover, the most possible analgesic mechanism is that the acetaminophen metabolite AM404 acts by activating TRPV1 and/or CB1 receptors. Our data also support a mechanism by which acetaminophen also induces analgesia in inflammatory pain conditions. These findings are applicable to clinical pain management with acetaminophen, but the analgesic mechanism of acetaminophen has not been elucidated completely. Therefore, further discussions and studies will be needed to understand the action of acetaminophen.

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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Morphine Antinociception Restored by Use of Methadone in the Morphine-Resistant Inflammatory Pain State

*Chizuko Watanabe, Asami Komiyama, Masaru Yoshizumi, Shinobu Sakurada and Hirokazu Mizoguchi**

Department of Physiology and Anatomy, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai, Japan

The antinociceptive effect of methadone in the morphine-resistant inflammatory pain state was described in the paw-withdrawal test using the complete Freund's adjuvant (CFA)-induced mouse inflammatory pain model. After intraplantar (i.pl.) injection of CFA, thermal hyperalgesia was observed in the ipsilateral paw. The antinociceptive effects of subcutaneous (s.c.) injection of morphine, fentanyl, and oxycodone against thermal hyperalgesia in the inflammatory pain state were reduced in the ipsilateral paw 7 days after CFA pretreatment. On the contrary, the antinociceptive effect of s.c. injection of methadone was maintained in the ipsilateral paw 7 days after CFA pretreatment. The suppressed morphine antinociception in the CFA model mice was bilaterally restored following s.c. treatment with methadone 20 min prior to or 3 days after CFA pretreatment. The suppressed morphine antinociception was also bilaterally restored by intraperitoneal treatment with MK-801 30 min prior to CFA pretreatment; however, the s.c. injection of morphine 30 min prior to CFA pretreatment failed to restore the suppressed morphine antinociception in the CFA model mice. The expression level of mRNA for μ-opioid receptors 7 days after i.pl. pretreatment was not significantly changed by i.pl. pretreatment with CFA or s.c. pretreatment with methadone. In conclusion, methadone is extremely effective against thermal hyperalgesia in the morphine-resistant inflammatory pain state, and restores suppressed morphine antinociception in the inflammatory pain state without altering the expression level of mRNA for μ-opioid receptors.

Keywords: antinociception, methadone, morphine, inflammatory pain, restore, μ-opioid receptor

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Luigi Antonio Morrone, University of Calabria, Italy

****Correspondence:***
Hirokazu Mizoguchi
mizo@tohoku-mpu.ac.jp

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INTRODUCTION

Inflammatory pain is the spontaneous hypersensitivity to pain that occurs in response to tissue damage and inflammation. The prominent features of inflammatory pain are edema, mechanical allodynia, thermal hyperalgesia, and mechanical hyperalgesia. Several experimental models of inflammatory pain have been developed in rodents, including formalin- (Hunskaar and Hole, 1987), carrageenan- (Miyake et al., 2019), and complete Freund's adjuvant (CFA)-based models (Ohsawa et al., 2000). Among the experimental models of inflammatory pain, the inflammatory pain model involving hind-paw inflammation caused by intraplantar (i.pl.) administration of CFA is

widely used to describe the mechanisms of inflammatory pain and the effectiveness of analgesics against inflammatory pain. Inflammatory pain in this model is distinct from pain in other chronic pain models where expression is restricted to the ipsilateral side. Mechanical allodynia in the CFA inflammatory pain model is observed bilaterally in both the ipsilateral (inflamed) and contralateral (non-inflamed) paw, whereas thermal hyperalgesia is observed only in the ipsilateral paw (Nagakura et al., 2003). Therefore, the mechanism underlying this inflammatory pain is considered to be more complicated than that underlying other chronic pain.

It is well established that the antinociceptive effect of morphine against both hyperalgesia and allodynia is suppressed in chronic pain, such as neuropathic pain (Narita et al., 2008), neuropathic cancer pain (Luger et al., 2002), and diabetic neuropathic pain (Zurek et al., 2001). Therefore, these types of pain are known as morphine-resistant intractable pain. Unlike morphine-resistant intractable pain, the antinociceptive effect of morphine is reported to be enhanced or retained against mechanical hyperalgesia (Maldonado et al., 1994; Fernández-Dueñas et al., 2007) and thermal hyperalgesia (Fernández-Dueñas et al., 2007) in the CFA-induced inflammatory pain state. However, in a clinical study, morphine was found to be less effective against inflammatory pain (Lillesø et al., 2000). We previously found that the antinociceptive effect of morphine was markedly suppressed against mechanical allodynia in the inflammatory pain state (Aoki et al., 2014b). In the present study, we further characterized the antinociceptive effect of narcotic analgesics, including morphine, against thermal hyperalgesia in the inflammatory pain state, and found a special effect of methadone in restoring suppressed morphine antinociception in the inflammatory pain state.

MATERIALS AND METHODS

All experiments were performed following the approval of the Ethics Committee for Animal Experiments at Tohoku Medical and Pharmaceutical University and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number and suffering of the animals used in the following experiments.

Animals

Male ddY mice (Japan SLC, Hamamatsu, Japan) weighing 18–24 g were used. Mice were housed in a temperature-(22–23°C) and humidity-controlled (50–60%) room with an alternating 12-h light/dark (lights on at 07:00 and off at 19:00) cycle. Food and water were available ad libitum.

Mouse Model for Inflammatory Pain

To produce the inflammatory pain model, mice underwent i.pl. injection of 50 μL of CFA (Sigma-Aldrich, St. Louis, MO) in the left hind paw using a syringe with a 26-gauge needle (Aoki et al., 2014a; Aoki et al., 2014b). Control mice underwent i.pl. injection of sterile saline.

Measurement of Thermal Hyperalgesia and Antinociception

The thermal hyperalgesia and antinociceptive effect of narcotic analgesics were measured using the paw-withdrawal test, using an automated tail-flick unit (Ugo Basile, Italy) (Mizoguchi et al., 2006). Mice were adapted to the testing environment for at least 1 h before stimulation. Each animal was restrained with a soft cloth to reduce visual stimulation, and a light beam was applied to the hind paw as a noxious radiant heat stimulus. The light beam focused on the plantar surface of the hind paw, and the latency of the paw withdrawal in response to noxious radiant heat stimulation was measured. The intensity of the noxious radiant heat stimulation was adjusted such that the pre-latency for the paw withdrawal response was approximately 6 s. The antinociceptive effect was expressed as a percentage of the maximum possible effect (% MPE), which was calculated using the following equation: $([T1 - T0]/[10 - T0]) \times 100$, where T0 and T1 are the pre- and post-drug latencies for the paw withdrawal response, respectively. To prevent tissue damage of the paw, noxious radiant heat stimulation was terminated automatically if the mouse did not lift the paw within 10 s.

Drugs

The drugs used were morphine hydrochloride (Takeda, Osaka, Japan), fentanyl citrate (Mallinckrodt Pharmaceuticals, St. Louis, MO), oxycodone hydrochloride (Mallinckrodt Pharmaceuticals), methadone hydrochloride (Mallinckrodt Pharmaceuticals), and (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*] cycloheptene-5,10-imine (MK-801) hydrogen maleate (Sigma-Aldrich Chemical Co.). All drugs were dissolved in sterile saline.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA in the mouse lumbar spinal cord was extracted using the RNeasy Lipid Tissue Mini Kit (QIAGEN K.K., Tokyo, Japan). The purified total RNA was quantified using a spectrophotometer at A260. RT-PCR amplification was performed using the SuperScript One Step RT-PCR system Platinum version (Life Technologies, Carlsbad, CA) (Aoki et al., 2014b). The synthetic forward and reverse primers for the μ-opioid receptor were 5'-CAG CCA GCA TTC AGA ACC ATG G-3' and 5'-ATG GTG CAG AGG GTG AAG ATA CTG G-3', respectively. The synthetic forward and reverse primers for β-actin were 5'-GCT CGT CGT CGA CAA CGG CTC-3' and 5'-CAA ACA TGA TCT GGG TCA TCT TCT T-3', respectively. The samples were heated to 50°C for 30 min for cDNA synthesis and to 94°C for 2 min for pre-denaturation, and then cycled 30 times through 94°C for 30 s for denaturation, 55°C for 30 s for annealing, and 72°C for 30 s for extension, and finally heated to 72°C for 7 min for final extension. The resulting PCR product was electrophoresed on a 0.8% agarose gel containing ethidium bromide. The agarose gel was photographed using a UV Imaging Analyzer FAS-III (Toyobo, Osaka, Japan); the PCR product for the μ-opioid receptor in agarose gel was then semi-quantified in relation to β-actin using a Lumino Imaging Analyzes FAS-1000 (Toyobo).

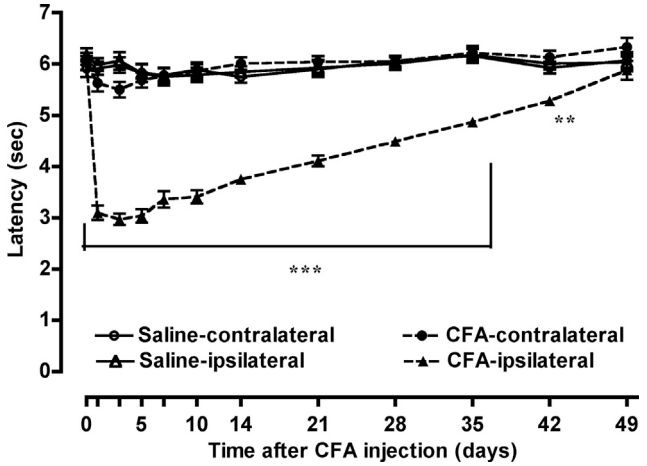

FIGURE 1 | CFA-induced thermal hyperalgesia. Groups of mice were injected i.pl. with 50 μL of saline or CFA in the left hind paw; the withdrawal threshold in the ipsilateral and contralateral paws in response to thermal stimulation of the plantar surface of each hind paw was measured for 49 days. Each value represents the mean ± S.E.M. for 12–14 mice. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. ipsilateral paw on the control model (i.pl. saline pretreatment) mice.

Statistical Analysis

The data are expressed as the mean ± SEM for at least eight mice. The statistical significance of differences between the groups was assessed with a one-way analysis of variance (ANOVA) or repeated measures two-way ANOVA followed by Bonferroni's test.

RESULTS

CFA-Induced Thermal Hyperalgesia

Groups of mice were injected i.pl. with 50 μL of saline or CFA in the left hind paw, and the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 49 days. After CFA injection, a decrease in the thermal withdrawal threshold (thermal hyperalgesia) was observed in the ipsilateral paw, but not the contralateral paw (**Figure 1**). Thermal hyperalgesia in the ipsilateral paw of the CFA-treated mice peaked 5 days after CFA treatment and was maintained for 42 days after CFA treatment. In contrast, the thermal withdrawal threshold was not altered in mice that received i.pl. saline treatment.

Antinociceptive Effect of Narcotic Analgesics in the Inflammatory Pain State

Groups of mice pretreated with i.pl. 50 μL of saline or CFA in the left hind paw, underwent subcutaneous (s.c.) injection of morphine (5 mg/kg) at 1, 3, 5, or 7 days after the i.pl. pretreatment, and the withdrawal threshold against thermal stimulation to the plantar surface of each hind paw was measured for 180 min. The potent increase in withdrawal threshold (antinociceptive effect) following morphine treatment was observed bilaterally in saline-pretreated mice at all time points (**Figure 2**). However, the antinociceptive effect of morphine in the ipsilateral paw was gradually suppressed after CFA pretreatment, and was significantly suppressed at 5 and 7 days after CFA treatment (**Figures 2C,D**). The antinociceptive effect of morphine in the contralateral paw also tended to be suppressed at 5 and 7 days after CFA treatment.

Another group of mice that underwent i.pl. pretreatment with 50 μL of saline or CFA in the left hind paw, underwent s.c. injection of fentanyl (0.08 mg/kg), oxycodone (1.5 mg/kg), or methadone (2.8 mg/kg) 7 days after i.pl. pretreatment; the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 60, 90, or 180 min, respectively. The potent antinociceptive effects of these narcotic analgesics was observed bilaterally in saline-pretreated mice (**Figure 3**). However, the antinociceptive effect of fentanyl was significantly suppressed bilaterally 7 days after CFA pretreatment (**Figure 3A**). The antinociceptive effect of oxycodone was significantly suppressed 7 days after CFA pretreatment in the ipsilateral paw, but not in the contralateral paw (**Figure 3B**). In contrast, the antinociceptive effects of methadone in both paws was not affected 7 days after CFA treatment (**Figure 3C**).

Recovery of Antinociceptive Effect of Morphine in Inflammatory Pain State

Groups of mice pretreated with 50 μL of saline or CFA i.pl. in the left hind paw, were injected s.c. with saline or methadone (11.2 mg/kg) 20 min prior, 3 days after, or 6 days after i.pl. pretreatment. Seven days after the i.pl. pretreatment, mice were injected s.c. with morphine (5 mg/kg), and the withdrawal threshold against thermal stimulation of the plantar surface of each hind paw was measured for 180 min. In the control model (i.pl. saline pretreatment) mice, morphine showed a bilateral potent antinociceptive effect with any s.c. pretreatment with saline or methadone (**Figures 4A,C,E**). In the CFA model (i.pl. CFA pretreatment) mice, the antinociceptive effect of morphine was bilaterally suppressed. However, the suppressed morphine antinociception in the CFA model mice was bilaterally restored following s.c. treatment with methadone 20 min prior to or 3 days after CFA pretreatment (**Figures 4B,D**). On the contrary, s.c. treatment with methadone 6 days after CFA pretreatment failed to restore the suppressed morphine antinociception in the CFA model mice (**Figure 4F**).

Another group of mice pretreated with 50 μL of saline or CFA i.pl. in the left hind paw, underwent s.c. injection of saline or morphine (20 mg/kg) or intraperitoneal (i.p.) injection of saline or MK-801 (0.25 mg/kg) 30 min prior to i.pl. pretreatment. Seven days after the i.pl. pretreatment, mice underwent s.c. injection of morphine (5 mg/kg), and the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 180 min. In control mice, morphine bilaterally showed a potent antinociceptive effect following s.c. pretreatment with saline or morphine (**Figure 5A**) or i.p. pretreatment with saline or MK-801 (**Figure 6A**). In the CFA

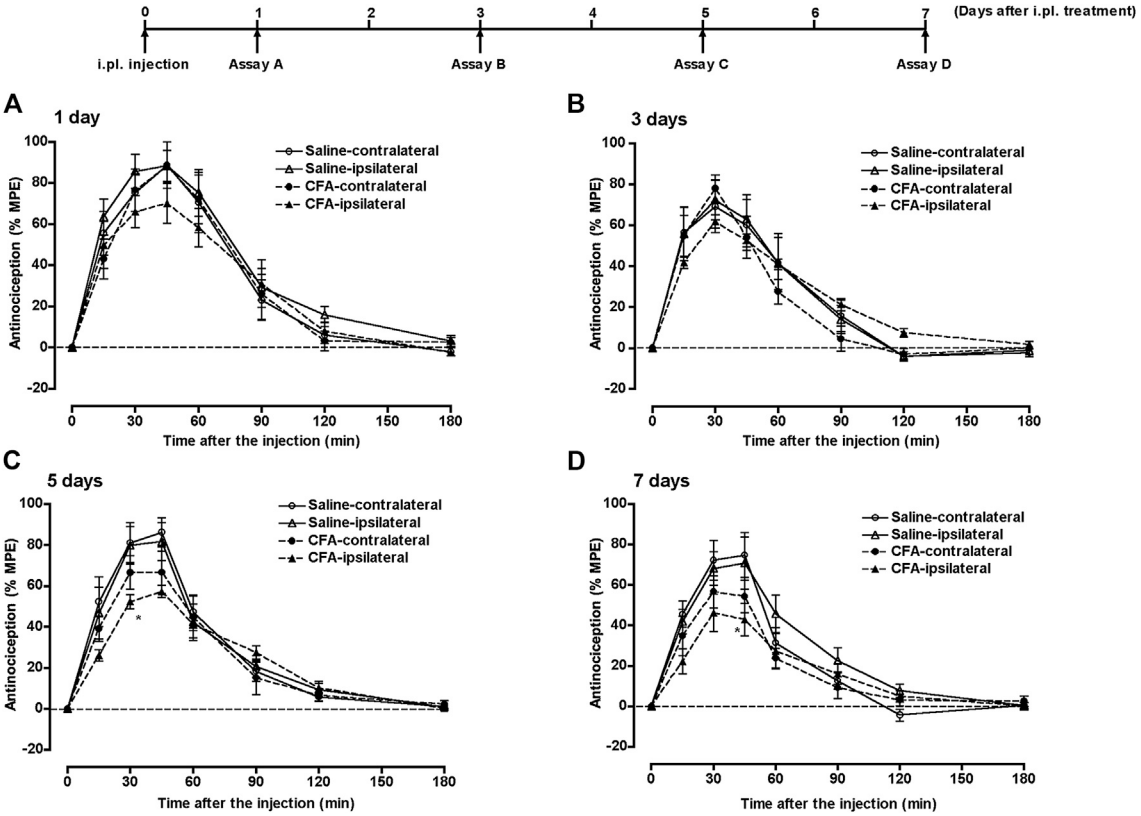

FIGURE 2 | The antinociceptive effect of morphine in the inflammatory pain state. Groups of mice pretreated i.pl. with 50 μL of saline or CFA in the left hind paw were injected s.c. with morphine (5 mg/kg) at 1 **(A)**, 3 **(B)**, 5 **(C)** or 7 days **(D)** after i.pl. pretreatment; the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 180 min. Each value represents the mean ± S.E.M. for eight mice. *$p < 0.05$ vs. ipsilateral paw in the control model (i.pl. saline pretreatment) mice.

model mice, the antinociceptive effect of morphine was bilaterally suppressed. The suppressed morphine antinociception was bilaterally restored following i.p. treatment with MK-801 30 min prior to CFA pretreatment (**Figure 6B**); however, s.c. treatment with morphine 30 min prior to CFA pretreatment failed to restore the suppressed morphine antinociception in the CFA model mice (**Figure 5B**).

mRNA Expression of μ-Opioid Receptors in the Inflammatory Pain State

The expression level of mRNA for μ-opioid receptors in the lumbar spinal cord on the ipsilateral and contralateral sides on the inflammatory pain was measured using semi-quantitative RT-PCR. The total RNA in the lumbar spinal cord of the ipsilateral side and contralateral side was extracted 7 days after CFA pretreatment, and the expression level of mRNA for μ-opioid receptors was measured using semi-quantitative RT-PCR. The expression level of mRNA for μ-opioid receptors in the lumbar spinal cord was not bilaterally altered by CFA pretreatment (**Figure 7**). Moreover, pretreatment with methadone did not bilaterally alter the expression level of μ-opioid receptor mRNA in either the control or CFA models.

DISCUSSION

We previously determined the antinociceptive effect of morphine against mechanical allodynia in the CFA-induced inflammatory pain state in mice using the von Frey filament test (Aoki et al., 2014b). In that study, the antinociceptive effect of morphine against mechanical allodynia was bilaterally suppressed at 1 and 4 days after CFA treatment. However, in the present study using the same inflammatory pain model, the antinociceptive effect of morphine against thermal hyperalgesia was not suppressed at 1 and 3 days after CFA treatment, and was significantly suppressed in the ipsilateral paw at 5 and 7 days after CFA treatment. Both mechanical allodynia and thermal hyperalgesia in the CFA-induced inflammatory pain state were quickly observed 1 day after CFA treatment and prolonged for approximately 40 days. This evidence clearly suggests that the antinociceptive mechanism of morphine against thermal hyperalgesia is different from that against mechanical allodynia, and both antinociceptive mechanisms are altered by CFA treatment at different times. This may be because there are controversial reports regarding the effectiveness of morphine in the inflammatory pain state (Maldonado et al., 1994; Fernández-Dueñas et al., 2007; Aoki et al., 2014b).

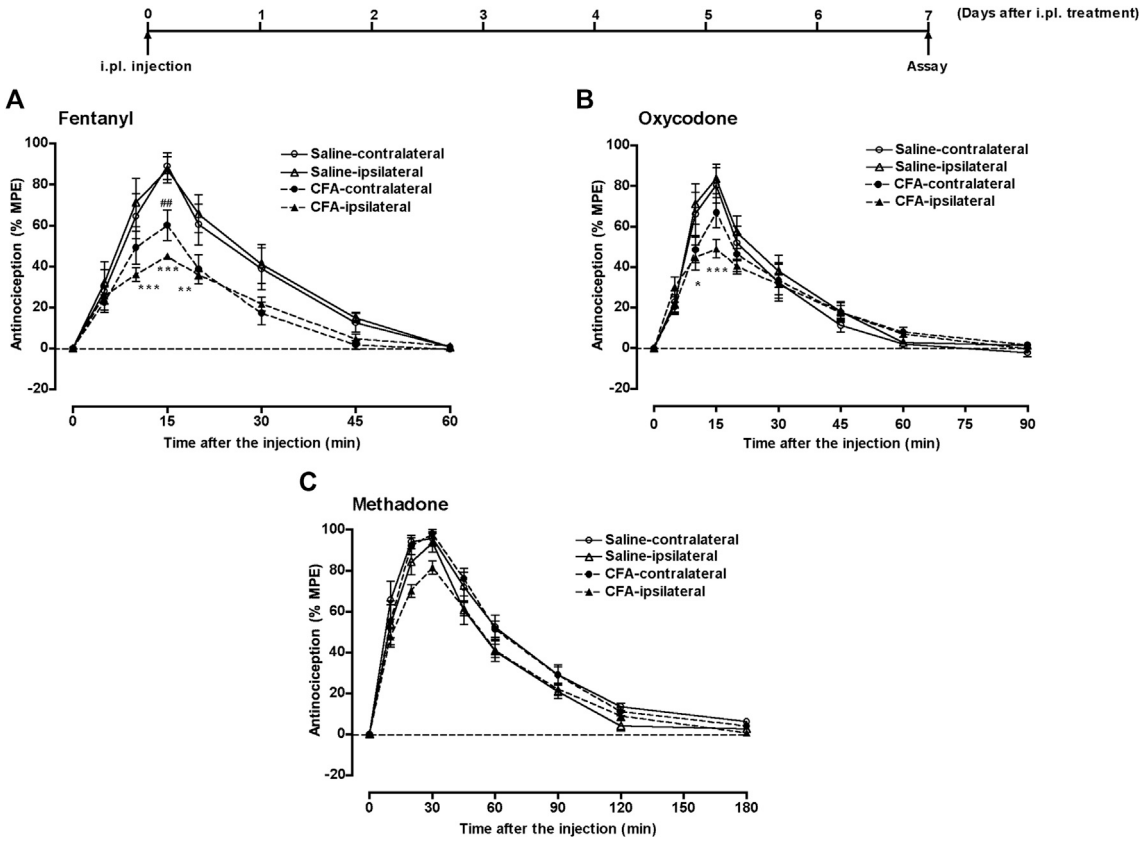

FIGURE 3 | The antinociceptive effect of narcotic analgesics in the inflammatory pain state. Groups of mice pretreated i.pl. with 50 μL of saline or CFA in the left hind paw, were injected s.c. with fentanyl **(A)** (0.08 mg/kg), oxycodone **(B)** (1.5 mg/kg), or methadone **(C)** (2.8 mg/kg) at 7 days after i.pl. pretreatment; the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 60, 90, or 180 min, respectively. Each value represents the mean ± S.E.M. for eight mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. ipsilateral paw in the control model (i.pl. saline pretreatment) mice. $^{\#\#}p < 0.01$ vs. contralateral paw in the control model (i.pl. saline pretreatment) mice.

In the present study, the antinociceptive effect of other narcotic analgesics, fentanyl and oxycodone, against thermal hyperalgesia was significantly suppressed in the ipsilateral paw 7 days after CFA treatment. However, the antinociceptive effect of methadone against thermal hyperalgesia was not affected 7 days after CFA treatment. We previously reported that the antinociceptive effect of fentanyl and oxycodone against mechanical allodynia was bilateraly suppressed 1 day after CFA treatment, whereas the antinociceptive effect of methadone against mechanical allodynia was suppressed only in the ipsilateral paw 1 day after CFA treatment (Aoki et al., 2014a). This evidence clearly suggests that among narcotic analgesics, methadone is relatively effective in the CFA-induced inflammatory pain state. Methadone is composed of *l*-methadone, which has agonistic activity against μ-opioid receptors and *d*-methadone, which has antagonistic activity against NMDA receptors (Inturrisi, 2005). The antagonistic activity of *d*-methadone against NMDA receptors may be involved in the retained antinociceptive effect of methadone against thermal hyperalgesia in the CFA-induced inflammatory pain state. However, in the present study, co-administration of fentanyl (0.08 mg/kg, s.c.) and MK-801 (0.25 mg/kg, i.p.), an NMDA receptor antagonist, failed to potentiate the antinociceptive effect of fentanyl against thermal hyperalgesia in the CFA-induced inflammatory pain state (data not shown). Therefore, the retained antinociceptive effect of methadone against thermal hyperalgesia in the CFA-induced inflammatory pain state may be mediated through a mechanism not related to the antagonism of NMDA receptors.

The most fundamental finding in the present study is that a single treatment of methadone restored the suppressed antinociceptive effect of morphine against thermal hyperalgesia in the CFA-induced inflammatory pain state. The suppressed antinociceptive effect of morphine against thermal hyperalgesia in the CFA-induced inflammatory pain state was completely restored by a single treatment with a high dose of methadone (four times higher dose of 90% antinociception) 20 min prior to or 3 days after, but not 6 days after, CFA pretreatment. The evidence suggests that a single treatment of high-dose methadone in the early phase of inflammation restores the suppressed morphine antinociception against thermal hyperalgesia in the CFA-induced inflammatory pain state. To investigate the mechanism of how methadone restored the suppressed morphine antinociception, the effect of a single treatment with

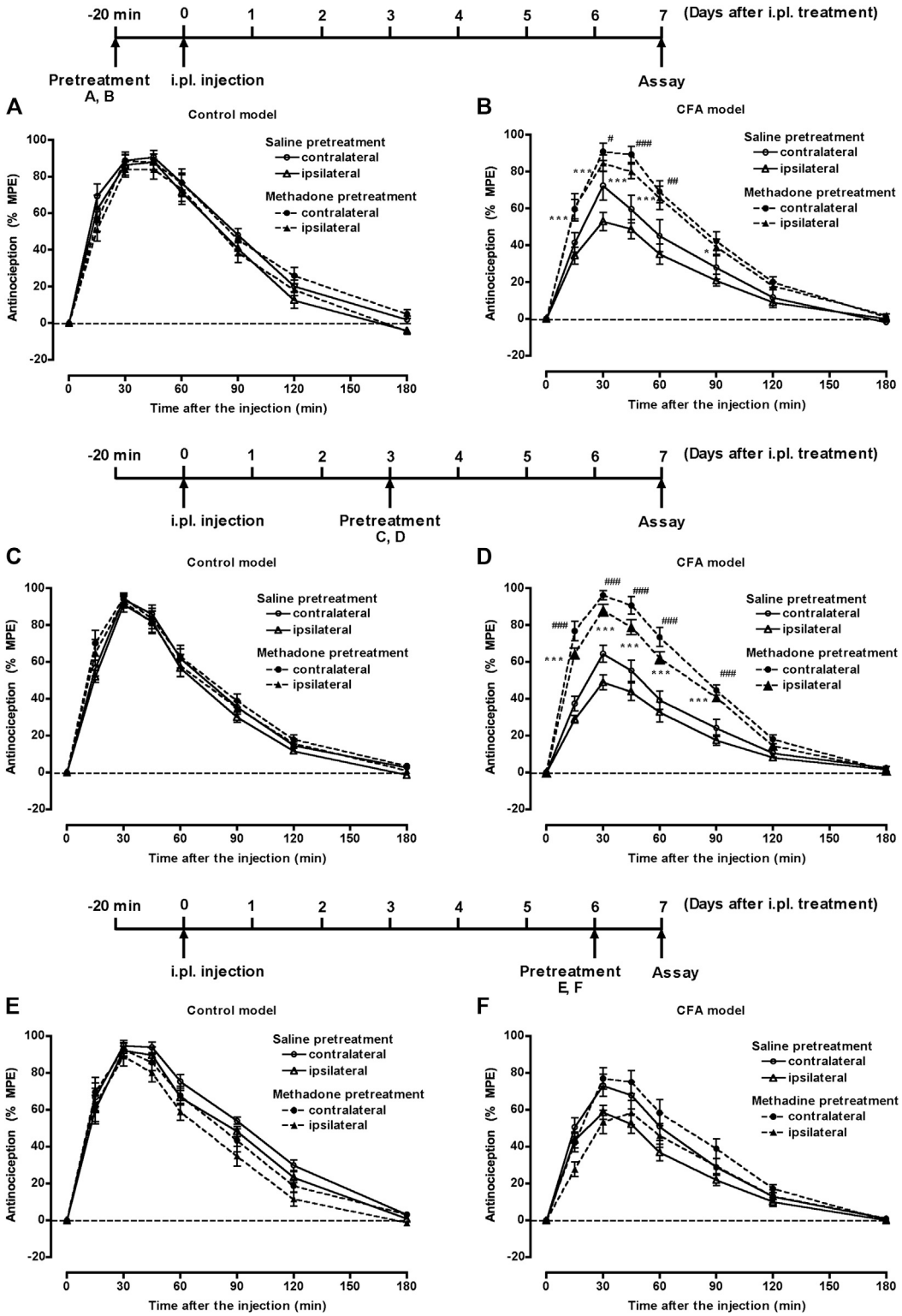

FIGURE 4 | The effect of methadone on suppressed morphine antinociception in the inflammatory pain state. Groups of mice pretreated i.pl. with 50 μL of saline **(A, C, E)** or CFA **(B, D, F)** in the left hind paw, were injected s.c. with saline or methadone (11.2 mg/kg) 20 min prior to **(A, B)**, 3 days after **(C, D)**, or 6 days after **(E, F)** i.pl. pretreatment. Seven days after i.pl. pretreatment, mice were injected s.c. with morphine (5 mg/kg), and the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 180 min. Each value represents the mean ± S.E.M. for eight mice. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. ipsilateral paw in the s.c. saline-pretreated mice. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. contralateral paw in the s.c. saline-pretreated mice.

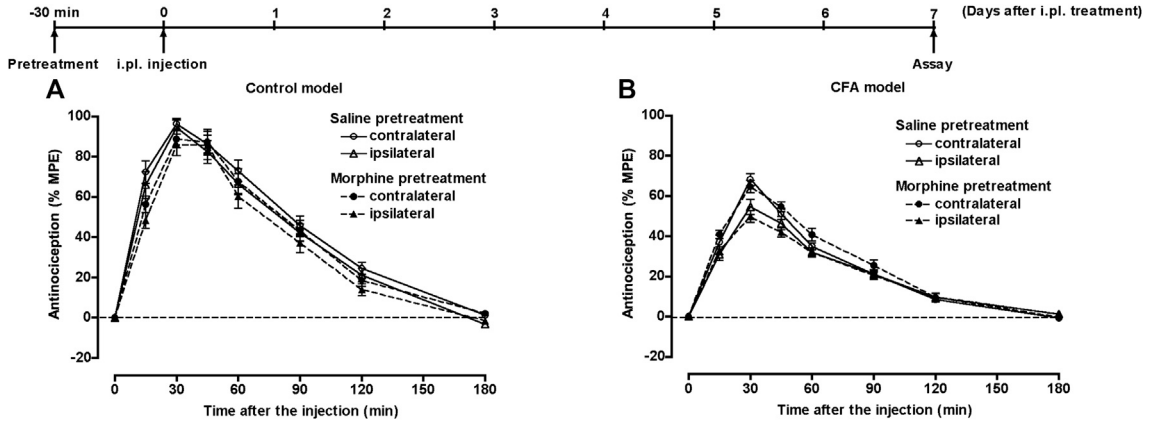

FIGURE 5 | The effect of morphine on suppressed morphine antinociception in the inflammatory pain state. Groups of mice pretreated i.pl. with 50 μL of saline **(A)** or CFA **(B)** in the left hind paw were injected s.c. with saline or morphine (20 mg/kg) 30 min prior to i.pl. pretreatment. Seven days after i.pl. pretreatment, mice were injected s.c. with morphine (5 mg/kg), and the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 180 min. Each value represents the mean ± S.E.M. for eight mice.

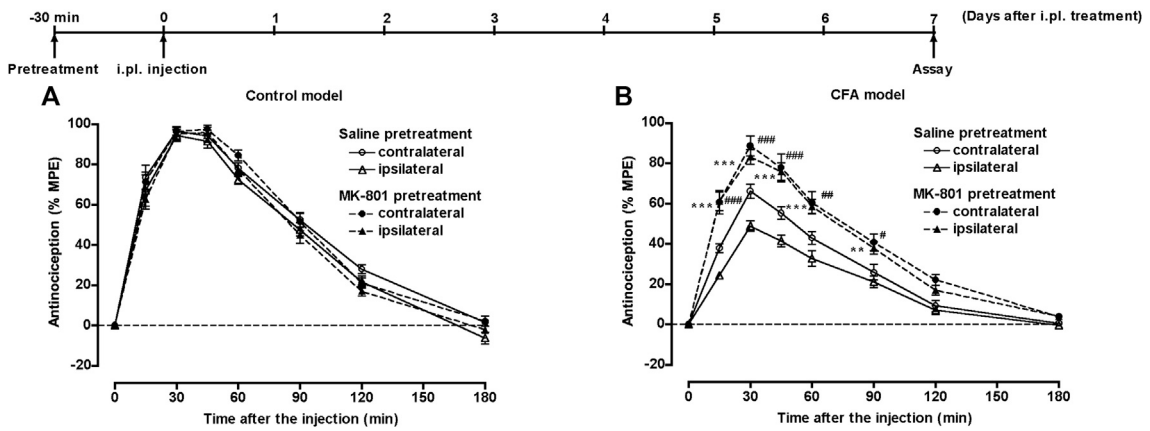

FIGURE 6 | The effect of MK-801 on suppressed morphine antinociception in the inflammatory pain state. Groups of mice pretreated i.pl. with 50 μL of saline **(A)** or CFA **(B)** in the left hind paw, were injected i.p. with saline or MK-801 (0.25 mg/kg) 30 min prior to i.pl. pretreatment. Seven days after i.pl. pretreatment, mice were injected s.c. with morphine (5 mg/kg), and the withdrawal threshold in response to thermal stimulation of the plantar surface of each hind paw was measured for 180 min. Each value represents the mean ± S.E.M. for eight mice. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. ipsilateral paw in s.c. saline-pretreated mice. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. contralateral paw in s.c. saline-pretreated mice.

morphine (20 mg/kg, s.c.) or MK-801 (0.25 mg/kg, i.p.) 30 min prior to CFA treatment on suppressed morphine antinociception against thermal hyperalgesia in the CFA-induced inflammatory pain state was examined. As a result, a single treatment with MK-801, but not morphine, restored the suppressed morphine antinociception against thermal hyperalgesia in the CFA-induced inflammatory pain state. The blockade of NMDA receptors by methadone in the early phase of inflammation may be involved in the restoration of suppressed morphine antinociception against thermal hyperalgesia in the CFA-induced inflammatory pain state.

We previously reported that suppressed morphine antinociception against mechanical allodynia in the ipsilateral paw in the early phase of CFA-induced inflammation (1 day after CFA treatment) is reflected in the reduced expression of the μ-opioid receptor mRNA in the ipsilateral dorsal root ganglion and ipsilateral lumbar spinal cord (Aoki et al., 2014b). Therefore, in the present study, the expression level of μ-opioid receptor mRNA was measured 7 days after CFA treatment. As a result, 7 days after CFA treatment, the expression level of μ-opioid receptor mRNA in the ipsilateral lumbar spinal cord was not significantly altered by CFA treatment. Moreover, the expression level of μ-opioid receptor mRNA was not altered by a single pretreatment with methadone. The evidence suggests that the expression of μ-opioid receptor mRNA in the ipsilateral lumbar spinal cord, which was once reduced in the early phase of CFA-induced inflammation, returns to normal levels 7 days after CFA treatment. A single injection of methadone may restore the

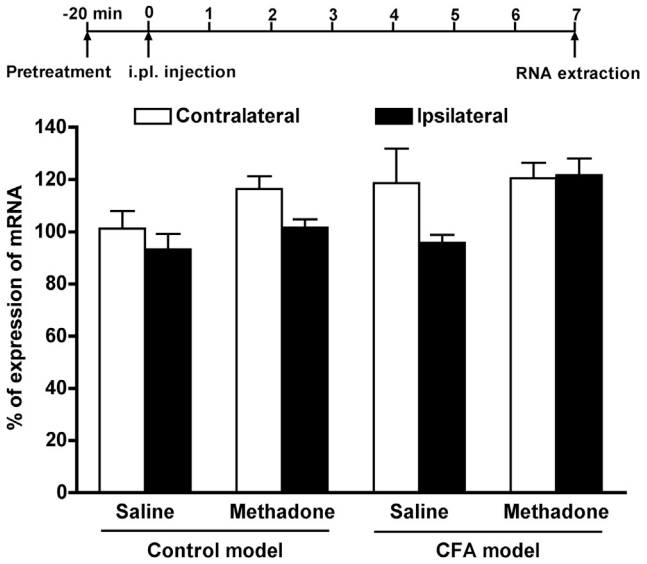

FIGURE 7 | The expression level of mRNA for μ-opioid receptors in lumbar spinal cord in the inflammatory pain state. Groups of control model mice and CFA model mice were injected s.c. with saline or methadone (11.2 mg/kg), and the expression level of mRNA for μ-opioid receptors in the lumbar spinal cord on the ipsilateral side and contralateral side 7 days after CFA pretreatment was measured using semi-quantitative RT-PCR. Data are expressed as the % of the mRNA level for μ-opioid receptors normalized to that of β-actin; each value represents the mean ± S.E.M. for duplicated three independent sets of experiments.

suppressed morphine antinociception against thermal hyperalgesia in the CFA-induced inflammatory pain state without altering the expression level of μ-opioid receptor mRNA in the ipsilateral lumbar spinal cord.

It has been well established that the phosphorylation of μ-opioid receptors by protein kinase C (PKC) enhances the desensitization of μ-opioid receptors (Bohn et al., 2004; Bailey et al., 2006). We previously identified that in addition to the reduced expression of μ-opioid receptor mRNA, the phosphorylation of μ-opioid receptors by PKC is also involved in the suppression of morphine antinociception against mechanical allodynia in the early phase of CFA-induced inflammation (Aoki et al., 2014b). In the chronic pain state, the activity of PKC in the dorsal spinal cord is enhanced by the activation of NMDA receptors in postsynaptic cells (Mao et al., 1995a; Mao et al., 1995b). Therefore, the activation of NMDA receptors causes the phosphorylation of co-localized μ-opioid receptors, which results in the desensitization of μ-opioid receptors (Sánchez-Blázquez et al., 2013). In fact, the development of hyperalgesia and allodynia in morphine-resistant chronic pain states is suppressed by NMDA receptor antagonists or PKC inhibitors (Mao et al., 1992; Mao et al., 1995a). Moreover, NMDA receptor antagonists prevent the development of antinociceptive tolerance to μ-opioid receptor agonists (Mao et al., 1998). Considering our previous finding that suppressed morphine antinociception against mechanical allodynia in the CFA-induced inflammatory pain state was partially restored by pretreatment with PKC inhibitor (Aoki et al., 2014b), blocking the NMDA receptors with a single treatment with methadone may prevent the phosphorylation of μ-opioid receptors by PKC in the CFA-induced inflammatory pain state.

CONCLUSION

In conclusion, methadone is extremely effective against thermal hyperalgesia in the morphine-resistant inflammatory pain state. Moreover, a single injection of methadone restores suppressed morphine antinociception in the inflammatory pain state without altering the expression level of mRNA for μ-opioid receptors.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee for Animal Experiments at Tohoku Medical and Pharmaceutical University.

AUTHOR CONTRIBUTIONS

HM organized the research and wrote the manuscript. CW, AK, and MY conducted the experiments. CW partially prepared manuscript. SS contributed for discussion.

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ORIGINAL RESEARCH
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Galanin Receptor 2 Is Involved in Galanin-Induced Analgesic Effect by Activating PKC and CaMKII in the Nucleus Accumbens of Inflammatory Pain Rats

Mengnan Li[1†], Xiaomin Zhang[1†], Chongyang Li[2], Yanan Liu[1], Shuang Yang[1] and Shilian Xu[1]*

[1] Department of Physiology, School of Basic Medicine, Kunming Medical University, Kunming, China, [2] Department of Oncology, Affiliated Hospital, Yunnan University, Kunming, China

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***Correspondence:**
Shilian Xu
xushilian@kmmu.edu.cn

[†] These authors have contributed equally to this work

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It has been reported that galanin has an analgesic effect via activating galanin receptors (GALRs). This study focused on the involvement of GALR2 in the galanin-induced analgesic effect and its signaling mechanism in the nucleus accumbens (NAc) of inflammatory rats. Animal models were established through injecting carrageenan into the plantar of rats' left hind paw. The results showed that GALR2 antagonist M871 weakened partially the galanin-induced increases in hind paw withdrawal latency (HWL) to thermal stimulation and hind paw withdrawal threshold (HWT) to mechanical stimulation in NAc of inflammatory rats. Moreover, the GALR2 agonist M1145 prolonged the HWL and HWT, while M871 blocked the M1145-induced increases in HWL and HWT. Western blotting showed that the phosphorylation of calcium/calmodulin-dependent protein kinase II (p-CaMKII) and protein kinase C (p-PKC) in NAc were upregulated after carrageenan injection, while p-PKC and p-CaMKII were downregulated after intra-NAc administration of M871. Furthermore, the CaMKII inhibitor KN93 and PKC inhibitor GO6983 attenuated M1145-induced increases in HWL and HWT in NAc of rats with inflammatory pain. These results prove that GALR2 is involved in the galanin-induced analgesic effect by activating CaMKII and PKC in NAc of inflammatory pain rats, implying that GALR2 agonists probably are potent therapeutic options for inflammatory pain.

Keywords: galanin receptor 2, inflammatory pain, analgesic effect, nucleus accumbens, CaMKII, PKC

INTRODUCTION

Pain is a dominating feature of inflammation, but the underlying mechanisms of inflammatory pain resolution are not fully understood. Thus, the treatment of inflammatory pain has always been a major issue in the clinic. Non-steroidal anti-inflammatory drugs are extensively used in the treatment of inflammatory pain associated with a number of diseases. However, there are safety

concerns regarding the use of these drugs, such as clinically relevant gastrointestinal, cardiovascular, and renal damage. Therefore, it is imperative to develop new drugs and tools that do not induce significant side effects.

In addition to endogenous opioids, which are important neuropeptides for the modulation of pain, other neuropeptides, such as substance P, are also involved in neurogenic inflammation (O'Connor et al., 2004). Galanin is an important neuropeptide that is widely distributed peptide in the peripheral tissues and central nervous system (CNS), participates in the regulation of nociceptive information (Amorim et al., 2015; Li et al., 2017). A study found that the hind paw withdrawal latencies (HWLs) were increased after intra-anterior cingulate cortex (ACC) administration of galanin in rats (Zhang et al., 2017b). Our studies also showed that intra-nucleus accumbens (NAc) administration of galanin increased the HWLs in rats (Xu S.L. et al., 2012; Yang et al., 2015; Zhang et al., 2019). Galanin plays a role by activating its specific receptors. Galanin receptors (GALRs), including GALR1-3, have been identified (Webling et al., 2012). It has been demonstrated that galantide, a non-selective GALRs antagonist, attenuates galanin-induced analgesic effect in rats (Xu S.L. et al., 2012; Yang et al., 2015). In this study, the GALR2-specific agonist M1145 (Runesson et al., 2009; Saar et al., 2013) and the GALR2-specific antagonist M871 (Sollenberg et al., 2006) were used to investigate the analgesic effect of GALR2 in rats with inflammatory pain. We hope to provide a new insight into the treatment of inflammatory pain.

The signaling pathways of the three GALRs are essentially different (Lang et al., 2015), and the signaling transduction mechanism of the analgesic effects of galanin and its receptors on inflammatory pain is not fully illustrated yet. Calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) belong to a family of serine/threonine kinases that are all phospholipase C (PLC)-dependent Ca^{2+}-related protein kinases. Activation of multiple PKC isoforms is important for the development of central and/or peripheral sensitization in persistent pain conditions (He and Wang, 2019). Besides, CaMKII also plays important roles in pain control (Crown et al., 2012; Kadic et al., 2014; He et al., 2016; Yao et al., 2016). N-methyl-D-aspartate (NMDA) receptors contribute to the up-regulation of inflammatory pain sensitization during inflammation (Tan et al., 2010), whereas KN93 (CaMKII inhibitor) and chelerythrine (PKC inhibitor) can decrease hyperalgesia and allodynia by regulating the phosphorylation of NMDA receptor subunits (Liu et al., 2014; Bu et al., 2015).

The NAc is an important central nucleus for pain modulation, which consists two regions: the core and shell (Baliki et al., 2013; Duan et al., 2015; Salgado and Kaplitt, 2015; Watanabe et al., 2018; Zhang et al., 2019). Our previous study showed that galanin in NAc had an analgesic effect and that this effect was blocked by non-specific GALRs antagonist galantide in rats with inflammatory pain (Yang et al., 2015). In this work we explored whether the CaMKII and/or PKC signaling pathways were implicated in the analgesic effect of GALR2 in NAc of inflammatory rats. Our results will improve the understanding of the molecular mechanism of galanin-induced analgesic effect.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180–250 g were supplied by the Experimental Animal Center of Kunming Medical University (Kunming, Yunnan, China). The rats were kept in cages with free access to food and water. The room temperature was kept at 22 ± 1°C, and the animals were housed under strictly controlled lighting conditions. All experimental protocols were tested in accordance with the approval of the "Animal Care and Use Committee at Kunming Medical University" and "National Institute of Health Guide for the Care and Use of Laboratory Animals."

Chemicals

Solutions for intra-NAc administration containing either 2 nmol rat galanin (Tocris, Bristol, United Kingdom); 0.1, 1, or 2 nmol M1145 (Tocris, Bristol, United Kingdom) were prepared in 1 μl of 0.9% sterilized saline; solutions containing either 6, 12, or 24 μg of KN93 ($C_{26}H_{29}ClN_2O_4S$; EMD Biosciences, Inc., La Jolla, CA, United States); or 12, 24, or 36 μg of GO6983 ($C_{26}H_{26}N_4O_3$; MCE, United Kingdom) were prepared in 1 μl of 1% dimethyl sulfoxide (DMSO). M871 (Tocris, Bristol, United Kingdom) was dissolved in 1 μl of 6% acetonitrile at a concentration of 2 nmol for intra-NAc administration. 2 mg of carrageenan (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in 0.1 ml sterilized saline, and was injected into the plantar of the left hind paw.

Antibodies

Table 1 shows a list of all antibodies used.

Behavioral Tests

The HWL to thermal stimulation and the hind paw withdrawal threshold (HWT) to mechanical stimulation were measured as previously described (Sun et al., 2003; Duan et al., 2015; Yang et al., 2015). The HWL to thermal stimulation was assessed by a Hot-Plate (YLS-6B, China), with the temperature kept at 52 ± 0.2°C. The unilateral hind paw was put on the hot-plate gently and ensured that the entire ventral surface of the hind paw touched the hot-plate. The time to hind paw withdrawal was measured in seconds (s) and recorded as the HWL to thermal stimulation. The HWT to mechanical stimulation was determined by a Randall-Selitto meter (Ugo Basile, 37215, Italy), that a wedge-shaped pusher with a loading rate of 30 g/s was applied to the dorsal surface of the rat's hind paw and the magnitude of the mechanical stimulation required to initiate the struggle response was measured as HWT.

Before the experiment, all rats were acclimated to behavioral tests for 4–5 days, the HWL to thermal stimulation was normally maintained between 3 and 6 s, and the HWT to mechanical stimulation was maintained between 4 and 7 g. When measuring HWL or HWT, if the rat did not withdrawal the hind paw after 15 s (or over 15 g), the paw would be lifted by the trier to avoid tissue damage.

TABLE 1 | A list of antibodies.

Antigen	Description of immunogen	Source, host species, catalog No., RRID	Concentration used
GAPDH	Monoclonal antibody is produced by immunzing animals with a synthetic peptide corresponding to residues near the carboxy terminus of human GAPDH	Cell Signaling Technology, Rabbit monoclonal Cat# 5174, RRID:AB_10622025	1:1000 (WB)
CaMKII (pan)	CaMKII (pan) (D11A10) Rabbit mAb detects endogenous levels of total CaMKII protein. The peptide sequence used as the antigen is 100% conserved between CaMKII-alpha, gamma and delta, and 88% conserved in CaMKII-beta. Synthetic peptide surrounding Val184 of human CaMKII-alpha	Cell Signaling Technology, Rabbit monoclonal Cat# 4436, RRID:AB_10545451	1:1000 (WB)
CaMKII (Thr286) Phosphate	recognizes endogenous levels of CamKII-α protein only when phosphorylated at Thr286. This antibody also recognizes endogenous levels of CamKII-β and CamKII-γ protein only when phosphorylated at Thr287 synthetic phosphopeptide corresponding to residues surrounding Thr287 of human CamKII-β protein	Cell Signaling Technology, Rabbit monoclonal Cat# 12716, RRID:AB_2713889	1:1000 (WB)
PKC alpha [Y124]	Synthetic peptide within Human PKC alpha aa 650 to the C-terminus. The exact sequence is proprietary	Abcam, Rabbit monoclonal Ab32376, RRID:AB_777294	1:5000 (WB)
PKC alpha (phospho T497)	Synthetic peptide (the amino acid sequence is considered to be commercially sensitive) within Human PKC alpha (phospho T497). The exact sequence is proprietary	Abcam, Rabbit monoclonal Ab76016, RRID:AB_1310584	1:5000 (WB)

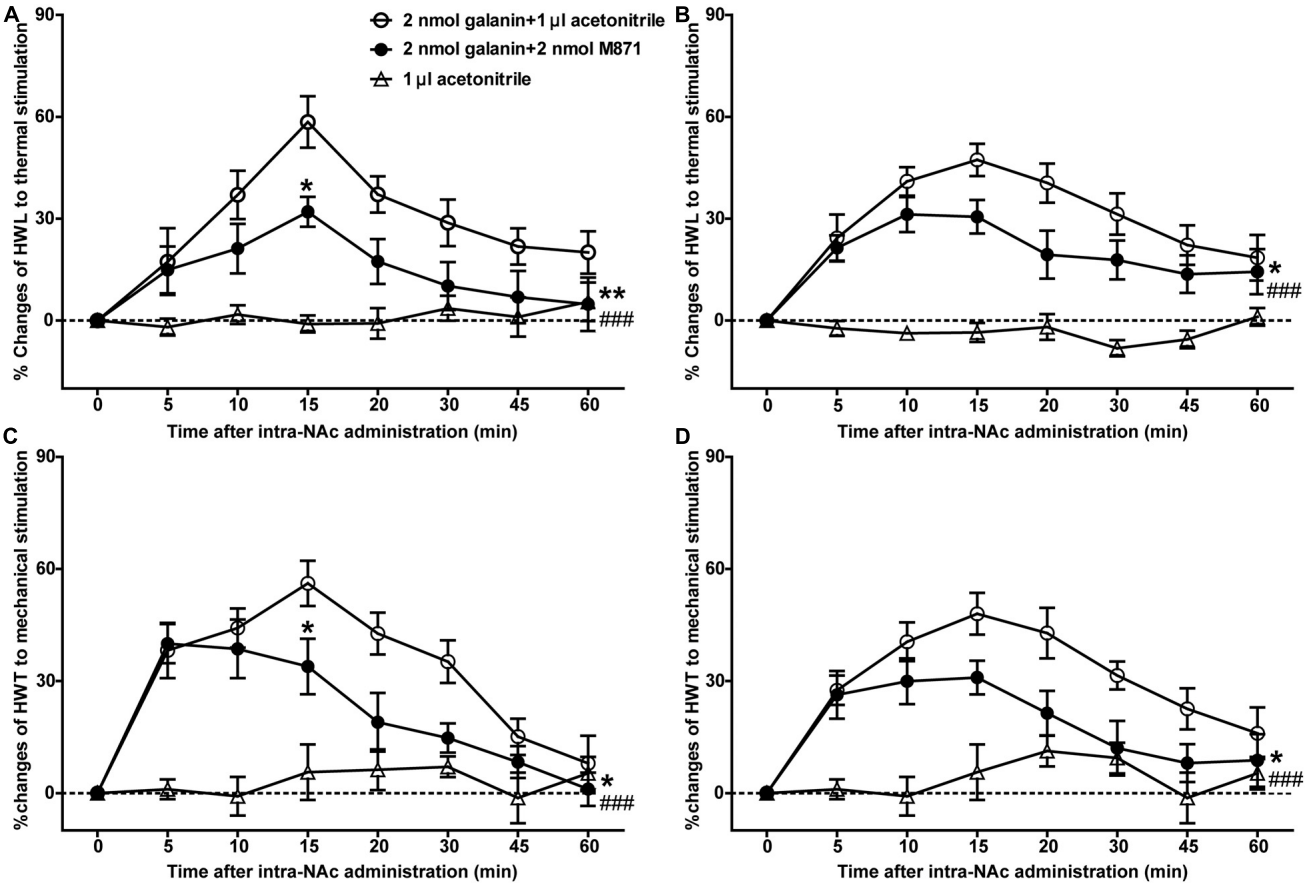

FIGURE 1 | Effect of M871 on galanin-induced increases in HWL and HWT in NAc of inflammatory pain rats **(A,C)**: left hind paw; **(B,D)**: right hind paw. 2 nmol galanin was bilaterally injected into NAc at 0 min, and 2 nmol M871 or 1 μl of acetonitrile as a control was bilaterally injected into NAc at 5 min. *Represents the difference between the group treated with galanin + M871 and the group treated with galanin + acetonitrile; #Represents the difference between the group treated with galanin + M871 and the group treated with acetonitrile alone. $^{*}P < 0.05$, $^{**}P < 0.01$, compared the galanin + M871-treated group with the galanin + acetonitrile-treated group. $^{\#\#\#}P < 0.001$ compared galanin + M871-treated group with the acetonitrile alone group.

Intra-NAc Catheter Implantation

Rats were first anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed on a stereotaxic instrument. Then a stainless-steel catheter with an outer diameter of 0.8 mm was implanted into the NAc (Bregma: +1.7 mm; Left or right of the midline: 1.6 mm; Ventrally to the surface of skull: 7.0 mm) (Paxinos and Watson, 1998) and was secured to the skull with dental acrylic. Rats were then allowed to recover for 2–3 days.

Carrageenan-Induced Inflammatory Pain Model

On the day of the experiment, an inflammatory pain model was established through subcutaneous injection of 0.1 ml of 2% carrageenan into the plantar of the left hind paw of rat (Sun et al., 2003; Xiong et al., 2005; Yang et al., 2015; Zhang et al., 2017a). The contralateral paw was untreated. Then, each animal model of inflammatory pain received an intra-NAc injection of the drugs.

Intra-NAc Injection

Three hours after carrageenan injection, each HWL and HWT were measured three times which were averaged to obtain a mean value as the baseline HWL and HWT. Each HWL or HWT test should be 5 min apart from the last test to prevent discomfort or injury.

Then, a stainless-steel needle with an outer diameter of 0.4 mm was inserted into the stainless-steel catheter and its tip exceeded the stainless-steel catheter by 1 mm for bilateral intra-NAc injection. The HWL and HWT were measured 5, 10, 15, 20, 30, 45, and 60 min after bilateral intra-NAc injection, and each HWL or HWT was expressed as percentage changes from the baseline HWL. The formula is as follows:

$$\mathrm{HWL\%} = \frac{\text{HWL measured after intra-NAc injection} - \text{baseline HWL}}{\text{baseline HWL}} \times 100\%$$

The drugs were injected into the core of NAc, and the location of the needle tip was verified at the end of each experiment. Only data from the animals with tip of the needle located in the NAc were used for statistical analysis.

Western Blot

Rats were deeply anesthetized with 4% isoflurane and euthanized, and tissue of bilateral NAc area was collected. The Western blot assay was operated as previously described (Duan et al., 2015; Yang et al., 2015). The expression of total-PKC (t-PKC) alpha,

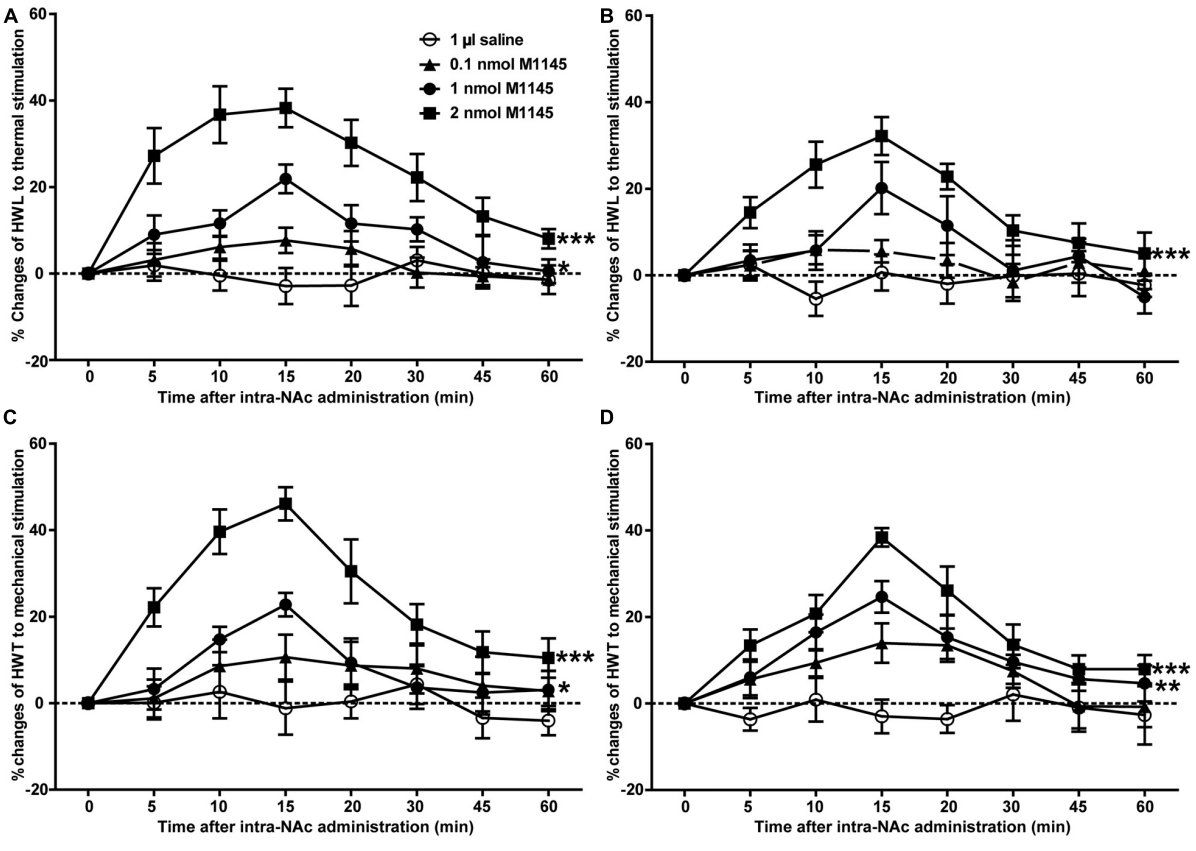

FIGURE 2 | Effect of M1145 on the HWL and HWT in NAc of inflammatory pain rats **(A,C)**: left hind paw; **(B,D)**: right hind paw. 0.1, 1, 2 nmol M1145 or 1 µl of saline as a control was bilaterally injected into NAc respectively at 0 min. The data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the saline group.

phospho-PKC (p-PKC) alpha, total-CaMKII (t-CaMKII) alpha and phospho-CaMKII (p-CaMKII) alpha were measured, and the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as internal control. The relative band density was assessed by ImageJ software. Western blotting of the protein samples was repeated at least three times.

Statistical Analysis

The data were analyzed using GraphPad Prism 5 software, and presented as the mean ± SEM. Western blot results were assessed by one-way ANOVA followed by Tukey's multiple comparison test, behavioral tests were analyzed by two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test, and $P < 0.05$ was considered as statistically significant.

RESULTS

The GALR2 Antagonist M871 Attenuated the Galanin-Induced Analgesic Effect in NAc of Inflammatory Pain Rats

Our previous study showed that galanin had an analgesic effect in NAc of inflammatory pain rats and that the expression of GALR2 was upregulated in NAc 3 h after injection of carrageenan (Yang et al., 2015). To explore whether this analgesic effect was mediated by GALR2, 3 h after carrageenan injection, two groups of inflammatory pain rats received an intra-NAc injection of 2 nmol galanin followed by an intra-NAc injection of 2 nmol GALR2 antagonist M871 (n = 8) or 1 μl of 6% acetonitrile as a control (n = 9) 5 min later. Compared to the group treated with galanin + acetonitrile, galanin-induced increases in HWL to thermal stimulation (Left hind paw: $F_{(1,75)} = 9.48$, $P = 0.0076$; Right hind paw: $F_{(1,75)} = 5.246$, $P = 0.0369$) and HWT to mechanical stimulation [Left hind paw: $F_{(1,75)} = 4.551$, $P = 0.0498$; Right hind paw: $F_{(1,75)} = 6.366$, $P = 0.0234$] were effectively blocked by the intra-NAc injection of GALR2 antagonist M871. And when comparing the HWL and HWT of the two groups at each time point, there was a significant difference in the left HWL and HWT after 15 min of galanin injection (**Figure 1**), which suggested that the analgesic effect of galanin on inflammatory pain might be mediated through the GALR2 activation in NAc of rats. The significance of the difference between the groups was determined by two-way ANOVA for repeated measurements followed by Bonferroni *post hoc* test.

In order to further study the blocking effect of M871 on galanin induced analgesia, inflammatory pain rats were

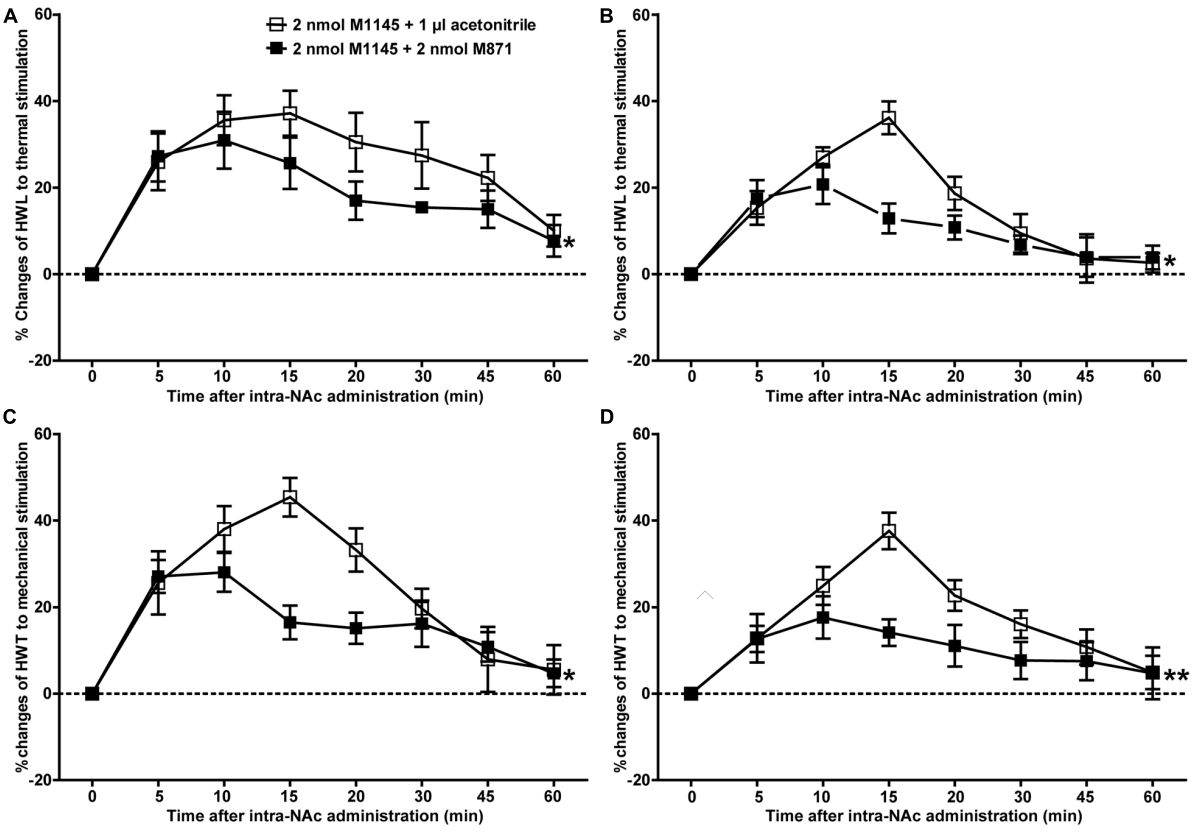

FIGURE 3 | Effect of M871 on M1145-induced increases in the HWL and HWT of inflammatory pain rats **(A,C)**: left hind paw; **(B,D)**: right hind paw. 2 nmol M1145 was bilaterally injected into NAc at 0 min, and 2 nmol M871 or 1 μl of acetonitrile as a control was bilaterally injected into NAc at 5 min. The data are presented as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ compared with the acetonitrile group.

given an intra-NAc injection of 6% acetonitrile (n = 8) alone. Compared to the group treated with acetonitrile alone, the HWL [Left hind paw: $F_{(1,70)}$ = 42.05, P < 0.0001; Right hind paw: $F_{(1,70)}$ = 29.82, P < 0.0001] and HWT [Left hind paw: $F_{(1,70)}$ = 22.15, P = 0.0003; Right hind paw: $F_{(1,70)}$ = 43.43, P < 0.0001] were increased in the group treated with galanin + M871, suggesting the antinociception of galanin was blocked partially by GALR2 antagonist M871 (**Figure 1**). This result implied that galanin in NAc had an analgesic effect on inflammatory pain by activating other GALRs.

Intra-NAc Injection of GALR2 Agonist M1145 Increased the HWL and HWT in Inflammatory Pain Rats

To investigate the analgesic effect of GALR2 activation on inflammatory pain, four groups of carrageenan-treated rats received an intra-NAc administration of 1 μl of either 0.1 nmol (n = 7), 1 nmol (n = 9), 2 nmol (n = 7) GALR2 agonist M1145 or 0.9% saline (n = 7) 3 h after carrageenan injection. Compared with those of saline group, the HWL to thermal stimulation significantly prolonged after intra-NAc administration of 1 nmol [Left hind paw: $F_{(1,84)}$ = 6.94, P = 0.0196; Right hind paw: $F_{(1,84)}$ = 2.02, P = 0.1796] and 2 nmol [Left hind paw: $F_{(1,72)}$ = 85.63, P < 0.0001; Right hind paw: $F_{(1,72)}$ = 46.59, P < 0.0001] M1145, but for rats received 0.1 nmol M1145, the HWL was not significantly increased [Left hind paw: $F_{(1,72)}$ = 2.15, P = 0.1683; Right hind paw: $F_{(1,72)}$ = 1.38, P = 0.2636] (**Figures 2A,B**). While the HWT to mechanical stimulation of rats treated with 1 nmol [Left hind paw: $F_{(1,84)}$ = 5.32, P = 0.0369; Right hind paw: $F_{(1,84)}$ = 11.31, P = 0.0046] and 2 nmol [Left hind paw: $F_{(1,72)}$ = 37.74, P < 0.0001; Right hind paw: $F_{(1,72)}$ = 31.85, P = 0.0002] M1145 significantly prolonged, but not with 0.1 nmol M1145 [Left hind paw: $F_{(1,72)}$ = 1.80, P = 0.2044; Right hind paw: $F_{(1,72)}$ = 4.46, P = 0.0564] (**Figures 2C,D**). The data were analyzed by two-way ANOVA (repeated-measures). The results confirmed that GALR2 activation had an antinociceptive effect in the NAc of rats with inflammatory pain.

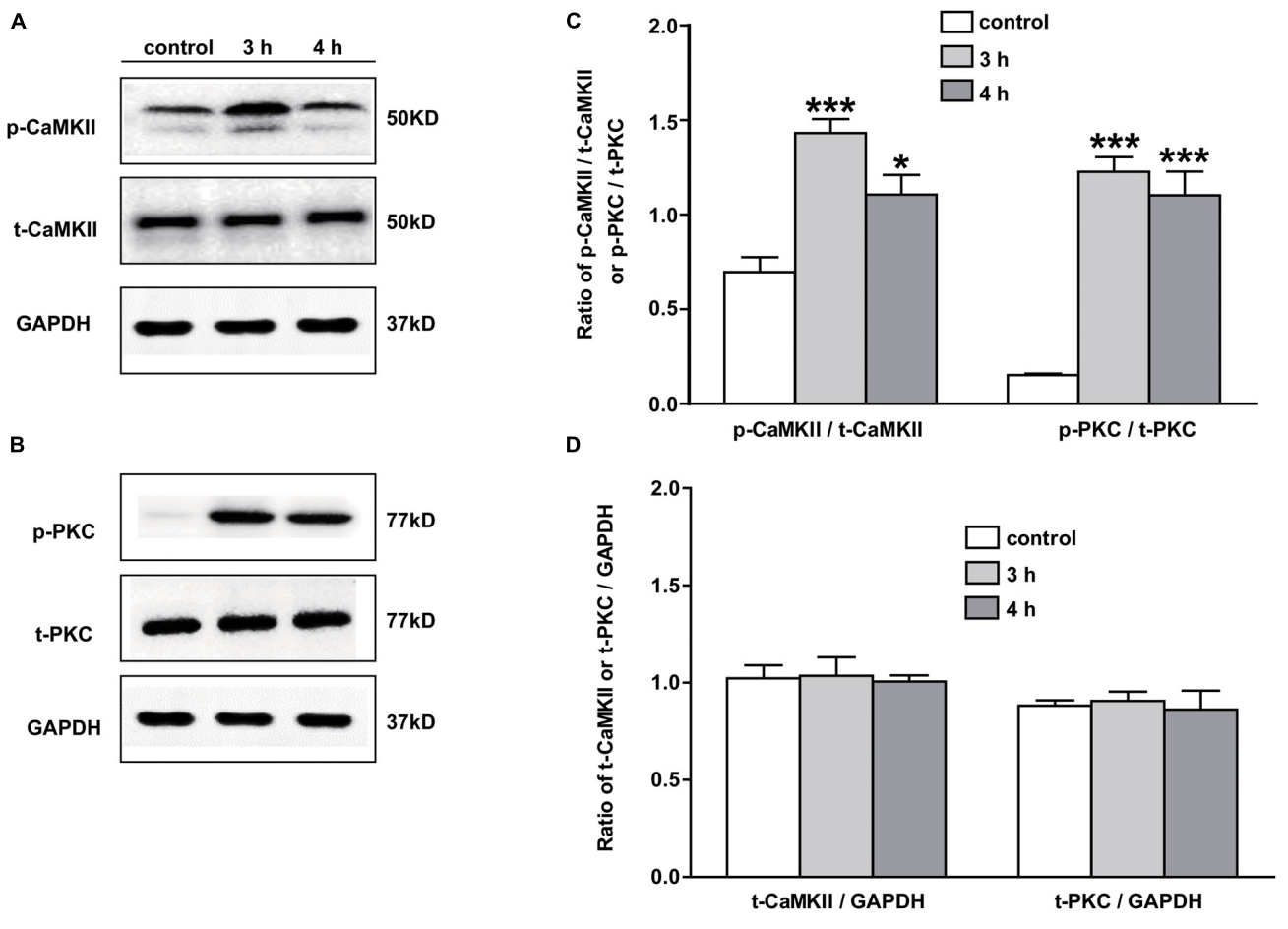

FIGURE 4 | The expression of PKC and CaMKII in NAc of inflammatory pain rats 3 h: 3 h after carrageenan injection; 4 h: 4 h after carrageenan injection. **(A,B)** Representative Western blots of p-CaMKII, t-CaMKII, p-PKC, t-PKC and GAPDH. **(C)** Histograms showing the p-CaMKII/t-CaMKII ratio and p-PKC/t-PKC ratio. **(D)** Histograms showing the t-CaMKII/GAPDH ratio and t-PKC/GAPDH ratio. The data are presented as the mean ± SEM. *P < 0.05, ***P < 0.001 compared with the saline group.

The GALR2 Antagonist M871 Reversed the M1145-Induced Increases in HWL and HWT in NAc of Inflammatory Pain Rats

To further confirm the analgesic effect of GALR2 activation in the NAc on inflammatory pain, carrageenan-treated rats received an intra-NAc administration of 2 nmol GALR2 agonist M1145, and 5 min later, rats received an intra-NAc injection of 2 nmol GALR2 antagonist M871 (n = 8) or 1 μl of 6% acetonitrile as a control (n = 8). Compared with those of M1145 + acetonitrile group, GALR2 antagonist M871 attenuated GALR2 agonist M1145-induced increases in the HWL to thermal stimulation [Left hind paw: $F_{(1,70)}$ = 5.64, P = 0.0324; Right hind paw: $F_{(1,70)}$ = 5.16, P = 0.0394] (**Figures 3A,B**) and HWT to mechanical stimulation [Left hind paw: $F_{(1,70)}$ = 6.00, P = 0.028; Right hind paw: $F_{(1,70)}$ = 9.10, P = 0.0092] (**Figures 3C,D**). The data were analyzed by two-way repeated-measures ANOVA. This result further implied that GALR2 activation had an analgesic effect on inflammatory pain in NAc of rats.

The Expressions of p-PKC and p-CaMKII Were Upregulated in NAc of Inflammatory Pain Rats

The signaling mechanism underlying the analgesic effect of galanin in NAc of inflammatory pain rats is unclear. In this study, the expressions of PKC and CaMKII were measured by Western blotting. The results showed that p-CaMKII in NAc was significantly upregulated 3 (n = 4, q = 8.44, $P < 0.001$) and 4 h (n = 4, q = 4.71, $P < 0.05$) after carrageenan injection, as shown in **Figures 4A,C**. Meanwhile, the p-PKC also significantly increased in NAc 3 (n = 4, q = 12.55, $P < 0.001$) and 4 h (n = 4, q = 11.08, $P < 0.001$) after carrageenan injection (**Figures 4B,C**).

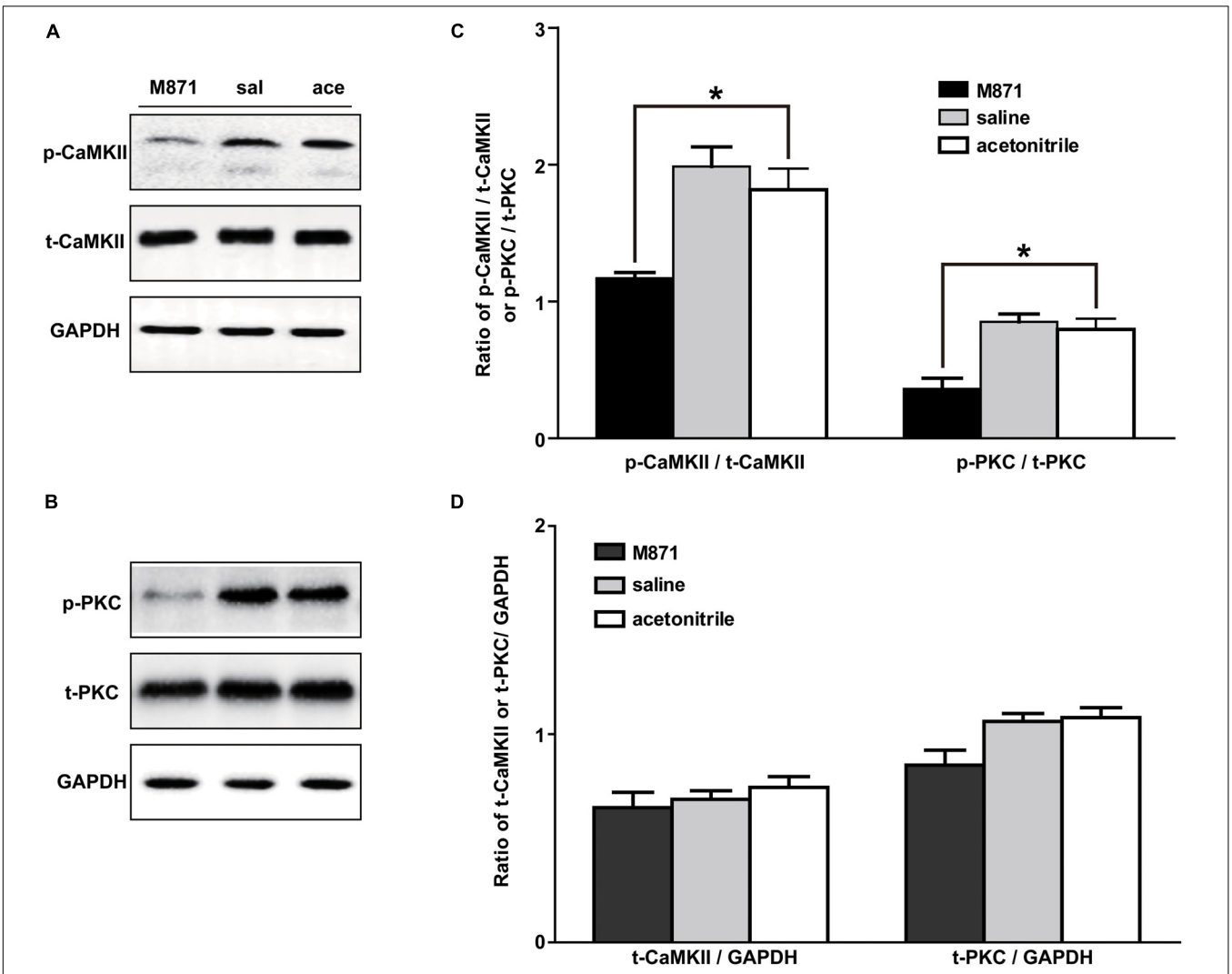

FIGURE 5 | Effect of M871 on PKC or CaMKII expression in NAc of inflammatory pain rats sal: saline; ace: acetonitrile. **(A,B)** Representative Western blots of p-CaMKII, t-CaMKII, p-PKC, t-PKC and GAPDH. **(C)** Histograms showing the p-CaMKII/t-CaMKII ratio and p-PKC/t-PKC ratio. **(D)** Histograms showing the t-CaMKII/GAPDH ratio and t-PKC/GAPDH ratio. The data are presented as the mean ± SEM. *$P < 0.05$ compared with the acetonitrile group.

But there were no significantly changes in the levels of t-CaMKII (**Figures 4A,D**) and t-PKC (**Figures 4B,D**) either 3 (t-CaMKII: $n = 4$, $q = 0.18$, $P > 0.05$; t-PKC: $n = 4$, $q = 0.39$, $P > 0.05$) or 4 h (t-CaMKII: $n = 4$, $q = 0.23$, $P > 0.05$; t-PKC: $n = 4$, $q = 0.32$, $P > 0.05$) after carrageenan injection, which suggested that the PKC and CaMKII signaling pathways in NAc might be involved in inflammatory pain in rats. The differences were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

The GALR2 Antagonist M871 Downregulated p-PKC and p-CaMKII Expressions in NAc of Inflammatory Pain Rats

We next investigated whether the PKC and/or CaMKII signaling pathways in NAc of inflammatory pain rats were mediated by GALR2 activation. Three hours after carrageenan injection, 2 nmol GALR2 antagonist M871, 1 μl of normal saline or 1 μl of 6% acetonitrile was injected into the NAc. Fifteen minutes after injection, proteins in bilateral NAc tissues were rapidly extracted, and the levels of PKC and CaMKII were determined by Western blotting. As shown in **Figure 5**, the intra-NAc administration of GALR2 antagonist M871 significantly downregulated the expressions of p-CaMKII ($n = 3$, $q = 5.20$, $P < 0.05$) and p-PKC ($n = 3$, $q = 6.09$, $P < 0.05$) compared with the acetonitrile-treated group, but the levels of t-PKC ($n = 3$, $q = 4.21$, $P > 0.05$) and t-CaMKII ($n = 3$, $q = 1.70$, $P > 0.05$) were not significantly different (**Figure 5**). The results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

These results showed that GALR2 antagonist M871 blocked GALR2 and then downregulated p-PKC and p-CaMKII expressions, suggesting that GALR2 activation exerted an analgesic effect through activating PKC and CaMKII in NAc of inflammatory pain rats.

The CaMKII Inhibitor KN93 and the PKC Inhibitor GO6983 Attenuated the Analgesic Effect of M1145 in NAc of Inflammatory Pain Rats

To further determine whether CaMKII is involved in GALR2-mediated analgesic effects, 3 h after carrageenan injection, 2 nmol GALR2 agonist M1145 was injected into the NAc to activate GALR2, and 5 min after intra-NAc injection of M1145, 24 μg ($n = 12$), 12 μg ($n = 8$), 6 μg ($n = 8$) of the CaMKII inhibitor KN93 or 1% DMSO as a control ($n = 12$) was injected into the NAc

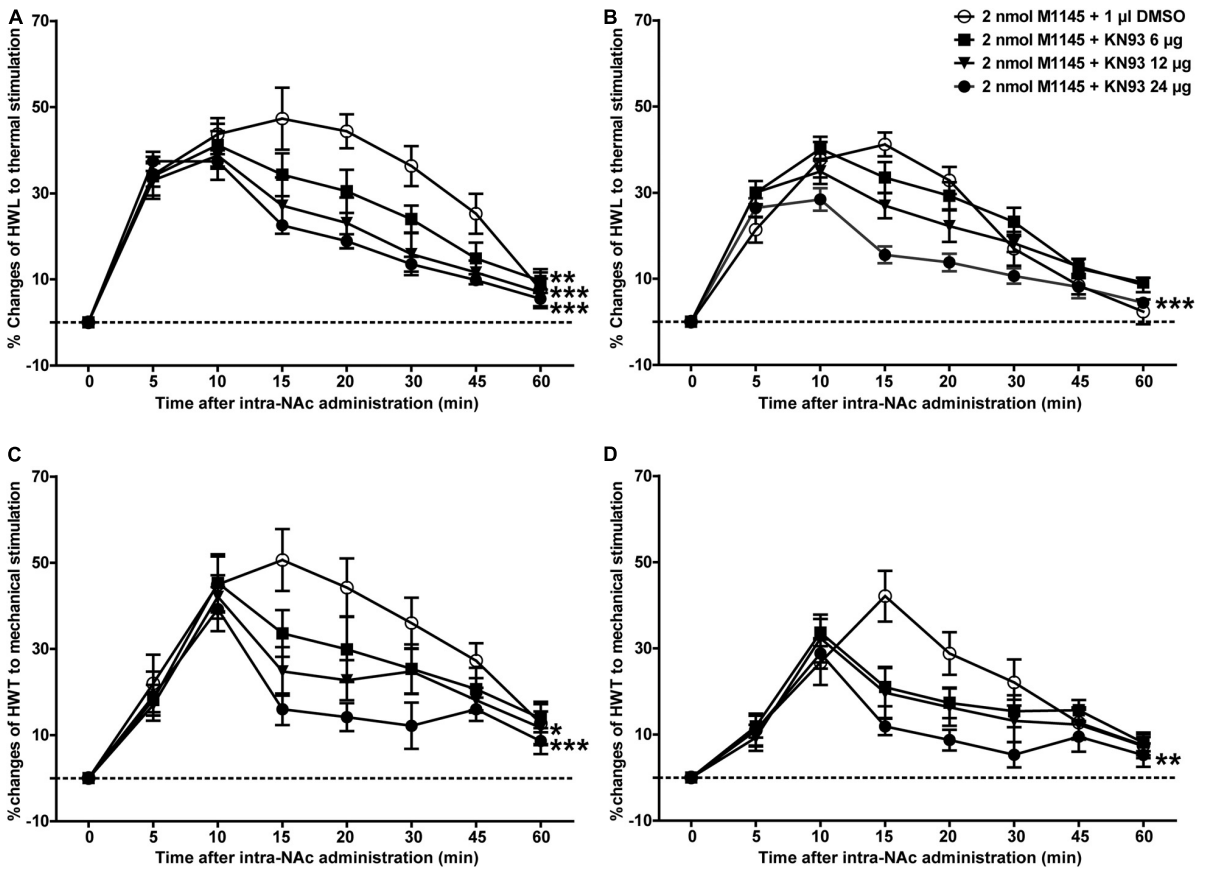

FIGURE 6 | Effect of KN93 on the M1145-induced increases in HWL and HWT in NAc of inflammatory pain rats **(A,C)** left hind paw; **(B,D)** right hind paw. 2 nmol M1145 was bilaterally injected into NAc at 0 min, and 6, 12, 24 μg KN93 or 1 μl of DMSO as a control was bilaterally injected into NAc respectively at 5 min. The data are presented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the DMSO control group.

respectively. Compared with that in the DMSO-treated group, the M1145-induced increase in HWL to thermal stimulation was attenuated in a dose-dependent manner by the intra-NAc administration of 24 μg [Left hind paw: $F_{(1,110)} = 40.05$, $P < 0.0001$; Right hind paw: $F_{(1,110)} = 19.36$, $P = 0.0002$], 12 μg [Left hind paw: $F_{(1,90)} = 16.43$, $P = 0.0007$; Right hind paw: $F_{(1,90)} = 2.78$, $P = 0.1486$] of CaMKII inhibitor KN93, but for rats received 6 μg of KN93, only left HWL was significantly increased [Left hind paw: $F_{(1,90)} = 11.39$, $P = 0.0034$; Right hind paw: $F_{(1,90)} = 0.07$, $P = 0.7990$] (**Figures 6A,B**). And the HWT to mechanical stimulation was also significantly attenuated by the intra-NAc administration of 24 μg [Left hind paw: $F_{(1,110)} = 17.78$, $P = 0.0004$; Right hind paw: $F_{(1,110)} = 8.58$, $P = 0.0078$], 12 μg [Left hind paw: $F_{(1,90)} = 5.00$, $P = 0.0383$; Right hind paw: $F_{(1,90)} = 1.53$, $P = 0.2322$] of KN93, but not 6 μg of KN93 [Left hind paw: $F_{(1,90)} = 1.39$, $P = 0.2541$; Right hind paw: $F_{(1,90)} = 0.81$, $P = 0.379$]. As shown in **Figures 6C,D**, the differences were analyzed by two-way repeated-measures ANOVA.

Meanwhile, to further study whether PKC is a downstream signaling molecule associated with the analgesic effect of GALR2 activation on inflammatory pain, 2 nmol GALR2 agonist M1145 was injected into the NAc of rats with inflammatory pain, and 5 min later, 12 μg ($n = 8$), 24 μg ($n = 8$), 36 μg ($n = 8$) of the PKC inhibitor GO6983 or 1% DMSO as a control ($n = 12$) was injected into the NAc, respectively. Compared with that in the DMSO-treated group, the M1145-induced increase in HWL to thermal stimulation was attenuated by the intra-NAc administration of 36 μg [Left hind paw: $F_{(1,90)} = 63.64$, $P < 0.0001$; Right hind paw: $F_{(1,90)} = 46.21$, $P < 0.0001$], 24 μg [Left hind paw: $F_{(1,90)} = 33.71$, $P < 0.0001$; Right hind paw: $F_{(1,90)} = 13.24$, $P = 0.0019$] or 12 μg [Left hind paw: $F_{(1,90)} = 10.00$, $P = 0.0054$; Right hind paw: $F_{(1,90)} = 4.84$, $P = 0.0411$] of PKC inhibitor GO6983 (**Figures 7A,B**). The M1145-induced increase in HWT to mechanical stimulation was also attenuated by the intra-NAc administration of 36 μg [Left hind paw: $F_{(1,90)} = 14.42$, $P = 0.0013$; Right hind paw: $F_{(1,90)} = 10.26$, $P = 0.0049$], 24 μg [Left hind paw: $F_{(1,90)} = 7.77$, $P = 0.0122$; Right hind paw: $F_{(1,90)} = 1.22$, $P = 0.2833$], but not 12 μg [Left hind paw: $F_{(1,90)} = 3.53$, $P = 0.0764$; Right hind paw: $F_{(1,90)} = 0.62$, $P = 0.4418$] of PKC inhibitor GO6983 (**Figures 7C,D**). The differences were analyzed by two-way repeated-measures ANOVA.

These results again proved that both PKC and CaMKII were involved in the analgesic effect of GALR2 in NAc of inflammatory pain rats.

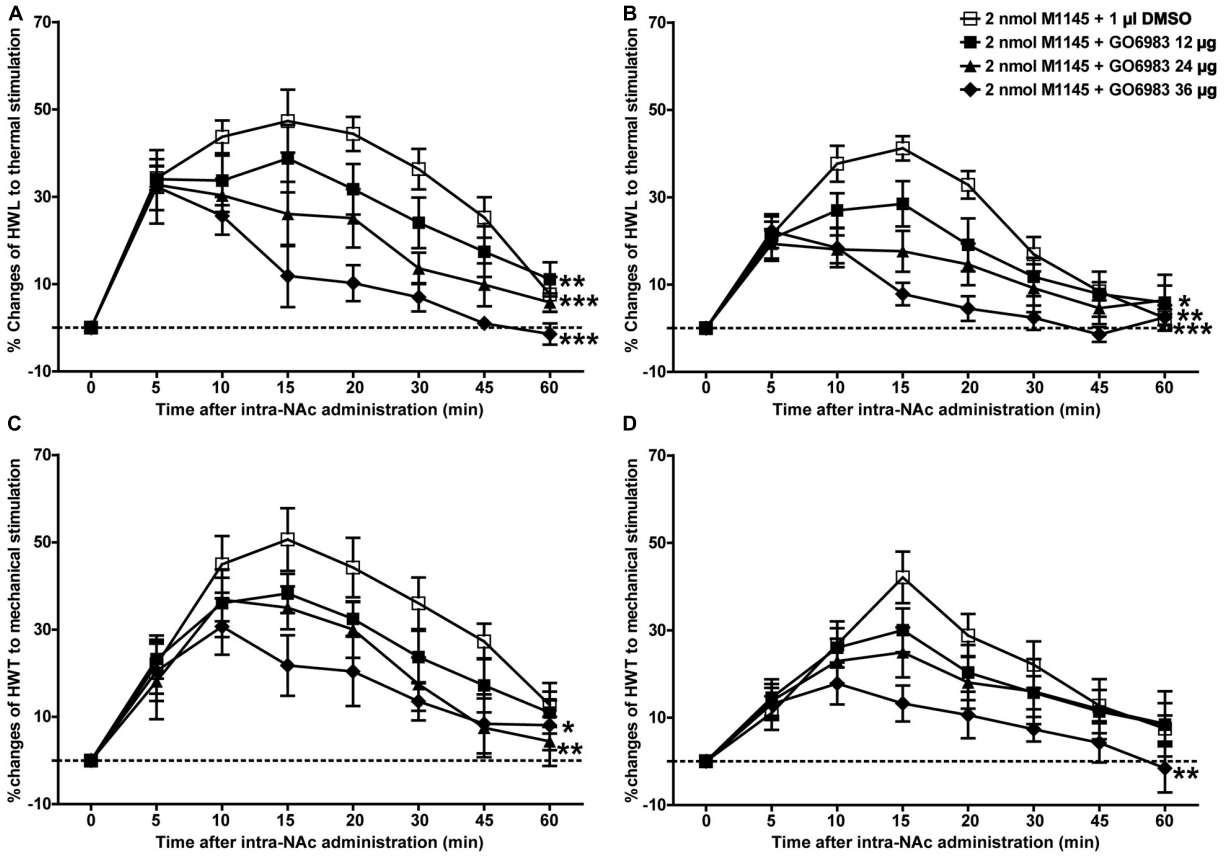

FIGURE 7 | Effect of GO6983 on the M1145-induced increases in HWL and HWT in NAc of inflammatory pain rats **(A,C)** left hind paw; **(B,D)** right hind paw. 2 nmol M1145 was bilaterally injected into NAc at 0 min, and 12, 24, 36 μg GO6983 or 1 μl of DMSO as a control was bilaterally injected into NAc respectively at 5 min. The data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the DMSO control group.

DISCUSSION

The role of neuropeptide galanin in pain modulation has been well-established, and it has been suggested that galanin may be a potential target for novel therapies. Some studies have recently proven that galanin has an analgesic effect on inflammatory pain (Yang et al., 2015; Zhang et al., 2017a). In this study, we aimed to use inflammatory pain rats to determine whether the analgesic effect of galanin is accomplished by activating GALR2 and to explore the underlying signaling mechanism. Some studies have demonstrated that the subcutaneous injection of carrageenan into the plantar region of the hind paw of animals can induce local inflammation and pain (Metcalf et al., 2015; Yang et al., 2015; Zhang et al., 2017a). The responses to noxious stimuli were enhanced during the inflammatory process, and bilateral HWL to noxious thermal stimulation and HWT to mechanical stimulation decreased 3 or 4 h after the injection of carrageenan (Sun et al., 2003; Xiong et al., 2005; Yang et al., 2015). In this study, animal models were established through injecting carrageenan into the plantar of left hind paw of rats.

An early study showed that thermal noxious or intense chemical stimulation could induce pain perception by an ascending nociceptive modulation and that this effect depended on both opioid and dopamine links in the NAc (Gear et al., 1999). The infusion of N-acetylaspartylglutamate into the NAc significantly attenuated the pain induced by activation of sensory nerves through optical stimulation (Watanabe et al., 2018). These studies suggested that the NAc plays an important role in mediating the suppression of tonic or persistent pain. As early as 1992, Kordower et al. (1992) reported that galanin-immunoreactive fibers were seen within the NAc in monkey, therefore the potential role of galanin in NAc on pain modulation is worth investigation.

Galanin and GALRs expressed at the sites of pain mediation. Xu et al. (2012) demonstrated that after sciatic nerve-pinch injury, GALR1 expression was up-regulated in spinal dorsal horn, whereas GALR2 was also up-regulated in both dorsal root ganglion and spinal dorsal horn. Our previous study showed that the expressions of both galanin and GALR1 were up-regulated in the NAc of rats with neuropathic pain (Duan et al., 2015; Zhang et al., 2019), while other studies showed that administration of GALR1 agonist M617 to intracerebroventricular (Fu et al., 2011), central nucleus of amygdala (Li et al., 2012), NAc (Duan et al., 2015) could induce a significant analgesic effect in rats, implying that GALR1 mediates the galanin-induced antinociceptive effect in rats. In addition, our previous study showed 3 h after carrageenan injection that the expressions of both GALR1 and GALR2 were significantly upregulated in NAc (Yang et al., 2015). Zhang et al. (2017a) also reported that the level of GALR2 in the ACC was increased in rats with acute inflammation (Zhang et al., 2017a). Compared with GALR1 and GALR2, there were fewer studies about the role of GALR3 in pain manipulation. Lv et al. (2019) reported that central spexin, a natural ligand for GALR2/3, produced an antinociceptive effect by activating GALR3 in the acute inflammatory pain models (Lv et al., 2019).

Whether GALR2 plays an antinociceptive role in the NAc of inflammatory rats was not examined until now. In the present study, we aimed to study the analgesic effect of GALR2 activation on inflammatory pain, and the first result found that galanin-induced analgesic effect was weakened by the intra-NAc administration of GALR2 antagonist M871 in rats with inflammatory pain. Consistent with this result, other studies also found that the administration of exogenous GALR2 antagonist M871 to the periaqueductal gray (PAG; Zhang et al., 2015), ACC (Zhang et al., 2017a) attenuated the galanin-induced analgesic effects in rats. These results together suggested that galanin might have an analgesic effect on inflammatory pain which was mediated by GALR2. But in this study, we also found that the antinociception of galanin was blocked partially by GALR2 antagonist M871, implying that galanin in NAc has an analgesic effect on inflammatory pain by activating other GALRs. In this study, to further prove the role of GALR2 on inflammatory pain, the GALR2 agonist M1145 was injected into the NAc of rats 3 h after carrageenan injection, the results showed that M1145 dose-dependently increased bilateral HWL to thermal stimulation and HWT to mechanical stimulation, and the M1145-induced analgesic effect was blocked by the intra-NAc administration of GALR2 antagonist M871. These results further confirmed that

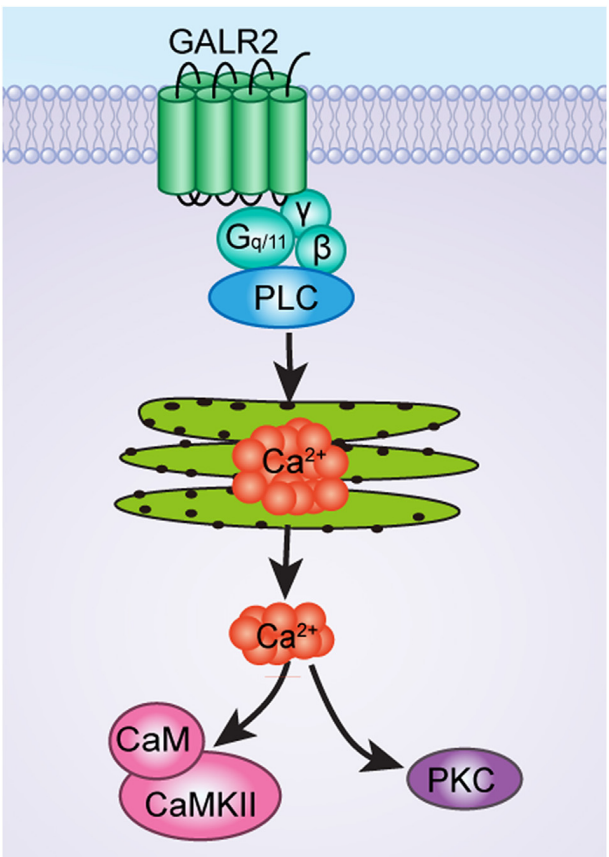

FIGURE 8 | The schematic diagram of CaMKII and PKC signaling pathways of GALR2-induced analgesic effect in NAc of inflammatory pain rats.

the analgesic effect of galanin on inflammatory pain was achieved through activating GALR2, although the underlying signaling mechanism is unclear.

Considerable evidences have shown that Ca^{2+}-mediated signaling pathways are important in nociception. A large amount of Ca^{2+} enters into the cell and then activates intracellular Ca^{2+}-dependent protein kinases, including CaMKII and PKC (Lisman et al., 2002). Former studies have reported that p-PKC is upregulated in dorsal root ganglion (DRG) neurons of rats with inflammatory arthritis pain (Koda et al., 2016; Bai et al., 2019). Consistently, the data of our present study showed that the expressions of both p-PKC and p-CaMKII in the NAc were upregulated 3 and 4 h after carrageenan injection in rats, which indicated that PKC and CaMKII in NAc were involved in the modulation of inflammatory pain of rats.

GALR2 mainly couples to $G_{q/11}$-type G-protein, therefore activation of GALR2 leads to phospholipase C (PLC) activation (Wittau et al., 2000), and causes calcium mobilization. Therefore, we conjected that the analgesic effect of GALR2 activation on inflammatory pain was achieved through the Ca^{2+}-mediated signal transduction pathways. Former studies showed that administration of the PKC inhibitor chelerythrine into cerebroventricular (Shi et al., 2011) or central nucleus of amygdala (Li et al., 2017) significantly inhibited galanin-induced analgesic effect in rats, while administration of the CaMKII inhibitor MAP into PAG also inhibited galanin-induced analgesic effect in rats (Zhang et al., 2015). Based on these studies, in this work, we focus on whether the PKC and/or CaMKII signaling pathways in the NAc underlay the GALR2 activation-induced analgesic effects in inflammatory pain rats. Interestingly, the results of this study showed that p-PKC and p-CaMKII in the NAc were downregulated after the administration of the GALR2 antagonist M871 in inflammatory pain rats, suggesting that PKC and CaMKII might be involved in the GALR2 activation-induced analgesic effect in the NAc of inflammatory pain rats. To further explore the underlying mechanism, in the present study, M1145 was injected into the NAc of rats with inflammatory pain, and the CaMKII inhibitor KN93 or PKC inhibitor GO6983 was injected into the NAc 5 min after the intra-NAc administration of M1145. The results showed that KN93 and GO6983 weakened the M1145-induced increases in the HWL and HWT of inflammatory pain rats. These results illustrated that GALR2 activation in the NAc had an analgesic effect on inflammatory pain via PKC and CaMKII signaling pathways in rats.

As has been mentioned earlier, GALR2 couples to G-protein ($G_{q/11}$-type), which causes PLC activation and calcium mobilization, and may then lead to the recruitment of Ca^{2+}-dependent PKC and CaMKII, as shown in **Figure 8**. Therefore, it is possible that after the injection of PKC and CaMKII inhibitors to block the PKC and CaMKII signaling pathways, GALR2-induced analgesic effect is then weakened.

Taken together, all the results of this study show that CaMKII and PKC are involved in inflammatory pain. GALR2 activation in the NAc results in an analgesic effect on inflammatory pain via the activation of PKC and CaMKII in rats. This finding implies that GALR2 agonists may be potent relievers of inflammatory pain.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee at Kunming Medical University.

AUTHOR CONTRIBUTIONS

SX: study concept and design. ML, YL and SY: acquisition of data. SX and CL: analysis and interpretation of data. XZ and SX: drafting and revision of the manuscript. SX and XZ: funding. SX and CL: study supervision. All authors contributed to the article and approved the submitted version.

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Small-Conductance Ca^{2+}-Activated K^+ Channels 2 in the Hypothalamic Paraventricular Nucleus Precipitates Visceral Hypersensitivity Induced by Neonatal Colorectal Distension in Rats

Ning-Ning Ji[1†], Lei Du[1,2†], Ying Wang[1], Ke Wu[1], Zi-Yang Chen[3], Rong Hua[4] and Yong-Mei Zhang[1*]*

[1]Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, China, [2]Anesthesiology Department of the Nanjing Children's Hospital, Nanjing, China, [3]Department of Anesthesiology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China, [4]Institute of Emergency Rescue Medicine, Xuzhou Medical University, Xuzhou, China

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Damiana Scuteri,
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Reviewed by:
Gongliang Zhang,
Lieber Institute for Brain Development, United States
Domenico Tricarico,
University of Bari Aldo Moro, Italy

***Correspondence:**
Yong-Mei Zhang,
zhangym700@xzhmu.edu.cn
Rong Hua
ilovezq@sohu.com

[†]These authors have contributed equally to this work

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Visceral hypersensitivity is one of the pivotal pathophysiological features of visceral pain in irritable bowel syndrome (IBS). Small-conductance Ca^{2+}-activated K^+ channel (SK) is critical for a variety of functions in the central nervous system (CNS), nonetheless, whether it is involved in the pathogenesis of visceral hypersensitivity remain elusive. In this study, we examined mechanism of SK2 in hypothalamic paraventricular nucleus (PVN) in the pathogenesis of visceral hypersensitivity induced by neonatal colorectal distension (CRD). Rats undergoing neonatal CRD presented with visceral hypersensitivity as well as downregulated membrane SK2 channel and p-PKA. Intra-PVN administration of either the membrane protein transport inhibitor dynasore or the SK2 activator 1-EBIO upregulated the expression of membrane SK2 in PVN and mitigated visceral hypersensitivity. In addition, 1-EBIO administration reversed the increase in neuronal firing rates in PVN in rats undergoing neonatal CRD. On the contrary, intra-PVN administration of either the SK2 inhibitor apamin or PKA activator 8-Br-cAMP exacerbated the visceral hypersensitivity. Taken together, these findings demonstrated that visceral hypersensitivity is related to the downregulation of membrane SK2 in PVN, which may be attributed to the activation of PKA; pharmacologic activation of SK2 alleviated visceral hypersensitivity, which brings prospect of SK2 activators as a new intervention for visceral pain.

Keywords: PKA, neonatal colorectal distension, visceral hypersensitivity, rats, hypothalamic paraventricular nucleus, small-conductance Ca^{2+}-activated K^+ channel 2

INTRODUCTION

Visceral hypersensitivity is one of the key pathophysiological features of irritable bowel syndrome (IBS) as well as other conditions with visceral pain (Chey et al., 2015). The mechanism underlying visceral hypersensitivity remains unclear on the grounds of absence of structural abnormalities detected in the internal organs. Our prior study demonstrated that re-exposure to CRD in adult rats having been subjected to neonatal CRD induced visceral hypersensitivity (Yu et al., 2014; Chen et al.,

2015; Zhang et al., 2016b; Song et al., 2018). The hypothalamic paraventricular nucleus (PVN) integrates multiple afferents to autonomously regulate visceral sensation (Li et al., 2014). Corticotropin-releasing factor (CRF) neurons in PVN increased corticosteroids and adrenocorticotropic hormone (ACTH) levels via the hypothalamic–pituitary–adrenal (HPA) axis, which can be disrupted by stress-induced PVN neuroplasticity and HPA axis dysregulation (de Kloet et al., 2005; Van den Bergh et al., 2008; Bravo et al., 2011; Green et al., 2011; Amath et al., 2012). Our prior studies confirmed that CRD induced visceral hypersensitivity as well as enhanced excitability of CRF neurons, with both CRF protein expression in PVN and plasma cortisol levels increased (Yu et al., 2014; Chen et al., 2015). The activation of CRF neurons in PVN leads to the CRF release, resulting in the undermined analgesia (Lariviere and Melzack, 2000) and exacerbated neuropathic pain (Fu et al., 2016), thereby implying the pivotal role of CRF neurons in PVN in modulating visceral hypersensitivity. However, the mechanism underpinning the elevated excitability of CRF neurons in PVN in the pathogenesis of visceral hypersensitivity remains elusive.

SK channels, which are an important membrane ion channels regulating neuronal excitability, consist of four subtypes (SK1, SK2, SK3 and IK1channels) and are widely distributed across the CNS (Kohler et al., 1996; Faber, 2009; Adelman et al., 2012; Deignan et al., 2012; Li et al., 2017). Activation of SK2 channel is prerequisite for the control of K^+ outflow and the consequent generation of the medium afterhyperpolarizations (mAHP) in response to the increase of the intracellular Ca^{2+} levels, with the firing rate decreased and the excitatory of neuron inhibited (Pedarzani et al., 2000; Strassmaier et al., 2005; Hammond et al., 2006; Chang et al., 2013). It is well known that abnormal neuronal excitability is one of the mechanisms of pain (Cheng et al., 2015; Fan et al., 2015) and SK channels are reportedly associated with nociception. Administration of the selective SK channel blocker UCL1848 increases neuronal responses to naturally evoked nociceptive stimuli. Conversely, administration of the selective SK channel activator 1-EBIO inhibits neuronal responses evoked by mechanical stimuli via the increased SK channel activity (Bahia et al., 2005). Administration of the selective SK2 channel blocker apamin increased excitability and enhanced excitatory synaptic transmission, as indicated by increased frequency of miniature EPSCs and action potentials, leading to hyperexcitability and pain hypersensitivity (Pagadala et al., 2013). SK channels in the amygdala mediate pain-inhibiting effects in a rat model of arthritic pain (Thompson et al., 2015). These findings on the multitude of effects associated with SK2 channel invited our exploration of the implications of SK2 channel in PVN in rat model of visceral hypersensitivity.

In this study, we examined the mechanism of SK2 channels underlying elevated excitability of CRF neurons in PVN in the pathogenesis of visceral pain. Our data suggested that visceral hypersensitivity is related to the downregulation of SK2 channel protein as well as inactivation of SK2 channel in PVN CRF neurons. Our findings might provide new molecular and neuronal insights into the precipitation of visceral hypersensitivity, thereby benefiting the medical intervention regimens with SK2 activators for visceral pain.

MATERIALS AND METHODS

Animals

Neonatal sucking male Sprague-Dawley rats (within 8 days) were provided from the Experimental Animal Center, Xuzhou Medical University (Xuzhou, China) and kept with the maternal rats until 21 days. After weaning, the young male rats were housed in fours in standard Plexiglas cages, with ad libitum access to food and water. Rats were checked daily and weighed weekly for 2 months or until body weight of 200–250 g prior to group designation. During the testing session, rats were maintained on a standard 12 h light–dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.), with constant temperature and humidity (22°C and 50%, respectively) and ad libitum access to food and water. Animal sample sizes (6 rats per group) were determined by the expected change of the experiments and previous experience from similar studies and were sufficient for all statistical tests (alpha = 0.05; power = 0.90; one-tailed test). Statistical analyses were performed with PASS version 15.0. The experimental protocol design was based on the Replacement, Reduction and Refinement principles described 2010/63/EU law on Animal Protection Used for Scientific Experiments. All procedures were conducted in accordance with the guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978) and the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee at Xuzhou Medical University.

Reagents

The reagents and antibodies were as follows: rabbit anti-KCa2.2 (APC-028) polyclonal Ab (Alomone Labs, Jerusalem, Israel); mouse anti-GAPDH (AC001) mAb (Abclonal, Woburn, MA, United States); alkaline phosphatase goat anti-rabbit IgG (ZB-2308); alkaline phosphatase horse anti-mouse IgG (ZB-2310); BCA protein assay kit (P0012); sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (P0015); BCIP/NBT alkaline phosphatase color development kit (C3206). Syn-PER™ Synaptic Protein Extraction Reagent (#87793) (Thermo Fisher Scientific, Waltham, MA, United States). AAV-CRH-EYFP-WPRE-pA (AAV-CRH-EYFP) was purchased from BrainVTA (Wuhan) Co., Ltd.

Visceral Hypersensitivity Model

Chronic visceral hyperalgesia model was established by repeated CRDs in neonatal rats. Neonatal rats were subjected to CRDs on postnatal days 8, 10, and 12, via an angioplasty balloon (20.0 mm in length and 3.0 mm in diameter) inserted into the rectum and descending colon. The balloon was distended at a pressure of 60 mmHg for 1 min prior to deflation and withdrawal, with the distention repeated daily and at an interval of 30 min. The neonatal rats were returned to the maternal rats immediately after each CRD procedure. Neonatal CRD, i.e.

model rats were weaned on day 21, and separated into different cages on day 30. CRD rats were routinely raised till the postnatal 8th week. By then, the adult rats underwent CRDs at 60 mmHg for 60 s, ten times at an interval of 15 s, so as to trigger visceral hyperalgesia.

Pain Threshold

Visceral sensitivity was assessed via pain threshold. Rats were placed in Lucite cubicles (20 × 8 × 8 cm) on an elevated Plexiglas platform and allowed for habituation for 15–30 min. Graded distension was exerted by rapid inflation of a balloon inside the descending colon and rectal region to a desired pressure (20, 40, 60, or 80 mmHg) for a duration of 20 s followed by a 4-min rest. The abdominal withdrawal reflex (AWR) was scored as: 0, no behavioral response to distension; 1, slight head movement followed by immobility; 2, contraction of the abdominal muscles; 3, lifting of the abdomen; 4, body arching and lifting of the derriere. The pain threshold by distension was defined as an AWR score of 3. For accuracy, each distension procedure was in triplicate. Naïve rats did not undergo any treatment.

Intra-Paraventricular Nucleus Microinfusion

Rats were anesthetized under 2% pentobarbital sodium (40 mg/kg) and mounted onto a David Kopf stereotaxic frame (Tujunga, CA, United States), with the cranium in a horizontal plane. With the scalp incised and holes drilled through the cranium for bilateral insertion of a microinjector needle (28 gauge) into the PVN region of the hypothalamus (A/P −1.5 mm, L/R ± 0.4 mm, D/V −7.7 mm from bregma), Dynasore (80 μM, 0.5 μl), EBIO (10 μg/0.3 μl), apamin (6.25 pmol/0.3 μl) or 8-Br-cAMP (100 mM, 0.1 μl) (Wang et al., 2012) was infused into PVN in 5 min, with the microinjector needle in place for an additional 5 min to allow for solution diffusion prior to the skin closure. 30 min thereafter, rats underwent behavioral tests or brain isolation. The site of the cannula track aiming at PVN was histologically verified for each brain, and rats with incorrect cannulation were excluded from data analysis. The virus (AAV-CRH-EYFP, AAV2/9, 2×10^{12} viral genome ml^{-1}, 300 nl) to label PVN CRF neurons was injected with identical procedure, and brain sections were sliced for electrophysiological recording 21 days thereafter.

HT-22 Hippocampal Neurons

Hippocampal HT22 cells were cultured in DMEM-HAMS F12, supplemented with 10% fetal bovine serum, L-glutamine (100 mM) and 1% antibiotics (penicillin, streptomycin) and incubated in humidified 5% CO_2 atmosphere at 37°C. At 80% confluence, cells were detached with trypsin-EDTA, rinsed and sub-cultivated in new flasks for 1–2 days prior to experimentation. The cells were then incubated at 37°C with 80 μmol/L dynasore for 30 min.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

The SK2 mRNA level in PVN was determined by means of qRT-PCR. Total cellular RNA was isolated from tissue samples via Trizol Reagent (15,596–026, Invitrogen, Carlsbad, CA, United States) as per the manufacturer's protocol. The RNA was quantified by spectrophotometry (OD 260/280). RNA was transcribed to cDNA using M-MLV Reverse Transcriptase (D263915) and dT primers. PCR amplification was performed with Taq polymerase using 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. The PCR primers for SK2 were 5′-TTG TGGAAGGGGCATAGGAGA-3′ (sense) and 5′-AATGGAGCAGATGA CTGGAGA-3′ (antisense), for GAPDH were 5′-TCTCTGCTCCTCCCTGTTC-3′ (sense) and 5′-ACACCGACCTTCACCATCT-3′ (antisense), which were synthesized by Invitrogen Biotech Co. Ltd. (Shanghai, China). The qRT-PCR was performed with a Rotor-Gene 3,000 real-time DNA analysis system (Corbett Research, Sydney, Australia) with real-time SYBR Green PCR technology. The reaction mixtures contained diluted cDNA, SYBR Premix Ex Taq II (2×; DRR081), 10 μM of each gene-specific primer, and nuclease-free water in a final volume of 10 μl. The cDNA results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) measured for the same sample.

Western Blotting

With the rats sacrificed and the brain quickly isolated in the ice bath, the PVN area was transferred into individual freezing storage tubes, followed by addition of Syn-PER™ Synaptic Protein Extraction Reagent (1 ml/100 mg) containing phosphatase inhibitors and PMSF. With the tissue fully homogenized by a homogenizer on ice, samples were centrifuged at 8,000 rpm for 10 min at 4°C. Following collection of the supernatant (containing cytoplasmic protein) and further centrifugation at 12,000 rpm for 20 min at 4°C, lysis buffer was added for dissolution of the deposition (containing membrane protein). Protein concentration was measured by the BCA Protein Assay kit (P0012). 25 μg of brain tissues was added and separated by 10% SDS-PAGE gel system for electrophoresis with the PageRuler Prestained Protein Ladder (26,616, Thermo). Thereafter, the protein was transferred onto the PVDF membrane at the constant voltage of 100 V and approximately 60 min with a wet transferometer. After the protein transference, the PVDF membrane was rinsed in washing buffer for 5 min, followed by addition of 5% skim milk, at room temperature (r/t) for 2 h. Membranes were then incubated in rabbit monoclonal anti-GAPDH (1:1,000, G9545, Sigma-Aldrich, Co. LLC, MO, United States) and rabbit anti-KCa2.2 (1:1,000, APC-028, Alomone Labs) or rabbit monoclonal anti-p-PKA (1:1,000) overnight at 4°C. On the following day, after 30 minutes of rewarming, the PVDF membranes were rinsed with washing buffer for 10 min in triplicate, and were then incubated in anti-rabbit IgG with alkaline phosphatase (1:1,000; A0208, Beyotime) on the shaking bed at r/t for 2 h, with BCIP/NBT alkaline phosphatase color development kit (C3206) employed

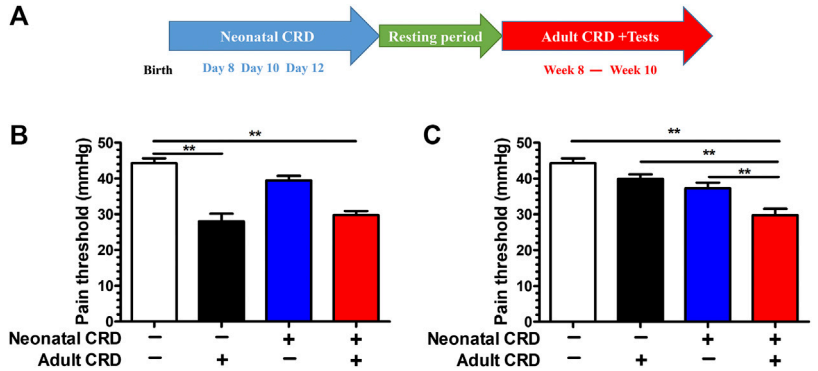

FIGURE 1 | Adult CRD precipitated the visceral hypersensitivity in rats experiencing neonatal CRD. **(A)** Rats undergoing neonatal CRDs on postnatal days 8, 10, and 12, and adult CRD in week 8. The behavioral testing was conducted in weeks 8–10. **(B)** Pain threshold was measured immediately after adult CRD (n = 6 rats each, $F_{3,20} = 25.83$, $p < 0.001$). **(C)** Pain threshold was measured 2 h after adult CRD (n = 6 rats each, $F_{3,20} = 16.34$, $p < 0.001$). Significance was assessed by ordinary one-way ANOVA with post hoc comparisons between groups. The data are expressed as the mean ± SEM. $^{**}p < 0.01$.

for protein coloration. ImageJ software was used for grayscale analysis.

Electrophysiological Recording

Transverse sections of the PVN area were taken at 250–300 mm in ice-cold slicing solution (mM): NaCl, 80; KCl, 3.5; $MgSO_4$, 4.5; $CaCl_2$, 0.5; NaH_2PO_4, 1.25; sucrose, 90; $NaHCO_3$, 25 and glucose, 10. Artificial cerebral spinal fluid (aCSF) (mM) consisted of NaCl, 126; KCl, 2.5; NaH_2PO_2, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 26; glucose, 10 and $CaCl_2$, 2.4. All solutions were saturated with 95% O_2 and 5% CO_2. The slices were incubated in cutting solution at 32°C for 15 min, and were then transferred into the aCSF at r/t for at least 1 h prior to submersion in recording chamber. Whole cell patch-clamp recording was conducted in CRF neurons with a glass pipette filled with an internal solution (mM): phosphocreatine-Tris, 10; 10 HEPES, 10 EGTA, 2 ATP-Mg, 0.5 GTPNa, 115 K gluconate, 20 KCl, 1.5 $MgCl_2$; with pH adjusted to 7.2 with KOH (285 mOsm). The resistance was 4–8 MΩ. Whole cell recoding: cells were maintained in the current-clamp mode at -60 mV and action potential firings in response to the injection of depolarizing current pulses were recorded with a patch-clamp amplifier (MultiClamp 700A, Axon Instruments, Union City, CA, United States). For measurement of SK currents, CRF neurons were held in voltage-clamp at a holding potential of −60 mV and 100 ms depolarizing pulse to 60 mV, which was employed to evoke an outward current. Cell-attached recordings were performed in voltage clamp with the current set around 0 pA. Data acquisition and analysis were performed using Clampex and Clampfit 10 (Axon Instruments, San Jose, CA, United States).

Statistical Analysis

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was employed. In the presence of statistical significance, post hoc Bonferroni or S–N–K multiple comparisons were applied. Independent samples Student's *t*-test was also used. All statistical tests were conducted using the SPSS 19.0 (IBM, Armonk, NY, United States) software package. Experimenters were blinded during the experiment and quantitative analysis was conducted to avoid biases. $p < 0.05$ was considered statistically significant.

RESULTS

Adult Colorectal Distension Precipitates the Visceral Hypersensitivity in Rats Undergoing Neonatal Colorectal Distension

To evaluate the effect of adverse early stress on visceral pain sensitivity in rats, we adopted the paradigm of neonatal CRD. Rats were subjected to neonatal CRDs on postnatal days 8, 10, and 12, and adult CRD in week 8. The behavioral test was performed in weeks 8–10, which revealed evident decrease of pain threshold in rat groups (adult CRD; neonatal + adult CRD) ($p < 0.01$). (**Figures 1A,B**). Intriguingly, 2 h after adult CRD procedure, the pain threshold returned to normal in the adult CRD group, whereas the pain threshold remained significantly lower in the neonatal + adult CRD group (**Figure 1C**), suggesting that rats experiencing neonatal CRD presented with underlying visceral hypersensitivity, which can be revived and amplified by subsequent CRD in adulthood.

Membrane SK2 Channel Protein and IAHP were Decreased in Rats Experiencing Neonatal Colorectal Distension

To address the physiological basis for visceral hypersensitivity in neonatal CRD rats, we next evaluated the SK2 expression and electrophysiological properties. Western blotting data revealed that the expression of PVN SK2 channel protein in membrane fraction was decreased in rats subjected to neonatal CRD (**Figure 2A**). Conversely, the PVN SK2 channel protein level in the cytoplasmic fraction was increased in rats undergoing neonatal CRD (**Figure 2B**). Moreover, Western blotting and

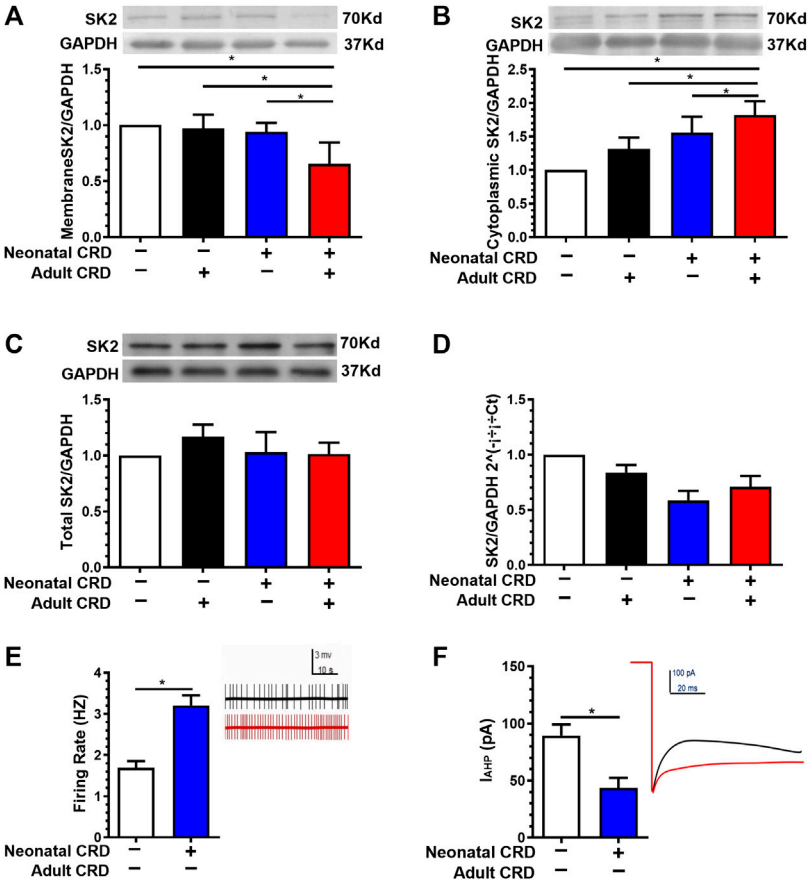

FIGURE 2 | Membrane SK2 channel protein and IAHP were decreased in rats undergoing neonatal CRD. **(A)** Western blotting data revealed that the PVN SK2 channel protein in membrane fraction was decreased in rats undergoing neonatal and adult CRD vs. rats undergoing adult CRD alone (n = 6 rats each, $F_{3,20} = 17.25$, $p < 0.0001$). **(B)** The PVN SK2 channel protein in the cytoplasmic fraction was increased in rats undergoing neonatal and adult CRD vs. rats receiving adult CRD alone (n = 6 rats per group, $F_{3,20} = 3.861$, $p = 0.0250$). **(C,D)** Western blotting and qRT-PCR data showed insignificant between-group difference in total PVN SK2 protein (n = 6 rats each, $F_{3,20} = 0.4638$, $p = 0.7108$ in **C**; n = 6 rats each, $F_{3,20} = 1.022$, $p = 0.4039$ in **D)**. **(E,F)** Rats undergoing neonatal CRD presented with an increase in CRF neurons firing rates vs. the naïve rats ($p < 0.05$, n = 10 neurons each); consistently, rats undergoing neonatal CRD presented with low IAHP vs. rats without neonatal CRD (n = 13 cells each, $t_{24} = 5.112$, $p < 0.0001$ in E; n = 9 cells each, $t_{16} = 6.913$, $p < 0.0001$ in **F)**. Significance was assessed by ordinary one-way ANOVA with post hoc comparisons between groups in A, B, C, D, two-tailed unpaired Student's *t*-test in E, **F**. The data are expressed as the mean ± SEM. *$p < 0.05$.

qRT-PCR data showed insignificant between-group difference in total PVN SK2 protein (**Figures 2C,D**). Rats with neonatal CRD exhibited an increase in spontaneous neuronal firing rates vs. the naïve rats (**Figure 2E**). Consistently, rats with neonatal CRD presented with lower IAHP compared to rats without neonatal CRD (**Figure 2F**). These findings suggested that neonatal CRD contributed to the internalization of SK2 channels.

Cell Membrane Protein Transport Inhibitor Dynasore Increased the Expression of Membrane SK2 Channel Protein and Alleviated Visceral Hypersensitivity

To address the contribution of member SK2 channel protein to visceral hypersensitivity, we next adopted cell membrane protein transport inhibitor dynasore (Dyn). We initially tested the effect of Dyn (80 μmol/L) on membrane SK2 channel protein at the cellular level. Given the effect of intracellular SK2 protein synthesis during inhibition of protein transport, we employed the protein synthesis inhibitor cycloheximide (Cyc, 20 μg/ml) to antagonize the synthetic effect. Western blotting results revealed that the Dyn significantly increased the expression of membrane SK2 channel protein in cultured HT22 hippocampal neurons *in vitro* (**Figures 3A,B**), indicative of its effective inhibition of membrane SK2 channel protein transfer from the membrane into the cytoplasm.

To verify the effect of Dyn on visceral hypersensitivity in neonatal CRD rats, Dyn was administered by intra-PVN injection 30 min before behavioral testing. We identified that Dyn precluded the decrease of membrane SK2 channel protein as well as the pain threshold in rats (**Figures 3C,D**), suggesting that downregulation of membrane SK2 channel protein precipitated visceral hypersensitivity in neonatal CRD rats.

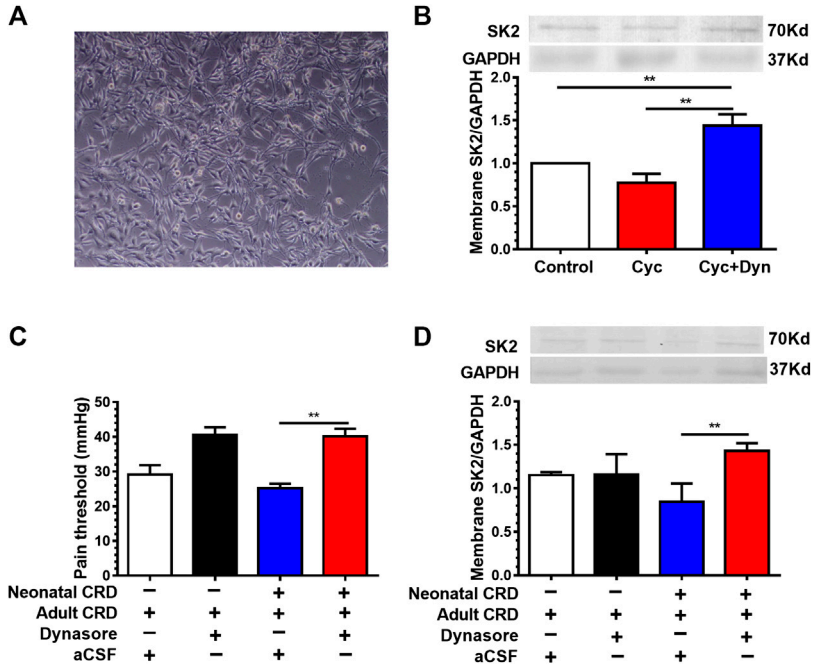

FIGURE 3 | Cell membrane protein transport inhibitor dynasore increased the expression of membrane SK2 channel protein and alleviated visceral hypersensitivity. **(A)** Representative images of cultured HT22 hippocampal neurons *in vitro*. **(B)** Effect of dynasore (Dyn) on membrane SK2 channel protein expression *in vitro*. Western blotting results showed that the Dyn significantly increased the expression of membrane SK2 channel protein in HT22 hippocampal neurons (n = 6 rats each, $F_{2,15}$ = 52.91, $p < 0.0001$). **(C,D)** Effect of intra-PVN injection of Dyn on pain threshold and membrane SK2 channel protein expression in neonatal CRD rats. Dyn precluded the decrease of membrane SK2 channel protein as well as the pain threshold in neonatal CRD rats (n = 6 rats each, $F_{3,20}$ = 12.62, $p < 0.0001$ in **C**; n = 6 rats each, $F_{3,20}$ = 3.637, $p = 0.0305$ in **D)**. Significance was assessed by ordinary one-way ANOVA with post hoc comparisons between groups. The data are expressed as the mean ± SEM. $^{**}p < 0.01$.

SK2 Channel Activator 1-EBIO Decreased the CRF Neuronal Firing Rates and Alleviated Visceral Hypersensitivity

To authenticate whether activation of SK2 channel mitigates visceral hypersensitivity in neonatal CRD rats, 1-EBIO (10 μg/0.3 μL) was injected (intra-PVN). 30 min after injection, 1-EBIO precluded the decrease of pain threshold. Notably, 1-EBIO even rescued the downregulation of membrane SK2 channel protein in neonatal CRD rats (**Figures 4A,B**). In addition, SK channel activator 1-EBIO (100 mM) reversed the increase of spontaneous firing rates of CRF neurons (**Figure 4C**). These results revealed that 1-EBIO could upregulate the activity of SK2 channel, thereby reducing the CRF neuronal excitability in PVN as well as alleviating visceral hypersensitivity.

To further explore the specific contribution of SK2 inhibition to visceral hypersensitivity in neonatal CRD rats, SK2 channel selective blocker apamin (6.25 pmol/0.3 μL) was administered (intra-PVN). We identified further reduction of membrane SK2 channel protein expression and the pain threshold in neonatal CRD rats (**Figures 4D,E**). Moreover, apamin increased the firing rates of CRF neurons (**Figure 4F**). These results indicated that SK2 inhibition precipitated visceral hypersensitivity in neonatal CRD rats.

Together, these findings demonstrated the potential of SK2 activator as an alternative agent for visceral pain.

PKA Activation Facilitated the Transfer of SK2 Channel Protein from the Cell Membrane into the Cytoplasm

On the grounds that SK2 channel regulates the pain threshold, we proceeded to explore the physiological basis for SK2 channel protein transfer from membrane into cytoplasm in neonatal CRD rats. Prior studies revealed that direct PKA phosphorylation of SK2 alters the distribution of SK2 channel proteins. Nevertheless, puzzles remain with respect to the potential correlations between PKA responses in CRF neurons and distribution of SK2 channel proteins as well as precipitation of behavioral abnormalities, such as visceral hypersensitivity in neonatal CRD rats. Intriguingly, no marked variations in the total PKA expression were evidenced (**Figure 5A**); whereas the expression of phosphorylated PKA in PVN was significantly increased in neonatal rats (**Figure 5B**). Intra-PVN administration of PKA agonist 8-Br-cAMP (100 mM, 0.1 μl) decreased membrane SK2 channel protein levels as well as pain threshold in neonatal CRD rats (**Figures 5C,D**). The results revealed that PKA was implicated in membrane translocation of SK2 channel in neonatal CRD rats.

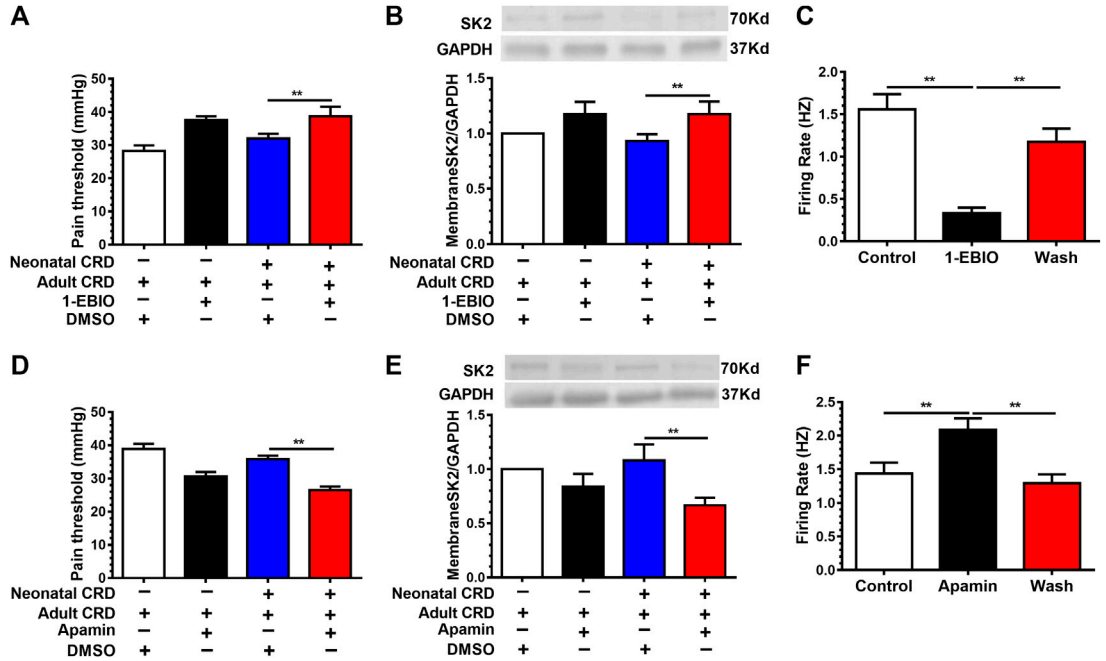

FIGURE 4 | SK2 channel activator 1-EBIO decreased the neuronal firing rates and alleviated visceral pain. Rats receiving intra-PVN injection of 1-EBIO (10 μg/0.3 μL) 30 min before behavioral tests. 1-EBIO prevented the decrease of **(A)** the pain threshold; **(B)** the membrane SK2 channel protein in rats undergoing neonatal CRD (n = 6 rats each, $F_{3,20} = 27.4$, $p < 0.0001$ in **A**; n = 6 rats each, $F_{3,20} = 5.387$, $p = 0.0070$ in **B**). **(C)** SK channel activator 1-EBIO (100 mM) reversed the increase of the CRF neurons firing rates (n = 10 cells each, $F_{2,27} = 41.91$, $p < 0.0001$). SK2 channel blocker apamin (6.25 pmol/0.3 μL) decreased **(D)** the pain threshold; **(E)** the expression of membrane SK2 channel protein in rats undergoing neonatal and adult CRD (n = 7 rats each, $F_{3,24} = 19.97$, $p < 0.0001$ in **D**; n = 6 rats each, $F_{3,20} = 8.901$, $p = 0.0006$ in **E**). **(F)** SK channel blocker apamin increased the CRF neurons firing rates (n = 9 cells each, $F_{2,24} = 7.95$, $p = 0.0022$). Significance was assessed by ordinary one-way ANOVA with post hoc comparisons between groups. The data are expressed as the mean ± SEM. $^{**}p < 0.01$.

DISCUSSION

In the present study, we validated that neonatal CRD contributed to visceral hypersensitivity. As per the assessment of the expression of SK2 channel protein that exhibited transfer from membrane into cytoplasm, and combination with the electrophysiological profiles of SK2 channel in CRF neurons, we postulated that decreased membrane SK2 expression precipitated abnormal neuronal physiology and visceral hypersensitivity. We also identified the efficacy of an SK2 activator in mitigating visceral hypersensitivity in neonatal CRD rats, demonstrating the potential of this approach as an intervention for visceral pain. Moreover, we disclosed PKA was the primary culprit for SK2 distribution anomalies in neonatal rats, which may provide novel targets for diseases caused by abnormalities of SK2 distribution including visceral pain.

Visceral hypersensitivity is among the key pathophysiological features of IBS as well as other disorders with visceral pain, for which no efficient therapies are available, thus rendering significant adverse effect on the quality of life (Wouters et al., 2012; Chey et al., 2015). Despite the obscure mechanism underlying visceral hypersensitivity, adverse early life stress may potentially contribute to the precipitation (Al-Chaer et al., 2000; Zhou et al., 2010; Larauche et al., 2012). Early life stress can predispose the developmental track of neuroendocrine system in the CNS, particularly the HPA axis, and precipitate the susceptibility to subsequent stressors and development of visceral hypersensitivity in adulthood (Ji et al., 2018). The hypothalamic CRF neurons are implicated in the activation of stress-induced HPA axis (Kusek et al., 2013), further supported by our prior report that visceral hypersensitivity is related to an increase of CRF mRNA and protein expression levels as well as CRF neuronal activation in PVN (Zhang et al., 2016a). Thus, it is essential to explore the CRF neuronal molecular mechanism underlying visceral hypersensitivity in PVN.

Our prior initial study focused on the first key relay stations of various visceral primary afferent information, i.e. dorsal horn (DH), and we found that a decrease in the number and function of membrane SK2 channels increased neuronal excitability in the spinal DH, leading to increased sensitivity to noxious stimuli and subsequent visceral hypersensitivity, which indicated the important role of SK2 in the ascending pathway (Song et al., 2018). Since it has been clarified that SK2 is involved in the regulation of chronic visceral pain, we want to further disclose the role of SK2 in the higher level key relay stations of various visceral primary afferent information and the hub of visceral sensation, i.e. PVN, and study the underlying mechanisms for regulating SK2 dynamic change in chronic visceral pain in this paper.

Coincidentally, we found that neonatal CRD rats presented with a downregulation of the membrane SK2 channel protein in PVN, and the expression of the membrane SK2 channel protein

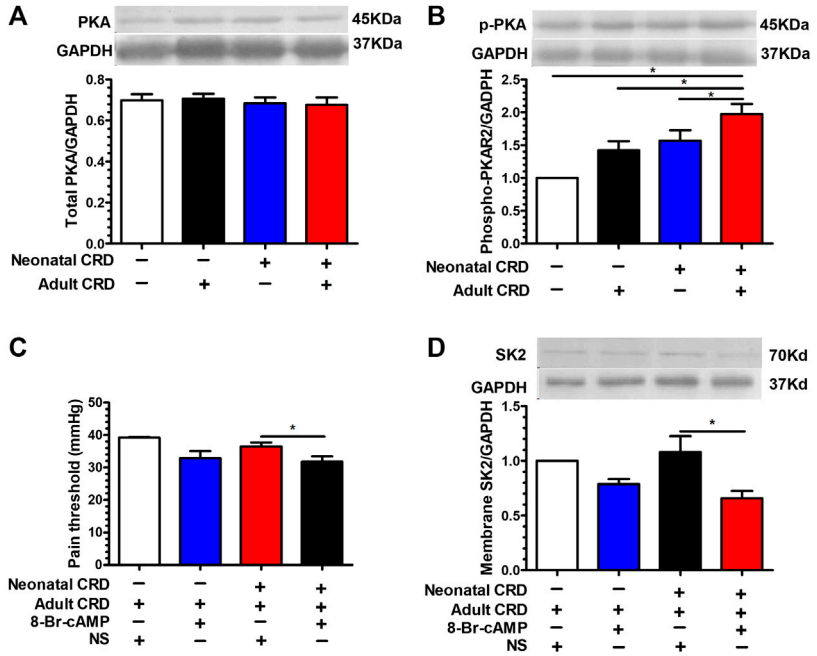

FIGURE 5 | PKA activation facilitated the transfer of SK2 channel protein from the cell membrane into the cytoplasm. **(A)** There was insignificant change in the total PKA expression (n = 6 rats each, $F_{3,20} = 0.3017$, $p = 0.8238$). **(B)** The expression of phosphorylated PKA in PVN was significantly increased in rats undergoing neonatal CRD vs. other groups (n = 6 rats each, $F_{3,20} = 30.44$, $p < 0.0001$). Intra-PVN administration of PKA agonist 8-Br-cAMP (100 mM, 0.1 μl) decreased **(C)** pain threshold as well as **(D)** membrane SK2 channel protein in Neonatal CRD group (n = 6 rats each, $F_{3,20} = 19.24$, $p < 0.0001$ in **C**; n = 6 rats each, $F_{3,20} = 15.33$, $p < 0.0001$ in **D**). Significance was assessed by ordinary one-way ANOVA with post hoc comparisons between groups. The data are expressed as the mean ± SEM. *$p < 0.05$.

was correlated with visceral hypersensitivity. Therefore, we hypothesized that SK2 channel in PVN is associated with visceral hypersensitivity. In addition, CRF neurons exhibited altered physiological properties, i.e. lower SK2 channel-mediated IAHP. Previous studies reported activation of SK2 channel is the prerequisite for the generation of medium afterhyperpolarization potential (mAHP) following single or multiple action potentials, leading to a decrease of the firing rate and inhibition of the excitatory of neurons (Pedarzani et al., 2005; Lu et al., 2009). Therefore, decreased SK2 channel-mediated IAHP may be attributed to the increased excitability of CRF neurons in PVN and aberrant modification of HPA axis in neonatal CRD rats with visceral hypersensitivity.

Given the contribution of the SK2 channel redistribution to visceral hypersensitivity, neonatal CRD rats should be well restored, provided SK2 is precluded from transference from the membrane into the cytoplasm. Indeed, this hypothesis was confirmed by the use of cell membrane protein transport inhibitor Dyn. This evidence further testifies the impact of abnormal SK2 distribution on visceral hypersensitivity, and may also render a therapeutic strategy for the diseases due to abnormal membrane protein internalization, including visceral pain. Our administration of SK2 channel activator 1-EBIO reversed the increase of neuronal firing rate in the CRF neurons in PVN and further relieved the visceral hypersensitivity in neonatal CRD rats, demonstrating the potential of this approach as an intervention for visceral pain.

Of note, membrane SK2 channel protein downregulation in neonatal CRD rats was not restricted to CRF neurons according to western blotting results. Despite our finding that SK2 channel activation in PVN mitigated the visceral hypersensitivity in neonatal CRD rats, whether SK2 channel activation in other neuronal populations can also serve as a therapeutic strategy for the treatment of neonatal CRD-induced visceral hypersensitivity awaits further exploration. Moreover, since 1-EBIO per se is not a selective SK2 activator, one cannot simply exclude the possibility of the involvement of other potassium channels in visceral hypersensitivity.

Nonetheless, the contributor of the downregulation of membrane SK2 channel protein in PVN remains unclear. Direct PKA phosphorylation of SK2 alters the distribution of SK2 channel proteins in the COS7 expression system, which may play a pivotal role in the regulation of the ImAHP and excitability of the nervous system (Ren et al., 2006; Abiraman et al., 2016). Activation of PKA leads to internalization of synaptic SK2 channel and enhances excitatory synaptic transmission and plasticity in amygdala and hippocampal neurons (Faber et al., 2008; Lin et al., 2008). Besides, activation of the cAMP-PKA signaling pathway in rat dorsal root ganglion and spinal cord contributes toward induction and maintenance of bone cancer pain (Zhu et al., 2014). Consistent with these reports, our present study confirmed that the PKA agonist 8-Br-cAMP increased the expression of phosphorylated PKA, which further downregulated the membrane SK2 channel

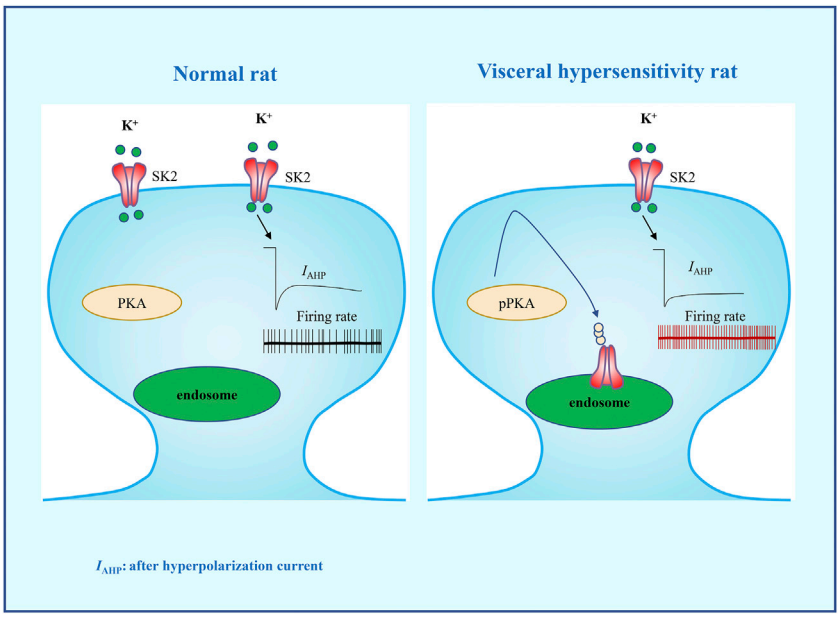

FIGURE 6 | A schematic diagram summarizing the hypothesis that PKA-regulated membrane SK2 channel in PVN precipitates neonatal CRD-induced visceral hypersensitivity. Activation of PKA facilitates the internalization of SK2 channel and the decrease of IAHP, thereby increasing the firing rate of CRF neurons in PVN, which precipitates neonatal CRD-induced visceral hypersensitivity.

protein and upregulated the cytoplasmic SK2 channel protein in PVN, facilitating the transfer of SK2 channel protein from the cell membrane into the cytoplasm, and reversely aggravated visceral hypersensitivity. A priori, CRD-induced visceral hypersensitivity decreased the membrane SK2 channel via the promoted PKA activity in PVN.

In this study, the redistribution of the SK2 channel in PVN participates in chronic visceral pain. The hypothalamic PVN integrates multiple afferents to precipitate autonomous output of pain and analgesia regulation (Wouters et al., 2012; Chen et al., 2014). SK channels, extensively distributed in the brain regions including PVN (Adelman et al., 2012; Deignan et al., 2012), modulate neuronal excitability, which further modulate neuropathic pain (Thompson et al., 2015). There is a wealth of evidence that the redistribution of the SK2 channel at the cell level is related to the phosphorylation of PKA in the membrane (Majekova et al., 2017; Authement et al., 2018). This study authenticated that visceral hypersensitivity is implicated in PKA-regulated membrane SK2 channel in PVN.

In conclusion, we evaluated the mechanism of the SK2 channels in PVN in the etiology of visceral pain. Our data confirmed that rats with visceral hypersensitivity present with a downregulation of membrane SK2 channel as well as functional inhibition in PVN, which may be related to PKA activation (**Figure 6**). Intra-PVN administration of SK2 channel activator 1-EBIO can reverse the visceral hypersensitivity. This study may provide an insight into the pathogenesis of visceral pain and pave a novel avenue for the development of therapeutic regimens for visceral pain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Xuzhou Medical University.

AUTHOR CONTRIBUTIONS

Participation in research design: Y-MZ and RH. Experimentation: N-NJ, LD, YW and KW. Data analysis: N-NJ and LD. Manuscript composition or revision: N-NJ, Z-YC, RH and Y-MZ. All authors contributed to the article and approved the submitted version.

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Analgesic Effects of Lipid Raft Disruption by Sphingomyelinase and Myriocin via Transient Receptor Potential Vanilloid 1 and Transient Receptor Potential Ankyrin 1 Ion Channel Modulation

Ádám Horváth[1,2], Maja Payrits[1,2], Anita Steib[1,2], Boglárka Kántás[1,2], Tünde Biró-Sütő[1,2], János Erostyák[2,3], Géza Makkai[2,3], Éva Sághy[1,2,4], Zsuzsanna Helyes[1,2] and Éva Szőke[1,2]*

[1]Deparment of Pharmacology and Pharmacotherapy, University of Pécs, Medical School, Pécs, Hungary, [2]János Szentágothai Research Centre and Centre for Neuroscience, University of Pécs, Pécs, Hungary, [3]Department of Experimental Physics, Faculty of Sciences, University of Pécs, Pécs, Hungary, [4]Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary

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***Correspondence:**
Ádám Horváth
horvatadam7@gmail.com

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Transient Receptor Potential (TRP) Vanilloid 1 and Ankyrin 1 (TRPV1, TRPA1) cation channels are expressed in nociceptive primary sensory neurons, and integratively regulate nociceptor and inflammatory functions. Lipid rafts are liquid-ordered plasma membrane microdomains rich in cholesterol, sphingomyelin and gangliosides. We earlier showed that lipid raft disruption inhibits TRPV1 and TRPA1 functions in primary sensory neuronal cultures. Here we investigated the effects of sphingomyelinase (SMase) cleaving membrane sphingomyelin and myriocin (Myr) prohibiting sphingolipid synthesis in mouse pain models of different mechanisms. SMase (50 mU) or Myr (1 mM) pretreatment significantly decreased TRPV1 activation (capsaicin)-induced nocifensive eye-wiping movements by 37 and 41%, respectively. Intraplantar pretreatment by both compounds significantly diminished TRPV1 stimulation (resiniferatoxin)-evoked thermal allodynia developing mainly by peripheral sensitization. SMase (50 mU) also decreased mechanical hyperalgesia related to both peripheral and central sensitizations. SMase (50 mU) significantly reduced TRPA1 activation (formalin)-induced acute nocifensive behaviors by 64% in the second, neurogenic inflammatory phase. Myr, but not SMase altered the plasma membrane polarity related to the cholesterol composition as shown by fluorescence spectroscopy. These are the first *in vivo* results showing that sphingolipids play a key role in lipid raft integrity around nociceptive TRP channels, their activation and pain sensation. It is concluded that local SMase administration might open novel perspective for analgesic therapy.

Abbreviations: CAPS, capsaicin; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; ECS, extracellular solution; Laurdan, 6-dodecanoyl-N,N-dimethyl-naphthylamine; MCD, methyl β-cyclodextrin; Myr, myriocin; PGE2, prostaglandin E2; RAMEB, random methylated β-cyclodextrins; RM, repeated measurement; RTX, resiniferatoxin; TRP, Transient Receptor Potential; TRPA1, Transient Receptor Potential Ankyrin 1; TRPC1, Transient Receptor Potential Canonical 1; TRPC3, Transient Receptor Potential Canonical 3; TRPV1, Transient Receptor Potential Vanilloid 1.

Keywords: lipid raft, myriocin, pain, sensory neuron, sphingomyelinase, transient receptor potential

INTRODUCTION

Cell physiology is influenced by lipid rafts, which are specialized cholesterol-, sphingomyelin- and ganglioside-rich plasma membrane microdomains, surrounding several receptors and ion channels (Simons and Ikonen, 1997). Membrane disintegration causes pathophysiological processes, such as neurodegeneration, neuropathy (Lee et al., 2014; Sonnino et al., 2014; Gambert et al., 2017) and synaptic transmission disruption (De Chiara et al., 2013). Modifying plasma membrane functions is a mechanism of action for pharmacological therapeutic interventions and/or side effects like the cytotoxic actions of anticancer drugs (Adinolfi et al., 2013), endocannabinoid-mediated analgesia (Rossi et al., 2012) and hyperalgesia (Dina et al., 2005). Our previous studies provided evidence that lipid raft disruption decreased the activation of Transient Receptor Potential (TRP) ion channels. These receptors are nonselective cation channels that open in response to temperature changes, binding of a broad range of exogenous and endogenous ligands, as well as other alterations of the channel protein (Gees et al., 2012; Vay et al., 2012). TRP "channelopathies" induce several diseases, such as skeletal muscle disorders, multiple kidney diseases and inherited pain syndrome (Moran et al., 2011; Nilius and Szallasi, 2014). Although pharmaceutical companies have put great efforts and investments into the development of TRP antagonists, only few drug candidates have reached the clinical stages of drug development (Szolcsányi and Sándor, 2012; Kaneko and Szallasi, 2014; Nilius and Szallasi, 2014).

TRP Vanilloid 1 (TRPV1) - expressed in the large population of polymodal nociceptors - is a nocisensor plasma protein gated by noxious heat (>43°C), protons (pH < 6.0), vanilloid-type agonists such as capsaicin (CAPS) and its synthetic analogs, resiniferatoxin (RTX), endogenous arachidonic acid or other fatty acid metabolites (Hwang et al., 2000; Smart et al., 2000; Welch et al., 2000; Raisinghani et al., 2005; Bianchi et al., 2006; Caterina and Park, 2006; Gavva, 2008; Myers et al., 2008; Szolcsányi, 2008; Cao et al., 2013; Aiello et al., 2016). Another similar TRP channel, TRP Ankyrin 1 (TRPA1), is often colocalized with TRPV1 on the CAPS-sensitive sensory nerves (Salas et al., 2009). Exogenous irritants, such as allyl-isothiocyanate (in mustard oil), cinnamaldehyde, allicin, 4-hydroxynonenal and mediators produced by inflammation or tissue injury, e.g., formaldehyde and methylglyoxal, as well as cold (below 17°C) and mechanical stimuli activate the TRPA1 receptor (Story et al., 2003; Bandell et al., 2004; Corey et al., 2004; Jordt et al., 2004; Macpherson et al., 2005, Macpherson et al., 2007; McNamara et al., 2007; Trevisani et al., 2007; Vilceanu and Stucky, 2010; Bautista et al., 2013; De Logu et al., 2019). Recent studies showed that both channels have some interactions with the endocannabinoid system and play an integrative role in regulating nociceptor and inflammatory functions (Akopian et al., 2008; Salas et al., 2009; Brizzi et al., 2014; Marrone et al., 2017). Pro-inflammatory neuropeptides, such as Substance P and calcitonin gene-related peptide (CGRP) released from the CAPS-sensitive fibres induce vasodilation, plasma protein extravasation and inflammatory cell activation (neurogenic inflammation) in the innervated area (Helyes et al., 2003, Helyes et al., 2009; Szolcsányi, 2004).

TRP channels are surrounded by lipid rafts of the plasma membrane modifying their functions (Liu et al., 2006; Morenilla-Palao et al., 2009; Szőke et al., 2010; Sághy et al., 2015). However, data are controversial about the outcomes of lipid raft disruption on TRP channel functions. Impaired TRP Canonical 1 (TRPC1) and TRP Canonical 3 (TRPC3) signaling was reported after methyl β-cyclodextrin (MCD) incubation depleting membrane cholesterol (Lockwich et al., 2000; Bergdahl et al., 2003; Graziani et al., 2006). In dorsal root ganglion neurons MCD treatment significantly reduced the CAPS-activated currents (Liu et al., 2006), but it had no effect on heat-evoked responses on TRPV1-transfected *Xenopus laevis* oocytes (Liu et al., 2003). MCD did not influence ^{3}[H]RTX binding to TRPV1 receptors on rat C6 glioma cells (Bari et al., 2005). Sphingomyelinase (SMase) hydrolyzes sphingomyelin (SM) to phosphocholine and ceramide (Kiyokawa et al., 2005; Kobayashi et al., 2006), thus influences the signaling through the cell membrane (Chao et al., 2011). The third mechanism to disrupt the lipid rafts besides MCD and SMase is to block the sphingolipid synthesis by inhibiting of serine palmitoyltransferase by myriocin (Myr) (Miyake et al., 1995).

Our previous results suggested that disrupting lipid rafts by pharmacologically depleting their various constituents, such as SM, cholesterol or gangliosides inhibited the CAPS-, and RTX-induced opening properties of TRPV1 and TRPA1 both on native sensory neurons and receptor-expressing cell lines (Szőke et al., 2010; Sághy et al., 2015). Fluorescence spectroscopy and filipin staining clearly supported the ability of MCD to deplete cholesterol from the cell membrane (Sághy et al., 2015). Besides the sensory neuronal cell bodies we also showed the ability of SMase to diminish TRPV1 and TRPA1 activation on the nerve terminals by measuring the release of the neuropeptide CGRP (Sághy et al., 2015).

Despite all these *in vitro* data showing that lipid raft disruption inhibits TRP channel activation, there are only few very recent reports investigating this phenomenon *in vivo*. Hyperalgesia responses in the RTX-evoked mouse neuropathy model (Lin et al., 2019) and prostaglandin E2 (PGE2) (Ferrari and Levine, 2015) administration were significantly attenuated by MCD. We recently reported the antihyperalgesic actions of MCD and a novel carboxamido-steroid compound in TRPV1 and TRPA1 activation-related mouse pain models (Horváth et al., 2020). Furthermore Myr exerted antitumor activity in a mouse melanoma model (Lee et al., 2011, Lee et al., 2012).

The aim of the present study is to examine the effects of SMase and Myr in mouse pain models of different mechanisms related to TRPV1 and TRPA1 activation.

MATERIALS AND METHODS

Ethics and Animals

Twelve- to Sixteen-week-old male NMRI mice were used in the formalin and RTX tests and male C57BL/6 mice of the same age in the CAPS-evoked wiping test. The animals were kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs. All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988). The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments and license was given (license no. BAI/35/702-6/2018).

Capsaicin-Evoked Acute Chemonocifensive Reaction

To characterize the effect of SMase and Myr - compared to the saline or dimethyl sulfoxide (DMSO) controls - on acute chemonociception, 30 μg/ml CAPS (20 μl, TRPV1 agonist) was instilled in the right eye of mice. Local pretreatments (20 μl) with 50 mU SMase or 1 mM Myr were performed 30 min or 24 h before the examination, respectively. CAPS-induced eye-wiping movements with the forelegs were counted in a 1-min period, as previously described (Szolcsányi et al., 1975; Szöke et al., 2002; Horváth et al., 2020). Only the one-leg movements were counted, washing-, or two-hand movements were not considered. CAPS administration was repeated in the second and third hours of the test.

Resiniferatoxin-Induced Thermal Allodynia and Mechanical Hyperalgesia

The effects of SMase and Myr were compared to the saline or DMSO controls in the RTX-induced (ultrapotent TRPV1 agonist) thermal allodynia and mechanonociceptive hyperalgesia model. RTX (0.1 μg/ml, 20 μl) was injected into right hindpaw of the animals, which evokes acute neurogenic inflammatory response with thermal allodynia and mechanical hyperalgesia (Meyer and Campbell, 1981; Pan et al., 2003). Baseline thermal- and mechanical threshold values were determined on two consecutive days, and these data were used for self-control comparisons. Intraplantar pretreatments (20 μl) with 50 mU SMase or 1 mM Myr were performed 30 min or 24 h before the RTX injection, respectively. RTX injection evoked an acute nocifensive reaction of paw licking, biting, lifting or shaking, but these behavioral changes lasted for less than a couple of minutes. The thermonociceptive threshold was measured by an increasing temperature Hot Plate (IITC Life Science, Woodland Hills, CA, United States) in the 10th, 20th and 30th min (Almási et al., 2003; Kántás et al., 2019; Horváth et al., 2020), and the mechanonociceptive threshold by a Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Italy) in the 30th, 60th and 90th min, as described earlier (Payrits et al., 2017; Kántás et al., 2019; Horváth et al., 2020).

Formalin-Evoked Acute Nocifensive Behavior

The effect of SMase and Myr compared to the saline- or DMSO-pretreated controls, were investigated on formalin-evoked (20 μl, 2.5%; into the right hindpaw) nocifensive behaviors. Intraplantar pretreatments (20 μl) with 50 mU SMase or 1 mM Myr were performed 30 min and 24 h before the examination, respectively. The duration of the nocifensive behaviors (hind paw licking, biting, shaking and holding) was monitored in two phase (0–5 min and 20–45 min) (Bölcskei et al., 2005; Horváth et al., 2020). The first phase is related to the direct chemical stimulation of nociceptors, e.g., TRPA1. There is a period of 10–15 min, when the animals show lack of nocifensive reactions. The second phase starts 15–20 min after the formalin injection, and it is referring to neurogenic inflammatory mechanisms. For more details see work of Tjolsen and co-workers (Tjølsen et al., 1992).

Fluorescence Spectroscopy to Determine Membrane Polarity Related to Lipid Raft Integrity

Native Chinese Hamster Ovary (CHO) cells were incubated with 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan) in 40 μM final concentration for 40 min at 37°C in a humidified atmosphere with 5% CO_2. The spectral shape and position of fluorescence emission and excitation spectra of Laurdan depend both on the speed of its dipolar relaxation and the polarity of its microenvironment in the membrane (Harris et al., 2002; Gaus et al., 2003). Cells were treated with 30 mU SMase or 100 nM Myr – dissolved in extracellular solution (ECS) - for 45 min at 37°C before Laurdan administration, then washed three times with phosphate-buffered saline (PBS) and scraped from the plates into 1 ml PBS.

Fluorescence excitation and emission spectra, excitation-emission matrices and anisotropy spectra, were measured by a HORIBA Jobin-Yvon Nanolog FL3-2iHR spectrofluorometer equipped with a 450-W xenon lamp. Samples were measured in a 4 mm path length quartz cuvette (Hellma 104F-QS) and kept at a constant 20°C using a Thermo Scientific circulating bath AC200-A25. Excitation-emission matrices consisting of a series of emission spectra recorded at different excitation wavelengths were measured to determine spectral changes. An excitation-emission matrix has one axis for the emission wavelengths, while the other includes the excitation wavelengths. At the intersection points, fluorescence intensity can be read as the value of the third axis. Steady-state emission anisotropy was measured in "L-format" arrangements to study the molecular mobility. Excitation was vertically polarized, while anisotropy was calculated from consecutively measured vertical and horizontally polarized emission intensities. Anisotropy $\langle r \rangle$ is defined as:

$$\langle r \rangle = \frac{I_{VV} - G^* I_{VH}}{I_{VV} + 2^* G^* I_{VH}},$$

where G is the spectrofluorometer's sensitivity factor given by:

$$G = \frac{I_{HV}}{I_{HH}},$$

where I_{HV} and I_{HH} are measured using horizontally polarized excitation and vertically and horizontally polarized emission, respectively. G value was automatically recalculated at each points of the anisotropy measurements.

Drugs and Chemicals

Myr from *Mycelia sterilia* (PubChem CID: 643894) (Sigma, St. Louis, MO, United States) was dissolved in DMSO (PubChem CID: 679) to obtain 5 mM stock solution. Further dilutions were made with ECS or DMSO to reach the final concentrations of 100 nM or 1 mM, respectively. SMase from *Bacillus cereus* (PubChem CID: 6476900) was purchased from Sigma in a glycerol buffered solution, and further dilutions were made with ECS or saline to reach the concentrations of 30 or 50 mU. CAPS (PubChem CID: 1548943) was purchased from Sigma and diluted with saline from a 10 mg/ml stock solution of 10% ethanol (PubChem CID: 702), 10% Tween 80 (PubChem CID: 5284448) in saline. RTX (PubChem CID: 5702546) was purchased from Sigma and was dissolved in ethanol to yield a 1 mg/ml stock solution. Further dilutions were made with saline to reach final concentrations of 30 and 0.1 μg/ml, respectively. Laurdan (PubChem CID: 104983) (Sigma) was dissolved in DMSO to obtain 10 mM stock solution, and further dilution was made with ECS to reach final concentration of 40 μM. Formalin (PubChem CID: 712) - dilution was made with PBS (PubChem CID: 24978514) to reach final concentration of 2.5% - was prepared from a 6% buffered formaldehyde stock solution (Molar Inc. Hungary).

Statistical Analysis

Fluorescence spectroscopy measurements were performed with four samples per group. All of the animal experiment data are presented as means ± SEM of six animals per group. Statistical analysis was performed by repeated measurement (RM) two-way ANOVA - the investigated factors were the pretreatment, time and their interaction (pretreatment x time) - with Bonferroni multiple comparisons post hoc test, in all cases $p < 0.05$ was considered statistically significant.

RESULTS

Sphingomyelinase and Myriocin Reduce the Number of Capsaicin-Evoked Eye-Wipings

The number CAPS-evoked eye-wiping movements within a 1-min period was 42.0 ± 1.8, 33.6 ± 1.7 and 28.0 ± 3.2 1, 2 and 3 h after local saline-pretreatment, respectively, in the control group showing desensitization in response to repeated CAPS administration. SMase pretreatment significantly decreased the number of wiping in the first hour, the corresponding values were 26.6 ± 2.5, 27.6 ± 2.2 and 24.6 ± 2.1 (**Figure 1A**).

Since Myr was dissolved in DMSO, this vehicle control was used in this experimental series. We also tested the DMSO in saline-pretreated animals compared to the DMSO-pretreated ones and we did not find significant difference (data not shown). In DMSO-pretreated animals CAPS administration induced 27.5 ± 2.8; 27.8 ± 1.4; 27.8 ± 2.4 eye wiping movements in the first, second and third hour, respectively. Myr pretreatment significantly diminished the number of eye-wipings in the first and second hour, these values were 16.2 ± 1.1; 19.0 ± 1.5; 23.5 ± 2.3, respectively (**Figure 1B**).

Sphingomyelinase and Myriocin Decrease the Resiniferatoxin-Induced Thermal Allodynia, and Sphingomyelinase Abolish Mechanical Hyperalgesia

The baseline heat threshold values of untreated mice were between 44 and 49°C. RTX induced 27.0 ± 2.8%; 20.9 ± 3.0% and 7.1 ± 3.8% drop of the thermonociceptive thresholds 10th, 20th and the 30th min after its intraplantar injection in the saline-pretreated control group. SMase pretreatment abolished the thermal allodynia in all measurement points with a decrease by 6.2 ± 1.8%; 0.8 ± 1.8% and an increase by 1.2 ± 0.4%, respectively (**Figure 2A**). The basal mechanonociceptive thresholds of intact mice were in a range of 9 and 10 g. RTX-evoked drop of the mechanonociceptive threshold values were 58.1 ± 3.9%, 55.6 ± 4.2%, 42.6 ± 2.5% 30, 60, and 90 min after the injection in the saline-pretreated control. SMase significantly alleviated the mechanical hyperalgesia in the 30th min, the respective values were 30.7 ± 2.5%, 39.4 ± 3.3%, 34.5 ± 8.2% (**Figure 2B**).

In case of Myr, the thermo- and mechanonociceptive threshold values of intact mice were between 44 and 49°C, 8 and 10 g, respectively. DMSO, as solvent of Myr was tested in saline-pretreated animals in contrast with DMSO-pretreated animals and significant desensitization effect was not revealed (data not shown). After RTX injection the thermonociceptive threshold in the DMSO-pretreated group decreased by 27.5 ± 3.2%; 21.0 ± 3.2% and 4.9 ± 3.8% in the 10th, 20th and 30th min, respectively, while Myr-pretreated group values decreased by 10.7 ± 5.2%; 6.3 ± 3.0% after 10 and 20 min its injection and increased by 5.5 ± 1.4% after 30 min. Myr pretreatment diminished the thermal allodynia in the 10th and 20th but not in the 30th min (**Figure 3A**). RTX-evoked mechanical hyperalgesia in the DMSO-pretreated control group were 43.9 ± 4.8%; 34.7 ± 4.3%; 28.6 ± 4.7% on the measurement points, compared to the Myr-pretreated group, these values were similar: 45.2 ± 8.7%; 25.4 ± 3.2%; 15.9 ± 6.9%. Myr pretreatment did not altered the mechanical hyperalgesia at any time point (**Figure 3B**).

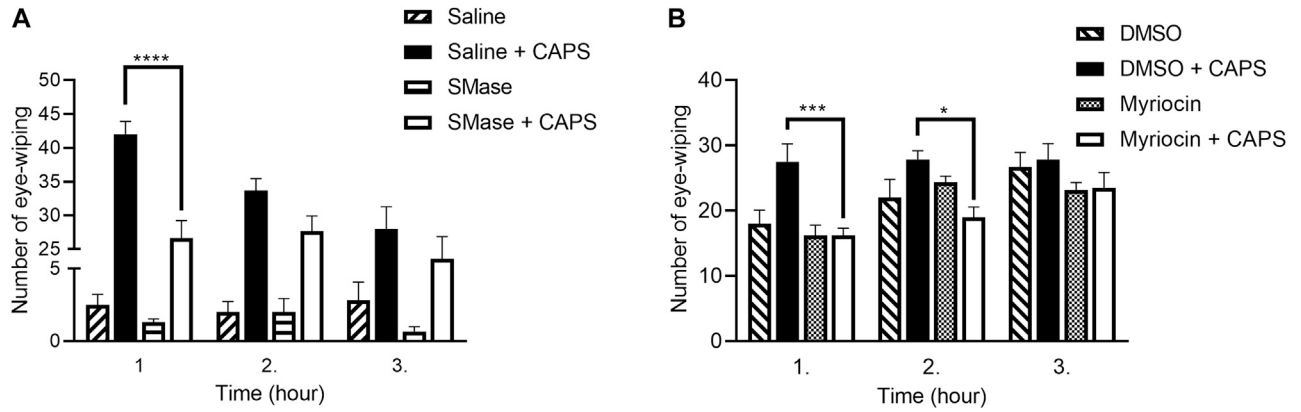

FIGURE 1 | Number of the eye-wiping movements in the CAPS-evoked acute chemonociceptive reaction. 50 mU SMase **(A)** and 1 mM Myr **(B)** also reduced the number of the eye-wiping movements. Data are means ± SEM of n = 6 animals/group. The corresponding F values of the pretreatment, time and their interaction were $F_{(3,20)} = 292.3$; $F_{(2,40)} = 5.240$; $F_{(6,40)} = 3.731$ in case of SMase pretreatment and $F_{(3,20)} = 8.182$; $F_{(2,40)} = 9.976$; $F_{(6,40)} = 1.598$ in case of Myr pretreatment, respectively. RM-Two-way ANOVA with Bonferroni post hoc test was used for statistical analysis (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ SMase/Myr pretreatment vs. saline/DMSO pretreatment).

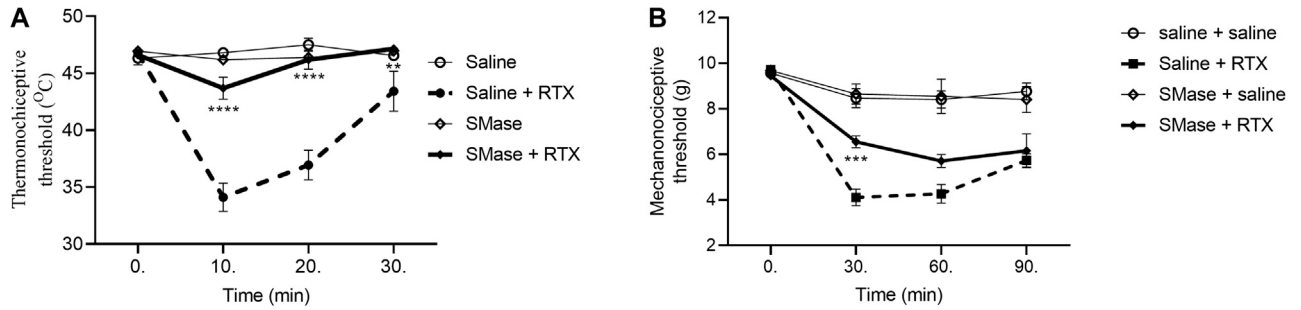

FIGURE 2 | Effect of 50 mU SMase in the RTX-induced thermal allodynia **(A)** and mechanical hyperalgesia **(B)**. Data are means ± SEM of n = 6 animals/group. Dashed and solid curves represent the saline and SMase pretreatments after RTX injection, respectively. The corresponding F values of the pretreatment, time and their interaction were F(3,20) 94.61; F(3,60) 19.76; F(9,60) 12.17 in the thermal allodynia and F(3,20) 22.88; F(3,60) 64.75; F(9,60) 9.859 in themechanical hyperalgesia respectively. RM-Two-way ANOVA with Bonferroni post hoc test was used for statistical analysis (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ SMase-pretreated group vs. saline-pretreated group).

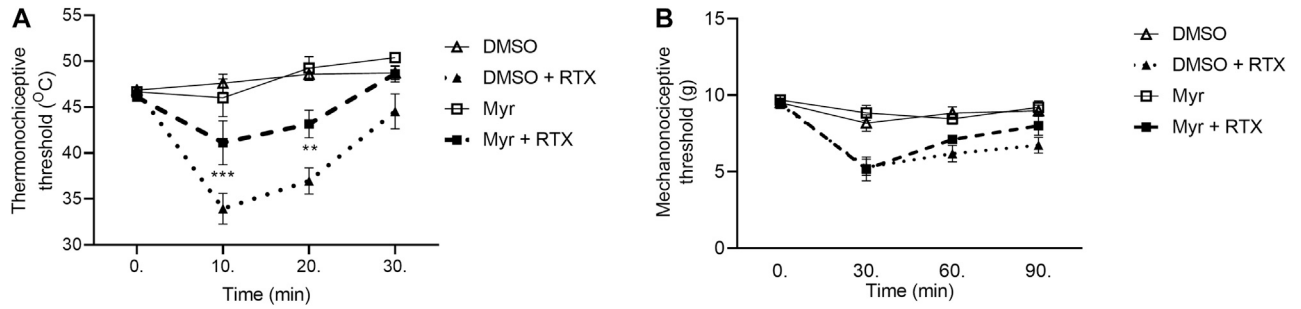

FIGURE 3 | Effect of 1 mM Myr in the RTX-induced thermal allodynia **(A)** and mechanical hyperalgesia **(B)**. Data are means ± SEM of n = 6 animals/group. Dotted and dashed curves represent the DMSO and Myr pretreatments after RTX injection, respectively. The corresponding F values of the pretreatment, time and their interaction were F(3,20) 20.80; F(3,60) 19.62; F(9,60) 6.617 in the thermal allodynia and F(3,20) 11.95; F(3,60) 35.96; F(9,60) 4.736 in the mechanical hyperalgesia respectively. RM-Two-way ANOVA with Bonferroni post hoc test was used for statistical analysis (**$p < 0.01$; ***$p < 0.001$ Myr-pretreated group vs. DMSO-pretreated group).

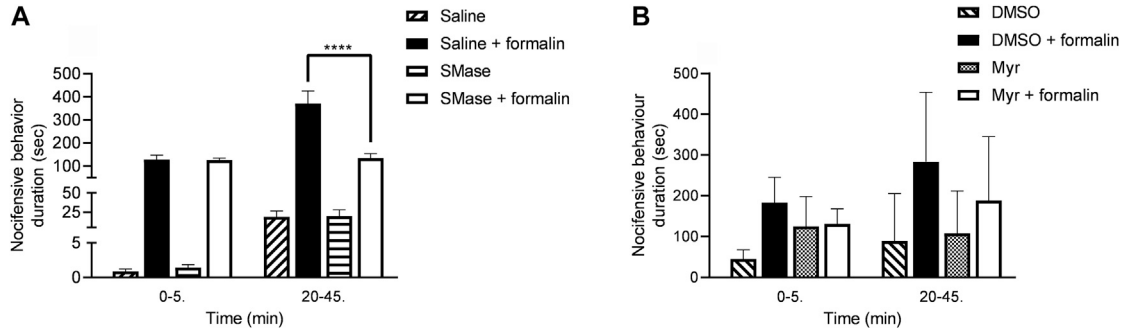

FIGURE 4 | Effects of 50 mU SMase **(A)** and 1 mM Myr **(B)** in the formalin-evoked acute nocifensive behaviors reaction. Data are means ± SEM of n = 6 animals/group. RM-Two-way ANOVA with Bonferroni post hoc test was used for statistical analysis (****$p < 0.0001$ SMase/Myr pretreatment vs. saline/DMSO pretreatment). The corresponding F values of the pretreatment, time and their interaction were $F_{(3,20)} = 37.02$; $F_{(1,20)} = 39.06$; $F_{(3,20)} = 24.68$ in case of SMase pretreatment and $F_{(3,20)} = 4.318$; $F_{(1,20)} = 3.032$; $F_{(3,20)} = 0.8396$ in case of Myr pretreatment, respectively.

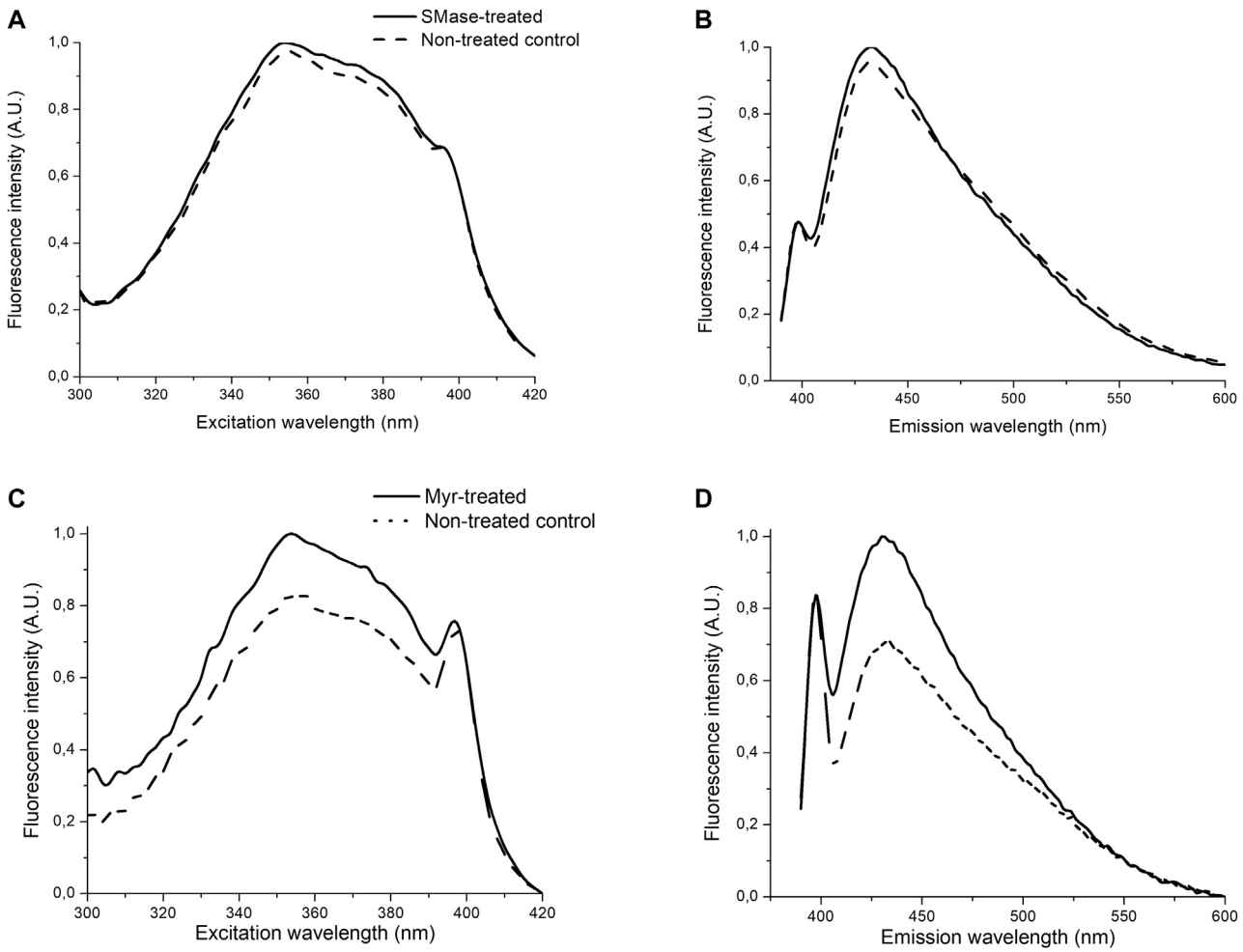

FIGURE 5 | Effects of 30 mU SMase or 100 nM Myr on normalized excitation- **(A,C)** or emission spectra **(B,D)** of Laurdan. Solid and dashed/dotted curves represent the values measured on the SMase/Myr-treated and non-treated control samples, respectively. Excitation wavelength: 350 nm, emission wavelength: 460 nm.

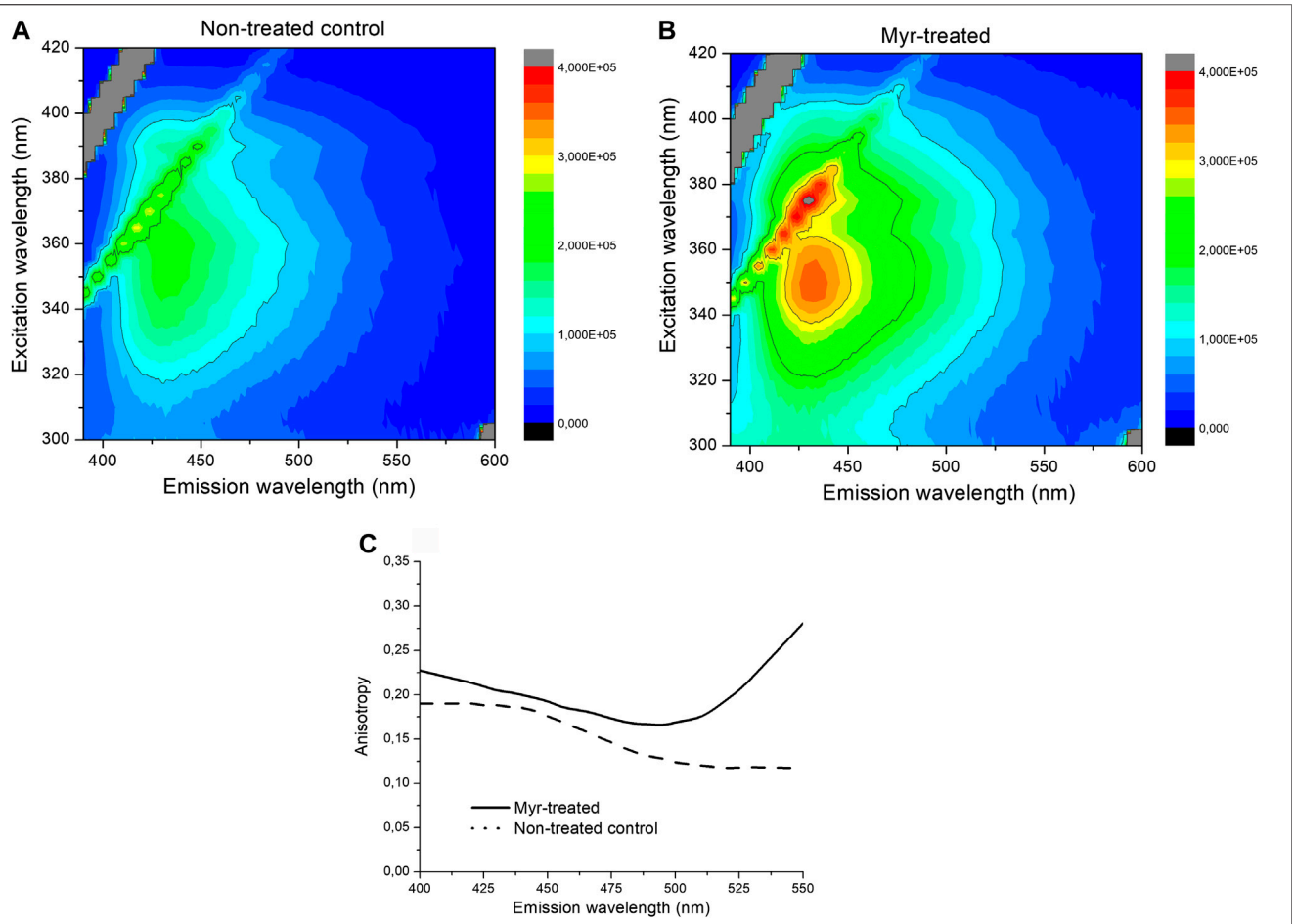

FIGURE 6 | Excitation-emission matricies of non-treated control **(A)** and 100 nM Myr-treated **(B)** samples, respectively. Emission anisotropy spectra of 100 nM Myr-treated (solid) and non-treated control (dotted) samples.

Sphingomyelinase Diminish Formalin-Evoked Acute Nocifensive Behaviors

The durations of formalin-induced paw lickings, liftings and shakings in the saline-pretreated control group were 127.2 ± 18.5 s and 371.1 ± 54.4 s in the first and second phases, respectively. SMase-pretreatment did not influence the acute chemonocifensive behavior in first phase due to direct activation of TRPA1 receptors on the sensory nerve endings, but induced significant inhibition in the second phase related to the acute neurogenic inflammatory reaction. The corresponding results were the following in the first and second phases, respectively: 125.9 ± 8.0 and 134.1 ± 20.1 s (**Figure 4A**).

In case of Myr, DMSO as its solvent was tested in saline-pretreated animals in contrast to DMSO-pretreated animals, and significant differences in the two groups was not found (data now shown). The formalin-evoked nocifensive behavior durations in the first phase were 183.1 ± 25.3 and 131.0 ± 15.2 s in the DMSO-pretreated control group and Myr-pretreated group, respectively. The durations of the second phase were 283.4 ± 69.4 and 188.7 ± 63.8 s in the DMSO control and Myr-pretreated animals. Myr had some biological effect, however this was not significant (**Figure 4B**).

Myriocin Changes the Membrane Polarity

SMase treatment did not influence the fluorescence spectroscopy picture as compared to the non-treated control. Spectral shift, broadening or changes in the shape of the spectra or intensity were not detected, as shown by no signal alterations of Laurdan. No changes were detected in the steady-state emission anisotropy measurements and transition from liquid-ordered to -disordered phase between the SMase-treated and non-treated cells (**Figures 5A,B**). In contrast to SMase, Myr treatment significantly modified the membrane microenvironment. The fluorescence emission was stronger in the Myr-treated samples than in the non-treated ones as shown by both excitation and emission spectra (**Figures 5C,D**). Excitation-emission matrices on the entire spectral region of Laurdan fluorescence also showed that the Myr-treated samples had higher fluorescence intensity than the non-treated control samples (**Figures 6A,B**). Emission anisotropy values of Myr-treated samples were significantly higher on the whole spectral range (**Figure 6C**).

DISCUSSION

We present here the first data on the analgesic effect of lipid raft disruption by SMase-induced SM hydrolization (Kiyokawa et al., 2005; Kobayashi et al., 2006) or Myr-induced glycosphingolipid synthesis blockade (Miyake et al., 1995). We proved that SMase and Myr decrease CAPS-evoked eye-wipings, as well as RTX-induced thermal allodynia, furthermore, SMase decreases the RTX-induced mechanical hyperalgesia and duration of the formalin-evoked -acute nocifensive behavior.

SMase and Myr significantly decreased TRPV1 activation-evoked eye-wiping movements by 37 and 41%, respectively. The decreasing response to repeated CAPS instillation was due to TRPV1 receptor desensitization (Sharma et al., 2013). Furthermore, SMase almost abolished TRPV1 stimulation-induced thermal allodynia and significantly reduced mechanical hyperalgesia. Myr had significant inhibitory effect on thermal allodynia induced predominantly by peripheral sensitization mechanisms, but not mechanical hyperalgesia involving central sensitization processes as well (Pan et al., 2003). TRPA1 activation-evoked acute neurogenic inflammatory nocifensive reactions were also significantly diminished by SMase (by 64%), while only non-significant decreasing tendency was induced by Myr.

These novel *in vivo* findings are well supported by our previous *in vitro* results demonstrating the ability of SMase and Myr to significantly and concentration-dependently inhibit TRPV1 receptor activation both on TRPV1 receptor-expressing cell line and primary cultures of trigeminal sensory neurons, similarly to MCD (Szőke et al., 2010). Furthermore, similar inhibitory actions of these lipid raft disruptors were described on the TRPA1, TRP Melastatin 8, but not the TRP Melastatin 3 cation channels (Sághy et al., 2015).

We provide the first direct evidence by fluorescence spectroscopy for the ability of Myr to induce transition from liquid-ordered to liquid-disordered phase indicating cholesterol depletion in the plasma membrane. Higher fluorescence intensity means less non-radiative processes of Laurdan and higher anisotropy reflects to more restricted motion of Laurdan after Myr treatment. These findings together indicate a more compact, closed membrane structure around Laurdan molecules. In contrast, SMase treatment did not influence the membrane polarity. We have previously proved that fluorescence spectroscopy is an appropriate technique to determine the plasma cholesterol content by comparing this method with the conventional filipin staining after MCD treatment in the same cell cultures (Sághy et al., 2015).

The formation of lipid rafts in the plasma membrane depends on the interaction of SM and cholesterol (Simons and Ikonen, 1997; Ridgway, 2000). Cholesterol content of the lipid membrane does not affect significantly the biosynthesis of SMs being involved in a variety of essential cellular functions (Ridgway, 2000). It has been reported that SM and cholesterol synthesis are independent processes in cultured human intestinal cells, but the amount of cholesterol and SM regulate their synthesis rates (Chen et al., 1993). It has been described that orally administered Myr decreases not only SM, but also cholesterol levels of the small intestinal epithelial cell plasma membrane (Li et al., 2009).

Although results of *in vitro* experiments provided evidence, that lipid raft disruption inhibited the activation mechanisms of TRP channels (Szőke et al., 2010; Sághy et al., 2015), there are only few recent animal experiments to prove this phenomenon *in vivo*. Cholesterol depletion by MCD induced antinociception in RTX-induced neuropathy in the mouse through phosphatidylinositol 4,5-bisphosphate hydrolysis (Lin et al., 2019). Furthermore, MCD and a novel carboxamido-steroid compound were able to exert antinociceptive effects by decreasing the activation of TRPV1 and TPRA1 ion channels in mice in distinct pain mechanisms (Horváth et al., 2020). The mechanical hyperalgesia induced by PGE2, but not cyclopentyladenosine was attenuated by MCD injection into the rat paw, suggesting that PGE2-evoked hyperalgesic effect is dependent on the lipid raft integrity (Ferrari and Levine, 2015). Complete Freund's adjuvant-induced thermal and mechanical hyperalgesia was attenuated by both local and systemic administration of random methylated β-cyclodextrins (RAMEB) in rats. The authors suggest that RAMEB capture the prostaglandin content and then decrease the inflammatory pain which might be a novel anti-inflammatory and analgesic tool (Sauer et al., 2017). In a very recent paper, the role of another components of lipid rafts, the gangliosides have been discussed in pain mechanisms (Sántha et al., 2020). Intraplantar injection of the ganglioside GT1b induced nociceptive responses and augmented formalin-evoked nocifensive reactions. Nevertheless, sialidase injection cleaving sialyl residues from gangliosides is able to diminish the nociceptive responses (Watanabe et al., 2011; Sántha et al., 2020).

The role of sphingolipids in pain sensation via the modulation of TRP channel activation is poorly understood and we had only *in vitro* experimental results (Szőke et al., 2010; Sághy et al., 2015). The present results are the first *in vivo* data providing clear evidence that sphingolipids play an important role in both TRPV1 and TRPA1-evoked nocifensive behaviors and hyperalgesia. Intraplantar pretreatments by both SMase and Myr inhibited thermal allodynia in the acute neurogenic inflammation model that develop mainly by peripheral sensitization of the sensory nerves. Furthermore, SMase injection into the paw even decreased mechanical hyperalgesia induced by both peripheral and central sensitization mechanisms (Pan et al., 2003), demonstrating a stronger inhibitory effect on the pseudo unipolar primary sensory neuron as compared to Myr. This observation together with the fact that SMase does not influence the membrane cholesterol composition support our conclusion on the key importance of sphingolipids over cholesterol in lipid raft integrity around TRPV1 and TRPA1 and consequent sensory nerve activation.

These *in vivo* results strongly suggest that membrane sphingolipid modification particularly by SMase might open novel analgesic opportunities. This innovative approach potently inhibits the activation of the sensory nerves via targeting pain sensing structures including TRPV1 and TRPA1. This might be particularly promising since it is more likely to inhibit the pathological activation, but not the

physiological functions of TRPV1 and TRPA1. Moreover, other main advantages are the opportunity for local administration and its rapid onset of action. Therefore, SMase seems to be a promising therapeutic tool with a good side effect profile (e.g. it could avoid the hyperthermic side effects of TRPV1 antagonists).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Research of Pécs University.

AUTHOR CONTRIBUTIONS

Conceptualization ÉSz and JS; methodology ÁH, BK, TB-S, MP, ÉSá, GM, JE, AS; formal analysis ÁH; investigation ÁH, BK, TB-S, MP, ÉSá, GM, JE, AS; resources ZH, ÉSz; writing—original draft preparation, ÁH; writing—review and editing ÉSz, ZH; visualization ÁH; supervision ÉSz, ZH; project administration ÁH, TB-S; funding acquisition ZH, ÉSz.

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REVIEW
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Addressing Pain for a Proper Rehabilitation Process in Patients With Severe Disorders of Consciousness

F. Riganello[1]*, *A. Soddu*[2] *and P. Tonin*[3]

[1]Research in Advanced NeuroRehabilitation, Istituto Sant'Anna, Crotone, Italy, [2]Department of Physics and Astronomy, Brain and Mind Institute, Western University, London, ON, Canada, [3]Research in Advanced NeuroRehabilitation, Istituto Sant'Anna, Crotone, Italy

Consciousness constitutes a fundamental prerequisite in the individual appraisal and experience of pain. In the same way, a person needs to be able to report on pain perception. Patients who suffered a severe brain injury with disorders of consciousness (DOC) represent a spectrum of pathologies affecting patients' capacity to interact with the external world. In these patients, the most relevant aspects in response to pain are physiologic and behavioral. The treatments and management of pain are challenging issues in these patients, arising serious ethical concerns and bringing emotional load among medical staff, caregivers, and relatives. In this review, we report the importance of having a correct pain management in DOC patients, to individuate the best pharmacological treatment that can make the difference in detecting a behavioral response, indicative of a change in the level of consciousness, and in planning a more effective rehabilitative approach.

Keywords: disorders of consciousness, pain, rehabilitation, assessment, pharmacotherapeutic approaches

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***Correspondence:**
F. Riganello
f.riganello@istitutosantanna.it

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INTRODUCTION

In 1979, the IASP approved the following definition of pain: *"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"* coupling the sensory and emotional dimensions of the experience, as well as the association between tissue injury and pain (IASP, 1979).

The emotional experience can be described by a complex system of interacting processes characterized by affective (i.e., subjective experienced feeling), expressive (e.g., mimics, behaviors), cognitive (e.g., thoughts), and physiological (e.g., heart rate) components (Scherer et al., 2001).

In 1999 McCaffrey and Pasero reported a similar definition: "Pain is *whatever the experiencing person says it is, existing whenever* the experiencing person *says it does*" denoting the subjectivity of the pain experience (McCaffrey and Pasero, 1999, p 63). Such definition implies not only that pain may be detected when a patient reports its manifestation but that consciousness constitutes a fundamental prerequisite in the individual appraisal and experience of pain. In 2007, at the Kyoto annual meeting, the publication of the modification of the IASP Basic Pain Terminology (Loeser and Treede, 2008) was approved, with the introduction of the terms nociceptive neuron, nociception, nociception stimulus, nociceptive pain, sensitization, peripheral and central sensitization (**Table 1**). Independently from a more accurate terminology, a key aspect of pain remains the subjective experience and the necessity to

TABLE 1 | International association for the study of the pain–terminology.

Pain: An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage.
• Pain and nociception are different phenomena. Pain cannot be inferred solely from activity in sensory neurons
• Pain is always a personal experience that is influenced to varying degrees by biological, psychological, and social factors
• Verbal description is only one of several behaviors to express pain; inability to communicate does not negate the possibility that a human or a nonhuman animal experiences pain

Nociception	The neural process of encoding noxious stimuli	Pain threshold	The minimum intensity of a stimulus that is perceived as painful
Nociceptive neuron	A central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli	Pain tolerance level	The maximum intensity of a pain-producing stimulus that a subject is willing to accept in a given situation
Nociceptive pain	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors	Paresthesia	An abnormal sensation, whether spontaneous or evoked
Nociceptive stimulus	An actually or potentially tissue-damaging event transduced and encoded by nociceptors	Sensitization	Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs
Nociceptor	A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli	Central sensitization	Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input
Noxious stimulus	A stimulus that is damaging or threatens damage to normal tissues	Peripheral sensitization	Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields
		Central neuropathic pain	Pain caused by a lesion or disease of the central somatosensory nervous system
		Peripheral neuropathic pain	Pain caused by a lesion or disease of the peripheral somatosensory nervous system
		Nociplastic pain	Pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain

Pain assessment in non-communicative patients.

report on it ("*. . .the experiencing person says it is . . .* "). The importance of reporting on the pain sensation is described in a study by Clarke and colleagues on the chronic pain in older adults, recommending the narrative approach to describe and discuss the experience of pain. If this approach could represent an useful tool to assess pain in subjects who are able to refer on it (Clarke et al., 2012), it highlights the issues in assessing pain in non-communicative patients.

Nociception Versus Pain

In the assessment of non-communicative patients, it is essential to discriminate a reflex from higher-order behavioral responses.

Noxious stimulation implies a response of the Autonomic Nervous System (ANS). Typical physiological responses are observable in the cardiovascular reactivity, respiration, skin conductance and pupil dilatation (Kyle and McNeil, 2014; Mischkowski et al., 2018).

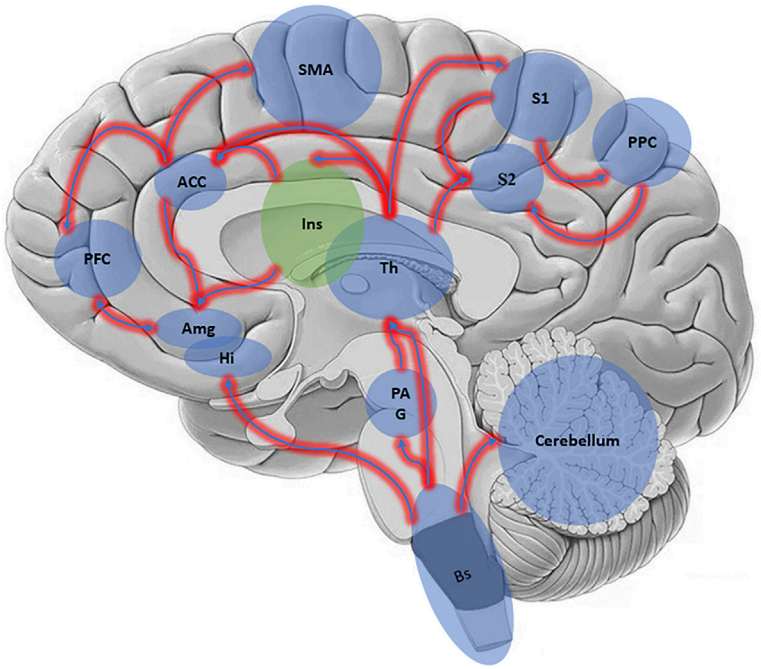
The nociception (i.e., the neural process of encoding noxious stimuli) refers to the perception (conscious or not) of nociceptive stimuli (an actually or potentially tissue-damaging event transduced and encoded by nociceptors) (Loeser and Treede, 2008), eliciting the activation of an extensive cortical network (i.e. somatosensory, insular, and cingulate areas, as well as frontal and parietal areas) (Coghill et al., 2003; Chatelle et al., 2014). The transmission of the information of the nociceptive stimulation follows the *via* spinothalamic tract to reach the thalamus and the cortex (Loeser and Treede, 2008; Morton et al., 2016). The reflex response is thought to be modulated by midbrain and thalamus (Morton et al., 2016), while part of the sensory–discriminative features of the pain processing entails the secondary somatosensory (S2) cortex, with the posterior insula (lateral network) (Ploner et al., 2002; Lockwood et al., 2013).

The conscious experience of pain requires a more complex network, generally called *Pain Matrix* (Iannetti and Mouraux, 2010; Salomons et al., 2016) (**Figure 1**).

The Pain Matrix involves two main subsystems: the Lateral Neuronal Network (LNN) and the Medial Network (MN). The LNN encompassing S2 cortex, lateral thalamus, and posterior insula (Mutschler et al., 2011) encodes the sensory discriminative information; the MN encompassing anterior cingulate cortex (ACC) and prefrontal cortex encodes affective-cognitive information (Medford and Critchley, 2010). Also, the cerebellum (Moulton et al., 2011) and motor areas (e.g., the striatum, cerebellum, and the supplementary motor area) (Barceló et al., 2012) are involved in pain perception and processing.

Several studies investigating the dynamics of activation (connectivity) of the pain matrix have shown that nociceptive input is first processed in the posterior insula, wherein it is coded in terms of intensity and anatomical location, and then transmitted to the anterior insula, where the emotional reaction to pain is elaborated (Ploner et al., 2002; Tracey, 2008; Frot et al., 2014).

For the "mind-body" theory, the pain experience necessitates of a body and a mental component (Duncan, 2000). The first encompasses the phenomena leading to perception and response,



Pain Matrix:

- **ACC**: Anterior Cingulate Cortex
- **Amg**: Amygdala
- **Bs**: Brainstem
- **Hi**: Hippocampus
- **Ins**: Insula
- **PAG**: Periaqueductal gray
- **PPC**: Posterior Parietal Cortex
- **PFC**: Prefrontal Cortex
- **S1**: Primary Somatosensory Cortex
- **S2**: Secondary Somatosensory Cortex
- **Th**: Thalamus
- **Cerebellum**

FIGURE 1 | Simplified scheme of the Pain Matrix.

such as pain pathway and central processing, while the second encompasses perception and interpretation of pain, including the cognitive and affective components (Sarno, 2001). The mind-body approach shows the impossibility to separate mind and body in the pain experience, then the importance of self-report. Nevertheless, the IASP stated that "Verbal description is the only one of several behaviors to express pain; inability to communicate does not negate the possibility that a human or a nonhuman animal experiences pain" (IASP, 2020).

In non-communicative patients, the most relevant aspects in response to pain are physiologic (i.e., modification in the vital parameters such as heart rate and respiration) and behavioral (i.e., modification in the facial expression, motor and visual response).

To assess pain in non-communicative patients several behavioral scales were developed, with each of them oriented to assess a specific typology of patients. As an example, the Behavioral Pain Scale (Payen et al., 2001) which is commonly used in trauma or post-operative care unit to assess pain in critically sedated and mechanically ventilated patients; the Faces, Legs Cry and Consolability scale (FLACC) (Merkel et al., 2002; Malviya et al., 2006), which was developed for the pediatric population to measure pain severity; or the Pain Assessment in Advanced Dementia scale (PAINAD) (Warden et al., 2003), developed for patients affected by dementia.

Disorders of Consciousness (DOC) represent a spectrum of pathologies affecting the capacity of patients to interact with the external world. It can be either due to a traumatic or a non-traumatic cause and sometime to a combination of both (Giacino et al., 2018).

Among the different definition of consciousness, the most accepted viewpoint refers to the brain's ability to form cognition of the world, by the perception of self and the environment. A requisite for conscious behaviors is the presence of adequate arousal (i.e., wakefulness) and awareness of content (i.e., sensory, cognitive, and affective experience) (Giacino et al., 2018). The first is referred to the level of consciousness and the second to the content of consciousness (Xie et al., 2017; Giacino et al., 2018).

The two possible conditions following the acquired brain injury (i.e. a terrible event disrupting the arousal and awareness systems, mediated respectively by the brainstem and cortex) are either the Vegetative State/Unresponsive Wakefulness Syndrome (UWS/VS) or the Minimally Conscious State (MCS) (Giacino and Kalmar, 2005; Laureys et al., 2010; Giacino et al., 2018). The first is characterized by spontaneous opening of the eyes and no sign of consciousness, with only residual reflexive responses to external stimuli; the second by minimal but discernible signs of non-reflex behaviors (i.e., response to visual, auditory, tactile, or noxious stimuli) which occur in a reproducible even if inconsistent manner (Giacino et al., 2002; Schnakers et al., 2009).

The clinical assessment is based on clinical consensus and behavioral scales such as the Coma Recovery Scale (Giacino et al., 2004; Seel et al., 2010).

For the assessment of pain in patients with DOC a specific scale, the Nociception Coma Scale (Schnakers et al., 2010; Riganello et al., 2014) has been developed. It is based on the observation of the motor response (non/flaccid, abnormal

posturing, flexion withdrawal, and localization to noxious stimulation), verbal response (non-verbalization, groaning, vocalization, and intelligible verbalization), visual response (none, startle, eyes movement and fixation) and facial expression (non-oral reflexive/startle response, grimace and cry), following a noxious stimulation (i.e., pressure on the fingernail bed using an algometer). Each subscale ranges from 0 (no response) to 3 (appropriate response), for a total score ranging from 0 to 12. A revised version, characterized by the absence of the visual subscale, was developed by Chatelle et al. (2016), but the two versions maintain the same clinimetric properties (Vink et al., 2017). Higher values for these scales indicate a more complex response to the noxious stimulus and content of consciousness.

A study by Sattin et al. (2018) reported lower pain pressure thresholds in DOC patients compared to healthy participants suggesting further investigations. Hyperestesia, hypoesthesia and anesthesia, conditions frequently present after acquired brain injury, may in fact alter responses to pain stimuli. Formisano and colleagues (Formisano et al., 2020) proposed for the evaluation of the response to painful stimuli by NCS and NCS-R, different and personalized stimuli (e.g., hand opening, upper limb abduction, head mobilization), because altered pain pathway may affect the searched responses by standard pressure on the fingernail bed.

Pain and Consciousness in Disorders of Consciousness Patients

The treatments and management of pain is a challenging issue in patients with DOC. The condition of suffering in DOC patients is a very controversial question. Generally, caregivers and relatives believe in the possibility that VS/UWS patients might feel pain, influencing end-of-life decisions. However, there is not a unanimous consensus about whether non-responsive patients might have a sufferance condition or might feel pain (Demertzi et al., 2013; Demertzi, 2018), implying increasing ethical questions (Riganello et al., 2016).

Neuroimaging studies have shown different processing of pain between UWS/VS and MCS patients (Boly et al., 2008; Chatelle and Thibaut, 2014; Garcia-Larrea and Bastuji, 2018). In a seminal Oxigen 15 ($O\text{-}H_2O$) PET study, pain induced activation of the midbrain, contralateral thalamus, and primary somatosensory cortex in UWS/VS patients (Laureys et al., 2002). Kassubek and colleagues, using the same PET technique in DOC patients, found the activation of the secondary somatosensory cortex, in the cingulate cortex contralateral to the stimulus, and the posterior insula ipsilateral to the stimulus (Kassubek et al., 2003). These findings suggest that DOC patients might have a residual perception and partial sensory-discriminative pain processing. However, the activation of the pain network resulted incomplete, with the primary somatosensory cortex functionally disconnected from the secondary somatosensory, bilateral posterior parietal, premotor, polysensory superior temporal, and prefrontal cortices (Laureys et al., 2004). The isolation of primary cortical activation from higher-order associative cortical activity suggests a non-integrated pain processing with a consequent less conscious experience (Boly et al., 2008).

Compared to the UWS/VS patients, MCS patients present higher metabolism in associative areas, principally in the precuneus/posterior cingulate cortex (Laureys et al., 2005), and a restoration of the correlation between these areas and the thalamus (Laureys et al., 2000). Boly and colleagues found similar brain area activation to noxious stimuli in MCS patients compared to controls (Boly et al., 2008) (i.e., thalamus, the primary somatosensory cortex, the secondary somatosensory cortex or insula, the posterior cingulate cortex/precuneus, and the anterior cingulate area). Another fMRI study performed by Markl and colleagues (Markl et al., 2013), demonstrated the significant activation of the sensory and affective components of the pain matrix in patients clinically diagnosed as UWS/VS, suggesting the possibility of a painful experience in some of these patients.

The neuroimaging, although it is a powerful tool of investigation, remains a complicated, expensive, time-consuming approach and of difficult use in the routine of clinical practice. In this frame, the behavioral pain assessment is still widely recognized as the most accessible and easiest approach. However, the risk of misdiagnosis remains high, considering that patients with DOC might not show any overt response to painful stimulation even if perceived (Schnakers et al., 2012; Chatelle and Thibaut, 2014; Calabrò et al., 2017; Cortese et al., 2020).

Different approaches of investigation based on Heart Variability Analysis (HRV), Galvanik Skin Response (GSR), or Laser Evocated Potential (LEP) have shown the possibility to observe pain processing in UWS/VS patients (de Tommaso et al., 2015; Riganello et al., 2018a; Cortese et al., 2020).

HRV is the fluctuation in the time intervals between adjacent heartbeats (interbeat interval - IBI) and represents the output of a complex brain-heart two-way interaction system (Riganello et al., 2012). The Central Autonomic Network (CAN), an integrative model where neural structures and heart function are involved and functionally linked in the affective, cognitive and autonomic regulation, describes this interaction (Benarroch, 2007; Thayer and Lane, 2009; Riganello, 2016). The principal neural structure of the CAN cover the brainstem (periaqueductal gray matter, nucleus ambiguous, and ventromedial medulla), limbic structures (amygdala and hypothalamus), prefrontal cortex (anterior cingulate, insula, orbitofrontal, and ventromedial cortex) and cerebellum (Benarroch, 2006, 2007; Lane et al., 2009; Thayer and Lane, 2009).

To describe the sympathovagal modulation, the HRV is generally analyzed in the time and frequency domains (Berntson et al., 1997). However, the physiological phenomena that characterize the biological events are dynamic and complex (Billman, 2011). For this reason, the non-linear analysis represents a useful approach to understand the brain-heart two-way interaction (Riganello, 2016). The HRV entropy quantifies the unpredictability and complexity of the IBI series. Higher and lower entropy indicate respectively higher or lower unpredictable IBI sequence, and correspondingly a higher or lower Heart-Brain two-way interaction (Riganello

et al., 2018b). In a study based on noxious and non-noxious stimuli, lower HRV entropy was observed in UWS/VS compared to MCS patients and lower in MCS patients compared to healthy controls (Riganello et al., 2018a). Cortese and colleagues, through the GSR and HRV entropy measures, observed a trace conditioning of the nociceptive stimulus (i.e., a conditioning protocol where the Conditioned Stimulus - pain - is presented, terminated, and followed after some intervening period by the Unconditioned Stimulus - Music -) in patients diagnosed as UWS/VS and without any oriented or reflex behavioral response to the nociceptive stimulation (Cortese et al., 2020). The trace conditioning is considered an appropriate method to assess consciousness's presence without a verbal report (Bekinschtein et al., 2009). The GSR is an indicator of psychological or physiological arousal, measured by the skin conductance that is controlled by the sweat glands, that are controlled by the sympathetic nervous system (Critchley et al., 2000; Cernat et al., 2017). The GSR signal, used to observe the presence of the trace conditioning, was observed only in patients with UWS/VS who changed the level of consciousness within thirty days from the first assessment (suggesting the possibility in these patients to perceive and learn the pain stimulus). Moreover, the HRV entropy was higher in these patients compared to those that remain with the diagnosis of UWS/VS (Cortese et al., 2020).

In two different LEP studies, authors found that brain-injured UWS/VS patients might process the painful stimuli (de Tommaso et al., 2013; de Tommaso et al., 2015). In a subsequent study by Naro et al. (2016) on MCS and UWS/VS patients, authors reported the modulation of the γ-band oscillation power induced by nociceptive repetitive laser stimulations and its correlation with the NCS-R. The results showed a strong positive correlation between γ-band oscillation power and NCS-R in all MCS and some of the UWS/VS patients, suggesting that, also in the presence of a lower NCS-R total score, the UWS/VS patients may have had a covert pain's experience. In a successive study, Calabrò and colleagues found γ-oscillations within the limbic system related to pain perception in some of the screened UWS/VS patients, evidencing that they might have perceived the affective component of pain (Calabrò et al., 2017).

Pain in Disorders of Consciousness and Treatment

The above-cited results put in evidence two relevant points: firstly, the assessment of nociceptive stimulation as mean to detect possible content of consciousness in patients diagnosed as UWS/VS; secondly, also if not capable of exhibiting oriented behavior to the painful stimuli, UWS/VS patients might perceive pain. In a recent work, Cortese and colleagues (Cortese et al., 2020) showed that the increase of the score in the NCS anticipates the increase of the score in the CRS-R. This finding highlights the importance of pain assessment in these patients, and how the behavioral response to pain could precede other responsive behavioral aspects. However, the oriented behavioral response to the nociceptive stimuli could be covered by a necessary pharmacotherapy for the treatments of the suspected pain condition (Pistoia et al., 2015).

Pain could be present in the acute phase and in the successive period of intensive rehabilitation (Schnakers et al., 2012; Schnakers and Zasler, 2015). The cause of pain might arise from multiple factors such as skin lesions, surgical wounds, neuropathic pain, or injury of various types (i.e., abdominal, chest, fractures) as well as nursing-maneuvers with devices used during the hospitalization period (i.e., percutaneous endoscopic gastrostomy, nasogastric tube, bladder catheter replacement, venous and arterial blood sampling) (Ivanhoe and Hartman, 2004; Crooks et al., 2007; Baron, 2009; Popernack et al., 2015; Bexkens et al., 2017). In the rehabilitation as well as in the chronic phase, pain can arise from peripheral nerve lesions, central pain, diffuse spasticity, joint limitations, bedsores, paraosteoarthropathy, constipation, post-traumatic headache (Olver et al., 1996; Khan et al., 2003; Sherman et al., 2006; Hoffman et al., 2007; Ofek and Defrin, 2007; Baron, 2009; Gironda et al., 2009).

The Central Nervous System damage might be the cause of chronic pain (e.g., thalamic pain following a traumatic brain injury with diffuse axonal injury (Munivenkatappa and Agrawal, 2016; Irvine and Clark, 2018)). These conditions may lead to changes in the central nervous system pain processing and to a Complex Regional Pain Syndrome (CPRS), a neuropathic pain disorder characterized by distinct clinical features including allodynia, hyperalgesia, sudomotor and vasomotor abnormalities, and trophic changes (Schnakers et al., 2012; Guthmiller and Varacallo, 2020). Mechanism underlying CPRS is multifactorial, involving abnormal neuronal transmission, autonomic dysregulation, and central sensitization. The proinflammatory and immunological response increase production of interleukins, bradykinin, substance P, and osteoprotegerin, with consequent peripheral sensitization, alteration of the sympathetic nervous system and increasing expression of adrenergic receptors on nociceptive fibers (Guthmiller and Varacallo, 2020).

The presence of painful symptoms might interfere with the rehabilitation processes limiting and/or delaying its effect. It is crucial to intervene with appropriate early measure to prevent the appearance of secondary damage associated with pain and functional limitation such as bedsore or muscle-tendon retraction (Schnakers and Zasler, 2015).

In DOC patients, there is no general agreement on pharmacological pain treatment (Bartolo et al., 2016). Generally, it should be administered in the presence of behavioral signs of pain. The accurate pharmacotherapy dosage is crucial to avoid interferences with the assessment and treatment plan for the recovery of consciousness. Ineffective control of pain could affect or inhibit the emergence of intentional behavioral responses, while over-treatment could limit cognitive recovery and attention (Fins et al., 2008; Bartolo et al., 2016).

Brain lesions in these patients are extensive, affecting the nervous system at the cortical, subcortical, intracortical and spinal level. It is essential to provide basic care, managing the insurgence of the secondary medical complication that could

increase the risk of further disability (Sazbon and Groswasser, 1990; Sazbon and Groswasser, 1991; Seel et al., 2013) and complicate their treatment and pain management.

Most of these patients are characterized by spasticity. The spasticity, due to a lesion of the pyramidal tract, is defined as "a motor disorder, characterized by a velocity-dependent increase in tonic stretch reflexes (muscle tone) with exaggerated tendon jerks, resulting from hyper-excitability of the stretch reflex as one component of the upper motor neuron syndrome." (Lance, 1980). It is present in the 89% of DOC patients (Thibaut et al., 2015) and associated with pain and other symptoms such as increased hypertonia and altered sensorimotor control and muscle spasms (Burke et al., 2013). Infiltration of botulinum is advised in case of focal spasticity and to treat severe or worsening cases (Childers et al., 2004; Verplancke et al., 2005). In the case of dystonia and diffuse spasticity, an improvement in their management was observed by the intrathecal baclofen (Pistoia et al., 2015). The improvement of the level of consciousness in DOC was associated to the use of the intrathecal baclofen (Margetis et al., 2014; Pistoia et al., 2015), due probably to the reduced overload of the dysfunctional sensory stimuli reaching the brain or to the stabilization of the circadian rhythms (Margetis et al., 2014).

The symptomatic pain treatment follows the criteria of proportionality and graduality, assessing the interaction with the current therapy (Bartolo et al., 2016). The therapies approaches are generally based on aspirin, paracetamol, nonsteroidal anti-inflammatory drugs, opioid and γ-aminobutyric acid (GABA)-ergic agents (Mura et al., 2013; Bartolo et al., 2016). Aspirin, paracetamol, and nonsteroidal anti-inflammatory drugs should be administered in case of presumed mild pain (Schnakers and Zasler, 2007).

In case of suspected moderate pain and neuropathic pain, it is suggested a high-dose of aspirin or paracetamol, oral NSAIDs, GABAergic agents (Czuczwar and Patsalos, 2001; Enna and McCarson, 2006; Schnakers and Zasler, 2015; Bartolo et al., 2016). GABAergic agents are also indicated in case of psychomotor agitation or opposition to mobilization associable to pain. GABA is widely distributed throughout the neuraxis playing a central role in mediating or modulating most central nervous system functions. $GABA_A$ and $GABA_B$ receptors and GABAergic neurons are present in spinal cord and brain areas associated with the mediation and perception of pain (Enna and McCarson, 2006). Behavioral and physiological responses to pain are regulated by GABAergic projections from the ventral tegmental area and substantia nigra to the ventrolateral periaqueductal gray and dorsal medullary raphe nucleus (Kirouac et al., 2004). Both inhibitors of GABA uptake and metabolism and GABA receptor agonists display significant antinociceptive activity in animal models of acute, inflammatory, and neuropathic pain (Malan et al., 2002; Sa et al., 2004). Further, the antinociceptive response was observed to be induced by the activation of $GABA_A$ receptors in the parafasciculus thalami (Reyes-Vazquez et al., 1986). The pharmacotherapy based on GABAergic agents may be accompanied by adverse effects such as drowsiness, fatigue, depression or constriction of the visual field (Czuczwar and Patsalos, 2001).

In the case of presumed severe pain, it is advised to consider the use of parenteral opioids, mixed agonists/antagonists, partial agonist opioids, antidepressants, anticonvulsants, and atypical agents (Schnakers and Zasler, 2015; Bartolo et al., 2016; Seal et al., 2018; Adams et al., 2020). The opioids act by binding proteins called opioid receptors that are widely distributed. Those involved in pain modulation are localized in the central and peripheral nervous system. These receptors also bind endorphins involved not only in pain modulation but also in other body functions such as reinforcement and reward mechanisms, mood and stress, mediated by deep structures of the brain (Russo and Nestler, 2013). The neural proliferation is also modulated by the opioid system (Sargeant et al., 2008) inducing, for example, neural degeneration (Atici et al., 2004; van Dijk et al., 2011) and apoptosis (Hu et al., 2002). Nevertheless, the use of opioids to treat analgesia may be accompanied by side effects, which will depend on the dose, such as somnolence, mental clouding, and respiratory depression (Rosenblum et al., 2008; Rogers et al., 2013) that might interfere with a correct diagnosis of the level of consciousness.

It is evident the current difficulty for pain treatment in patients with DOC, and the impossibility for the patient to refer on the pain perception makes the choice of the correct pharmacological approach a challenge.

At the light of these concerns, the guideline of the physicians should be based on the cost/benefit, intended as to follow the ethical principle of nonmaleficence/beneficence of the treatments.

CONCLUSION

Pain is not only a perceptual phenomenon. The initial injury, cause of the pain, disrupts the body's homeostatic systems which, in turn, produce stress. Pain involves a dynamic interaction among biological, psychological, and social factors. These components may modulate pain perception and disability (Duncan, 2000; Gatchel and Kishino, 2008).

The assessment and management of pain in patients with a DOC remain a challenge. The perception of pain in these patients arises rehabilitative problems with ethical issues extending beyond the boundaries of end-of-life decisions (Miller-Smith et al., 2019; Wolf-Meyer, 2020). To date, the correct assessment of DOC patients has a high rate of misdiagnosis (Bosco et al., 2010; van Erp et al., 2015), and the misinterpretation of the behavioral signs may lead to a non-fully appropriate rehabilitative approach.

The correct pain management and the capability to individuate the best pharmacological treatment can make the difference in detecting a behavioral response indicative of a change in the level of consciousness in DOC patients, and in planning a more effective rehabilitative approach.

AUTHOR CONTRIBUTIONS

All authors contributed and agreed on the final version of the manuscript.

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REVIEW
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The Endocannabinoid System Contributes to Electroacupuncture Analgesia

Iona J. MacDonald[1] *and Yi-Hung Chen*[1,2,3]*

[1] Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan, [2] Chinese Medicine Research Center, China Medical University, Taichung, Taiwan, [3] Department of Photonics and Communication Engineering, Asia University, Taichung, Taiwan

The extensive involvement of the endocannabinoid system (ECS) in vital physiological and cognitive processes of the human body has inspired many investigations into the role of the ECS and drugs, and therapies that target this system and its receptors. Activation of cannabinoid receptors 1 and 2 (CB_1 and CB_2) by cannabinoid treatments, including synthetic cannabinoids, alleviates behavioral responses to inflammatory and neuropathic pain. An increasing body of scientific evidence details how electroacupuncture (EA) treatments achieve effective analgesia and reduce inflammation by modulating cannabinoid signaling, without the adverse effects resulting from synthetic cannabinoid administration. CB_1 receptors in the ventrolateral area of the periaqueductal gray are critically important for the mechanisms of the EA antinociceptive effect, while peripheral CB_2 receptors are related to the anti-inflammatory effects of EA. This review explores the evidence detailing the endocannabinoid mechanisms involved in EA antinociception.

Keywords: cannabinoid receptors, electroacupuncture, endocannabinoid system, analgesia, pain

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University of Insubria, Italy

****Correspondence:***
Yi-Hung Chen
yihungchen@mail.cmu.edu.tw

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INTRODUCTION

Since its discovery in the 1990s, the complex signaling network of the endocannabinoid system (ECS) has increasingly been seen to be a key player in the regulation of numerous vital physiological and cognitive processes, such as female reproductive events, pain sensation, mood, and in mediating the pharmacological effects of cannabis (Wang et al., 2006; McPartland et al., 2007; Aizpurua-Olaizola et al., 2017; Toth et al., 2019).

The ECS contains two major G-protein-coupled type 1 (CB_1) and type 2 (CB_2) cannabinoid receptors that are activated by the psychoactive ingredient of cannabis, Δ^9-tetrahydrocannabinol (THC). The main basic mechanisms triggered by CB_1 and CB_2 receptors are mediated by G proteins that are mostly of the $G_{i/o}$ type, resulting in inhibition of activity of adenylate cyclases, the cAMP cascade and voltage-gated calcium (Ca^{2+}) channels, and stimulation of mitogen-activated protein kinase (MAPK) activity (Di Marzo and Petrocellis, 2006). CB_1 receptors also inhibit voltage-activated Ca^{2+} channels, stimulate inwardly rectifying potassium (K^+) currents and activate both phospholipase C (through G protein $\beta\gamma$ subunits) and PI-3-kinase (Di Marzo and Petrocellis, 2006).

CB_1 is mainly expressed in the cerebral cortex, basal ganglia, cerebellum, and hippocampus; lower levels of CB_1 expression are found in the peripheral and autonomic nervous system, as well as the heart, lung, thymus, spleen, and reproductive system (Fulmer and Thewke, 2018). CB_1 is also expressed in immune cells, at levels of up to 100-fold lower than those of CB_2 (Fulmer and Thewke, 2018). In the central nervous system (CNS), activation of CB_1 inhibits excitatory and inhibitory neurotransmission, and the modulation of cognitive, memory, and motor functions, as well as analgesia (Chiurchiu et al., 2015).

CB_2 is expressed predominantly by cells of hematopoietic origin in the peripheral immune system (in bones, spleen, and skin); low levels of CB_2 expression are found in other cell types, including epithelial cells, osteogenic cells, cardiomyocytes, fibroblasts, and vascular smooth muscle cells (Fulmer and Thewke, 2018). Evidence of CB_2 expression in the CNS is controversial and requires further confirmation (Fulmer and Thewke, 2018). Upregulation of CB_2 is implicated in chronic inflammation of the nervous system, as well as with several cardiovascular and bone disorders (Chiurchiu et al., 2015).

Two endogenous lipophilic molecules, anandamide (AEA, *N*-arachidonoylethanolamide) and 2-arachidonoylglycerol (2-AG), are capable of activating CB_1 and CB_2, and are considered to be the main endocannabinoids (Aizpurua-Olaizola et al., 2017; Maccarrone, 2017). These endocannabinoids, as well as various enzymes involved in the biosynthesis and/or degradation of endogenous lipid ligands, comprise the ECS, a complex enzyme and transporter apparatus that affects virtually all central and peripheral systems in mammals (Aizpurua-Olaizola et al., 2017; Toth et al., 2019). With ongoing research identifying more bioactive lipids with cannabimimetic properties, it is reasonable to expect that investigations will gradually offer more insights into the ECS (Aizpurua-Olaizola et al., 2017; Maccarrone, 2017; Toth et al., 2019).

TARGETING THE ENDOCANNABINOID SYSTEM (ECS) FOR TREATING PAIN AND INFLAMMATION

The involvement of the ECS in several physiological regulation pathways makes it an attractive target for drugs and therapies in the management of pain and inflammation. Activation of endocannabinoids alleviates behavioral responses to acute, inflammatory and neuropathic pain (Palazzo et al., 2010). The CB_1 receptor is found in high densities in the superficial layers of the spinal dorsal horn (SDH), the dorsal root ganglia, the descending pathway of pain modulation, and the peripheral terminals of primary afferent neurons (Palazzo et al., 2010). The cellular location of CB_1 receptors is predominantly the presynaptic axon terminals of both γ-aminobutyric acid (GABA)ergic, and glutamatergic neurons (Campolongo and Fattore, 2015). Smaller quantities of CB_1 receptors are expressed in astrocytes, oligodendrocytes, and microglia, where the receptors regulate synaptic transmission (Zou and Kumar, 2018). CB_2 receptors predominantly reside in the periphery and represent a target in inflammatory pain processing (Palazzo et al., 2010).

AEA and 2-AG are synthesized on demand and function as retrograde messengers after their release from postsynaptic neurons in areas related to the descending pain modulatory pathway, from where they are transported to the CNS and peripheral terminals of primary afferent neurons, inhibiting neurotransmitter release from presynaptic terminals (Gondim et al., 2012; Kano, 2014). By activating CB_1 receptors in astrocytes, 2-AG triggers the release of glutamate, which activates the *N*-methyl-D-aspartate receptor (NMDAR) on pyramidal neurons (Zou and Kumar, 2018). Dense populations of endocannabinoid signaling molecules surround synapses in various brain regions including the periaqueductal gray (PAG) region, the hippocampus, cerebral cortex, amygdala, dorsal and ventral striatum, hypothalamus, cerebellum, and spinal cord, all of which are considered to be responsible for the neural functions that depend upon endocannabinoid signaling (Kano, 2014). Experimental data show that retrograde endocannabinoid signaling governs various aspects of neural signaling including learning and memory, anxiety and mood, addiction, feeding behavior, motor learning, and analgesia (Kano, 2014). Activation of CB_1 and CB_2 receptors inhibits established inflammatory hypersensitivity and swelling in animal models of inflammatory hyperalgesia (Gondim et al., 2012). Human samples of osteoarthritis and rheumatoid arthritis synovium tissue contain CB_1 and CB_2 receptors as well as AEA and 2-AG, indicating that the ECS may be closely related to pain and inflammation associated with arthritic disease (Gondim et al., 2012).

Cannabinoid treatments have proven efficacy in chronic pain and symptom control in palliative/supportive care. These compounds include the plant-derived cannabinoid tetrahydrocannabinol/cannabidiol (THC/CBD) oromucosal spray (nabiximols), and the synthetic THC analogs nabilone, dronabinol, and ajulemic acid, as well as the CBD oral solution Epidiolex®, all of which achieve their biological effects by activating cannabinoid receptors. However, while synthetic cannabinoids are generally devoid of many of the side effects of opiates, such as their large abuse potential and the life-threatening side effect of respiratory depression, use of synthetic cannabinoids has been linked to severe illness, intensive care admission, and death (Kasper et al., 2015). Synthetic cannabinoids can also affect the cardiovascular system, with case reports describing adverse outcomes including cardiogenic shock, acute respiratory depression and cardiopulmonary resuscitation (Khan S. P. et al., 2019).

The ECS maintains bodily homeostasis by influencing physiological processes such as cannabinoid signaling in the skin (Toth et al., 2019), emotional stasis (Stampanoni Bassi et al., 2018), immune homeostasis in the gut (Acharya et al., 2017), and the regulation of appetite, food intake, and energy balance (Pagotto et al., 2006), all of which depend on CB_1 and CB_2 receptors, among others (Maccarrone, 2017). The present review provides a brief general overview of

acupuncture and electroacupuncture (EA) and highlights the therapeutic potential in EA-induced modulation of cannabinoid signaling for effective analgesia and reductions in inflammation. Importantly, these therapeutic outcomes are produced without the adverse effects associated with synthetic cannabinoids.

MANUAL ACUPUNCTURE AND ELECTROACUPUNCTURE ANALGESIA

Traditional manual acupuncture has a long history, whereas EA was introduced into clinical practice as recently as the 1950s (Napadow et al., 2005; White et al., 2008). During manual acupuncture, needles are inserted into historically and empirically defined acupoint locations and are then manually manipulated by twisting, lifting or thrusting movements (Napadow et al., 2005). The EA technique involves the insertion of two needles that serve as electrodes for passing an electric current (Napadow et al., 2005). At least one of the needles is positioned at an acupoint (Napadow et al., 2005).

Both manual acupuncture and EA have demonstrated clinically relevant effects in chronic pain conditions (Scharf et al., 2006; Endres et al., 2007; Haake et al., 2007; Selfe and Taylor, 2008; Chen et al., 2017). The use of acupuncture within TCM is supported by a wealth of data demonstrating its therapeutic effects in numerous clinical conditions, including pain, such as the treatment of general chronic pain (Vickers et al., 2012), migraine prophylaxis (Linde et al., 2016; Musil et al., 2018) and treatment (Zhao et al., 2005), the treatment of chronic low back pain (Yuan et al., 2008; Chou et al., 2017), fibromyalgia (Deare et al., 2013; Mist and Jones, 2018), and osteoarthritis (Manheimer et al., 2010; Lin et al., 2016). An individual patient data meta-analysis that used data from 29 randomized controlled trials (RCTs) including a total of 17,922 patients found that acupuncture (whether it was manual acupuncture or EA) was significantly superior to both sham and no acupuncture control for all four chronic pain conditions investigated (back and neck pain, shoulder pain, chronic headache, and osteoarthritis) for the efficacy of acupuncture (Vickers et al., 2012). These findings were confirmed in a subsequent update of this meta-analysis (Vickers et al., 2018). The updated review included an additional 13 RCTs of acupuncture (manual acupuncture or EA) compared with either sham acupuncture or no acupuncture control for non-specific musculoskeletal pain, shoulder pain, chronic headache, or osteoarthritis; data were analyzed from a total of 20,827 patients (Vickers et al., 2018).

Manual acupuncture is associated with the activation of all types of afferent fibers (Aβ, Aδ, and C), while the EA current has to be sufficiently intense to excite Aβ-type and some of the Aδ-type afferent fibers for eliciting analgesia (Zhao, 2008). Adding manipulation to EA reportedly produces more potent analgesia compared with EA alone (Kim et al., 2000). An oft-stated advantage of EA for clinical practice or research is that it can objectively and quantifiably define stimulus frequency and intensity (Napadow et al., 2005). Functional magnetic resonance imaging (fMRI) investigations have revealed that EA (2 and 100 Hz) is associated with more widespread increases in fMRI signaling compared with manual acupuncture, while all acupuncture needling is associated with more widespread responses in the brain compared with the placebo-like tactile control stimulation (Napadow et al., 2005). Differential brain activation is observed between manual acupuncture and EA, which has also been noted by other fMRI and positron emission tomography (PET) investigations into acupuncture analgesia in the human brain, suggesting that different brain networks or different brain mechanisms are involved during manual acupuncture and EA (Kong et al., 2002; Napadow et al., 2005; Yang J. et al., 2012).

Electroacupuncture (EA) is used by traditional Chinese medicine (TCM) practitioners and acupuncturists for many different therapeutic conditions (Sidhu et al., 2017). Some acupuncturists contend that manual needling is sufficient to achieve the desired therapeutic results, whereas others argue that EA provides a unique role, especially in patients with chronic nociceptive pain (White et al., 2008). Some research suggests that EA may provide more effective pain relief than manual acupuncture, with the addition of the electric current optimizing the effects elicited by manual acupuncture (Wan et al., 2001; Barlas et al., 2006). Furthermore, low-frequency, high-intensity EA has been associated with a significantly larger hypoalgesic effect compared with placebo needling, whereas low-intensity stimulation was not significantly different from placebo needling in hypoalgesic responses (Barlas et al., 2006).

THE DESCENDING PAIN MODULATORY PATHWAY IN ACUPUNCTURE ANALGESIA

The pathophysiology of pain has been thoroughly researched by many in-depth papers and reviews (Loeser and Melzack, 1999; Raffaeli and Arnaudo, 2017; Chen et al., 2020). Among the brain and spinal cord areas, the descending pain modulatory pathway is critical in pain perception and acupuncture analgesia (Lv et al., 2019). This system arises in the PAG, where transmitters are activated that contact the rostroventromedial medulla and project to the raphe nuclei in the brainstem, and to inhibitory synapses in the SDH (Lai et al., 2019; Lv et al., 2019). Local administration of GABA agonists into the ventrolateral area of the PAG (vlPAG) promotes pain, while local administration of GABA antagonists produces antinociceptive effects by reducing inhibitory neurotransmission (Zhu et al., 2019). The GABA disinhibition hypothesis proposes that tonically active GABAergic interneurons within the PAG release GABA, which acts via $GABA_A$ receptors to inhibit spinally projecting output neurons (Zhu et al., 2019). According to this hypothesis, opioids and cannabinoids indirectly suppress the inhibitory influence of local GABAergic interneurons and effectively disinhibit the antinociceptive pathway of the

neuronal output descending to the spinal cord (Zhu et al., 2019). Thus, cannabinoids are capable of producing analgesia via a central action in the descending pain modulatory pathway, direct spinal action, or peripheral nerve action (Gondim et al., 2012).

EXPLORATIONS INTO ACUPUNCTURE- AND ELECTROACUPUNCTURE (EA)-INDUCED ANALGESIA

Endogenous Opioid Involvement

In 1973, Chinese investigators performed a clinical study involving healthy volunteers that demonstrated time-dependent analgesic effects of manual acupuncture at acupoint LI4 (Research Group of Acupuncture Anesthesia, 1973). The increase in pain threshold after manual acupuncture at LI4 was blocked by pretreatment with local anesthetic 2% procaine injected deep below LI4, indicating the importance of nerve innervations embedded in structures deep below the acupoint (Research Group of Acupuncture Anesthesia, 1973). When they then treated the affected limbs of hemiplegic and paraplegic patients with acupuncture, the researchers found no effect on pain threshold on the unaffected side, supporting the involvement of peripheral sensory nerves and the afferent nerve pathway in the spinal cord (Research Group of Acupuncture Anesthesia, 1973). The significance of pain relief achieved with the use of LI4 has been highlighted by recent investigations showing immediate, significant relief from inflammatory pain in mice injected with complete Freund's adjuvant (CFA) in the hind paws (Yen et al., 2019).

In the late 1970s, researchers discovered that the opiate receptor antagonist naloxone attenuated acupuncture analgesia in humans (Mayer et al., 1977) and in mice (Pomeranz and Chiu, 1976). This analgesic action was attributed to the release of a morphine-like substrate in the CNS. In the early 1980s, the purification of β-endorphin and enkephalin implicated these opiates as key players in acupuncture in humans and animals (Clement-Jones et al., 1980; Pert et al., 1981; Kiser et al., 1983). The research revealed that acupuncture increased levels of plasma enkephalin and cerebrospinal fluid β-endorphin in humans after acupuncture treatment.

Investigations into the relationship between different levels of EA analgesia have found that met-enkephalin, β-endorphin, and endomorphin are preferably stimulated by low-frequency (2 Hz) EA, while dynorphin is the only opioid peptide that responds to high-frequency (100 Hz) EA stimulation (Han, 2004). Further experimental research has shown that a dense-and-disperse (DD) mode of EA stimulation alternating 2 Hz with 100 Hz, each lasting for 3 s, evokes the simultaneous release of both enkephalins and dynorphins, resulting in a synergistic interaction that maximizes the therapeutic effect (Han, 2004). These findings have been substantiated in clinical research (Han, 2011).

Interference With the Central Sensitization Process

The general consensus among scientists in the pain research field is that chronic pain states in humans are driven by three core mechanisms: nociceptive and neuropathic pain mechanisms, as well as central sensitization (Harte et al., 2018). The term central sensitization conveys the understanding that CNS mechanisms are implicated in the amplification of pain, independently of peripheral injury or inflammation (Harte et al., 2018). Effective pain management relies upon the recognition of central sensitization and whether it is resulting from ongoing nociceptive input or is occurring despite no obvious peripheral driver (Harte et al., 2018).

The analgesic effects of manual acupuncture and EA are produced through multiple pathways and interfere with the central sensitization process by reducing levels of inflammatory mediators in the peripheral tissue, including substance P, interleukin (IL)-1β, IL-8, IL-10, and tumor necrosis factor (TNF)-α (Zhang et al., 2012; Lai et al., 2019). Possible processes whereby acupuncture reduces central sensitization include the release of the endogenous opioid, adrenergic, and 5-hydroxytryptamine (5-HT, serotonin) receptors, the *N*-methyl-D-aspartate/α-amino-3-hydroxy-5-methyl-4-isozazolepropionin acid/kainate (NMDA/AMPA/KA) pathways, and afferent segmental inhibition (Lai et al., 2019). Segmental acupuncture analgesia postulates that high-frequency, low-intensity EA activates myelinated afferent A-fibers that reduce nociceptive transmission via inhibitory interneurons in the spinal dorsal root, reducing the response to painful stimuli within the same spinal segment (Baeumler et al., 2014, 2019).

The Role of the Adenosine A_1 Receptor

Researchers have noted that the hypothesized activation of centrally acting, endogenous opioid peptides in the CNS in response to acupuncture stimulation cannot completely explain why acupuncture needling is conventionally applied to an acupoint adjacent to the area of pain (Goldman et al., 2010). In mouse models of inflammatory pain, Goldman et al. (2010) found that analgesic effects from manual acupuncture are mediated by the release of the transmitter adenosine. Injecting the mice after acupuncture treatment with deoxycoformycin, an adenosine deaminase inhibitor, prolonged the accumulation of adenosine and the antinociceptive effects of acupuncture. The researchers suggest that activation of the adenosine A_1 receptor is necessary for acupuncture analgesia (Goldman et al., 2010).

Non-opioid Mechanisms

Conversely, other research using human subjects failed to demonstrate naloxone reversal of EA analgesia, calling into question the premise that endorphins play a significant role in acupuncture analgesia (Chapman et al., 1983). In that study, 14 healthy adult volunteers who demonstrated EA analgesia during electrical stimulation of the LI4 acupoint were randomly assigned to naloxone 1.2 mg or normal

saline (Chapman et al., 1983). Naloxone failed to reverse pain thresholds elevated by acupuncture, suggesting that other mechanisms are involved in acupuncture-induced analgesia (Chapman et al., 1983).

THE ROLE OF THE ENDOCANNABINOID SYSTEM (ECS) IN ELECTROACUPUNCTURE (EA) ANALGESIA

Greater understanding of the ECS has encouraged investigations into the role of the ECS in acupuncture analgesia. This research is discussed below.

EA-Induced Analgesia and Anti-inflammatory Effects Depend on CB_1 Receptors and/or CB_2 Receptors

EA analgesia in inflammatory pain relates to the dopamine system and CB_1 receptors in the striatum (Shou et al., 2013). In the complete Freund's adjuvant (CFA)-induced model of arthritis in mice, EA significantly increased CB_1 receptor expression in the striatum and prolonged paw withdrawal latency; both effects were attenuated by the CB_1 receptor antagonist AM251, indicating an important role for the CB_1 receptor in EA-induced analgesia (Shou et al., 2013). The study researchers also reported that EA upregulated dopamine D_1 and D_2 receptor mRNA expression in the corpus striatum, which was effectively blocked by AM251 (Shou et al., 2013). They suggested that EA analgesia in inflammatory pain is due to cross-modulation between the dopamine system and CB_1 in the striatum (Shou et al., 2013).

CB_1 and CB_2 receptors contribute to antinociceptive and anti-inflammatory effects of EA in arthritis of the rat temporomandibular joint. In one study, researchers found that EA significantly inhibited mechanical hypernociception in rats with acute arthritis induced by zymosan in the temporomandibular joint (TMJ) (Gondim et al., 2012). This effect was reversed by AM251, although AM251 failed to affect the EA-induced anti-inflammatory response in the TMJ (Gondim et al., 2012). In contrast, the CB_2 receptor antagonist AM630 reversed the EA-induced anti-inflammatory effect but did not alter its antinociceptive effect (Gondim et al., 2012). These findings are supported by another study implicating the involvement of CB_1 receptors in EA-induced orofacial antinociception among rats exposed to noxious facial heat (Almeida et al., 2016). In this experimental study, EA alone at acupoint ST36 induced antinociception that was blocked by pretreatment with AM251, but not by AM630 (Almeida et al., 2016). Moreover, the antinociceptive effects of EA were prolonged and intensified by pretreatment with an endocannabinoid metabolizing enzyme inhibitor (MAFP) or an anandamide reuptake inhibitor (VDM11) (Almeida et al., 2016). Acupuncture at ST36 (located below the knee) is generally used to treat dysfunctional gastrointestinal activity (Cheong et al., 2013) and rarely for diseases of the head and mouth (Yang Y. et al., 2012). However, the study by Almeida et al. (2016) revealed that ST36 is also related to the ECS.

CB_1 Receptors in the vlPAG Are Critically Important for the Mechanisms of the EA Antinociceptive Effect

The vlPAG midbrain region is an important site of action in cannabinoid-induced antinociception and a likely supraspinal site of orexin antinociception (Ho et al., 2011). Orexin A and B are hypothalamic peptides that activate postsynaptic orexin 1 and orexin 2 receptors (OX_1Rs and OX_2Rs), which have many roles in physiological processes including energy homeostasis, stress processing, visceral functions, reward seeking behavior, cognition, endocrine functions, arousal, and pain modulation (Razavi and Hosseinzadeh, 2017). OX_1Rs and OX_2Rs are densely distributed in the PAG (Ho et al., 2011). In particular, orexin A can induce an opioid-independent analgesic mechanism that is mediated by OX_1R-initiated 2-AG signaling in the vlPAG (Ho et al., 2011; Chen et al., 2018). Subsequent research using murine pain models has demonstrated how low-frequency median nerve stimulation (MNS) using acupuncture needles at the PC6 acupoint (EA-PC6) induces analgesia involving both CB_1 receptors and OX_1Rs (Chen et al., 2018). MNS-PC6-induced antinociception (a procedure equivalent to EA-PC6) was prevented by systemic blockade of OX_1Rs or CB_1 receptors, but not by opioid receptor antagonists (Chen et al., 2018). Systemic blockade of OX_1Rs or CB_1 receptors also prevented the EA-PC6-induced reduction in GABA levels in the vlPAG microdialysate (Chen et al., 2018). Notably, EA-PC6-induced analgesia was reduced by intra-vlPAG inhibition of 2-AG synthesis, implicating a cannabinoid (2-AG)-dependent mechanism, which was supported by the finding that EA-PC6-induced antinociception was markedly attenuated in *Cnr1*$^{-/-}$ mice, which lack the CB_1 receptor (Chen et al., 2018). These findings suggest that PC6-targeting low-frequency MNS activates hypothalamic orexin neurons, releasing orexins that activate postsynaptic OX_1Rs in the vlPAG to generate 2-AG, which inhibits GABA release through CB_1 receptors in the vlPAG and induces analgesia by disinhibiting vlPAG outputs (Chen et al., 2018). These findings have been reinforced in further investigations by these researchers, who report that repeated EA-PC6 treatments remained fully effective in morphine-tolerant mice with neuropathic pain caused by chronic constriction injury (CCI) of the sciatic nerve, via a mechanism involving OX_1Rs and CB_1 receptors (Lee et al., 2020).

In other research involving mice with knee osteoarthritis, EA increased the levels of CB_1 receptors and 2-AG in the vlPAG to reduce chronic knee osteoarthritis pain; the effect of EA on pain hypersensitivity was reversed when AM251 was injected into the vlPAG (Yuan et al., 2018b). Subsequent research by the same group discovered that knocking out the CB_1 receptor on GABAergic neurons abolished most of the antinociceptive effects of EA in mice with CCI or knee osteoarthritis, while knocking out the CB_1 receptor on glutamatergic neurons in the vlPAG only partly reduced the effects of EA (Zhu et al., 2019). The researchers

proposed that inhibition of GABAergic neurons and activation of glutamatergic neurons in the vlPAG through CB_1 receptors contribute to EA-induced analgesia (Zhu et al., 2019).

Counterintuitively, while some research has shown that low doses of μ-opioid receptor antagonists levonaloxone, naltrexone, cyclazocine, and diprenorphine block EA analgesia (Cheng and Pomeranz, 1980), other investigations have demonstrated that ultra-low-dose naltrexone (0.001–0.004 mg/day) enhances opiate-induced analgesia while also reducing opioid tolerance and dependence in chronic pain conditions (Chindalore et al., 2005; Webster, 2007). Some researchers have hypothesized that low-dose naltrexone (3–4.5 mg/day) could synergistically enhance acupuncture-induced pain relief (Hesselink and Kopsky, 2011). In support of this hypothesis, low-dose naltrexone appears to exert hypoalgesic effects in healthy human volunteers exposed to noxious electrocutaneous stimulation (France et al., 2007), blocks acute tolerance to morphine and attenuates morphine-elicited thermal hyperalgesia in rats (McNaull et al., 2007), and enhances cannabinoid-induced antinociception in rats (Paquette and Olmstead, 2005). Preclinical research has effectively illustrated interactions between the cannabinoid system and opioid antinociception, showing that AM251 can completely block morphine-induced peripheral and central antinociception in a rat model of inflammatory pain and in the tail-flick test in mice, while AM630 is only partially effective or fails to antagonize antinociception induced by morphine (Pacheco et al., 2008, 2009). These researchers have therefore suggested that μ-opioid-induced antinociception involves peripheral (Pacheco et al., 2008) or central (Pacheco et al., 2009) activation of CB_1 and/or CB_2 receptors that potentiate the opioid-induced antinociceptive mechanisms. Other evidence suggests that a regimen of low-dose THC combined with low-dose morphine enhances opioid analgesic potency without increasing undesirable side effects (Cichewicz, 2004). Further support for considerable crosstalk between the opioid and endocannabinoid pathways comes from studies involving transgenic mice with inactivated CB_1 or CB_2 receptors [*cnr1* and *cnr2* knockout (KO) mice], which exhibit marked reductions in peripheral or spinal morphine analgesia (Desroches et al., 2014). This loss in morphine analgesia was not explained by downregulation in μ-opioid spinal expression nor by altered binding properties or G protein coupling of the μ-opioid receptor in *cnr1*KO and *cnr2*KO mice (Desroches et al., 2014).

CB_2 Receptors in the Peripheral Tissue Mediate the Anti-inflammatory Effects of Acupuncture

It is possible that the roles of CB_1 and CB_2 receptors differ according to the type of pain condition. For instance, EA significantly increased levels of AEA in inflamed skin tissue and produced antinociceptive effects by activating peripheral CB_2 receptors in a rat model of CFA-induced inflammatory pain (Chen et al., 2009), while CB_1 receptors appeared to mediate the anti-inflammatory effect of EA in a rat model of migraine (Zhang et al., 2016). In the CFA model of inflammatory pain induced by the local injection of CFA into the hind paw of rats, local pretreatment with AM630 significantly attenuated the antinociceptive effect of EA, whereas local pretreatment with AM251 had no significant effect on EA analgesia (Chen et al., 2009). In a follow-up study using the same inflammatory pain model, the researchers found increased numbers of CB_2 receptors on macrophages, T-lymphocytes, and keratinocytes in the epidermis and dermis in response to CFA injections; further increases were observed in all of these cells expressing CB_2 receptors in the inflamed skin of rats in the CFA plus 2 and 100 Hz EA groups (Zhang et al., 2010). CFA plus 2 Hz EA and CFA plus 100 Hz EA also significantly increased mRNA and protein levels of CB_2 receptors in the inflamed skin tissue (Zhang et al., 2010). In another study involving rats with CFA-induced inflammatory pain, EA appeared to reduce pain by inhibiting activation of the NLRP3 inflammasome in inflamed skin tissue through CB_2 receptor stimulation (Gao et al., 2018). Similarly, other researchers found that EA-induced activation of the CB_2 receptor inhibited chronic pain in mice with knee osteoarthritis (Yuan et al., 2018a). In their study, EA treatment was associated with significant increases in levels of CB_2 receptor expression in fibroblasts and significant reductions in IL-1β-positive cells in the knee meniscus; knockout of the CB_2 receptor blocked EA analgesia and EA had no effect upon IL-1β expression in $CB2^{-/-}$ mice (Yuan et al., 2018a).

EA analgesia in inflammatory pain may also be due to an interaction between peripheral CB_2 receptors and endogenous opioids (Su et al., 2011). β-Endorphin, the endogenous ligand of the opioid peptide, is derived from the precursor proopiomelanocortin (POMC). In a rat model of inflammatory pain, mRNA levels of POMC and protein levels of β-endorphin were significantly increased in inflamed skin tissues after rats were treated with the CB_2 receptor agonist AM1241 or EA, while AM630 significantly reduced these effects (Su et al., 2011). Percentages of β-endorphin-immunoreactive keratinocytes, macrophages, and T-lymphocytes were also significantly increased by AM1241 or EA, whereas pretreatment with AM630 blocked these effects (Su et al., 2011). Thus, a peripheral interaction between μ-opioid receptors and CB_2 receptors is implicated in EA analgesia in inflammatory pain.

The same group of researchers subsequently reported that EA appears to reduce inflammatory pain and proinflammatory cytokine expression by activating CB_2 receptors in CFA-induced skin inflammation (Su et al., 2012). EA at GB30 and GB34 acupoints and also local injections of AM1241 significantly decreased thermal hyperalgesia and mechanical allodynia in inflammatory skin tissue; the antinociceptive effect of EA was blocked by pretreatment with AM630 (Su et al., 2012). EA or AM1241 treatment also significantly reduced IL-1β, IL-6, and TNF-α mRNA and protein levels in inflamed skin tissue; these inhibitory effects were reversed by AM630 pretreatment (Su et al., 2012).

The placebo effect in acupuncture analgesia is well-recognized (Musial, 2019). The ECS can also play an important role in placebo analgesia. Research has revealed the secretion of

TABLE 1 | Reports published between 2009 and 2020 (cited in ascending date order) describing the involvement of the ECS in EA analgesia.

Pain model	Acupuncture treatment	Major findings	Conclusions	References
Inflammatory pain/CFA injection	2 or 100 Hz EA (1 mA, 30 min) at GB30 and GB34, on days 2, 4, and 6 after CFA injection	• EA at 2 and 100 Hz significantly reduced thermal hyperalgesia and mechanical allodynia associated with CFA injections. • EA significantly increased endogenous levels of AEA in inflamed skin tissue. • EA-induced antinociception was significantly reduced by AM630, but not significantly altered by AM251. AM251 and AM630 were each given as subcutaneous injections into the dorsal surface of the left hind paw 5 min prior to sham EA or EA treatment.	EA appears to enhance the local release of endogenous AEA from inflamed skin tissue. EA analgesia in inflammatory pain involves the activation of peripheral CB_2 receptors.	Chen et al., 2009
Inflammatory pain/CFA injection	2 or 100 Hz (1 mA, 30 min) at GB30 and GB34 once every other day, starting from the second day after CFA injection, for 7 days	• EA at 2 and 100 Hz significantly increased CB_2R mRNA and protein expression in inflamed skin tissue. • EA 2 and 100 Hz significantly increased quantities of CB_2R-immunoreactive keratinocytes, macrophages, and T-lymphocytes in inflamed skin tissue.	EA upregulates CB_2 receptor expression in keratinocytes and inflammatory cells in inflamed skin tissue.	Zhang et al., 2010
Inflammatory pain/CFA injection	2 Hz EA (1 mA, 30 min) at GB30 and GB34, on days 2, 4, and 6 after CFA injection	• EA or AM1241 significantly reduced thermal hyperalgesia and mechanical allodynia; pretreatment with β-funaltrexamine (a selective μ-opioid receptor antagonist) attenuated these effects. • EA or AM1241 significantly increased POMC mRNA and β-endorphin protein levels in inflamed skin tissues; these effects were significantly reduced by pretreatment with AM630. AM1241 or AM630 was injected subcutaneously into the dorsal surface of the left hind paw 5 min before each session of sham EA or EA treatment. • EA also significantly increased the percentage of β-endorphin-immunoreactive keratinocytes, macrophages, and T-lymphocytes in inflamed skin tissue; AM630 prevented these effects.	EA increases endogenous opioid expression in keratinocytes and infiltrating immune cells at the inflammatory site by activating peripheral CB_2 receptors.	Su et al., 2011
Inflammatory pain/CFA injection	2 Hz EA (1 mA, 30 min) at GB30 and GB34, on days 2, 4, and 6 after CFA injection	• EA or AM1241 treatment significantly reduced thermal hyperalgesia and mechanical allodynia after CFA injection. • AM630 significantly attenuated EA antinociception. AM1241 or AM630 was injected subcutaneously into the dorsal surface of the left hind paw 5 min before each session of sham EA or EA treatment. • EA or AM1241 treatment significantly reduced IL-1β, IL-6, and TNF-α mRNA and protein levels in inflamed skin tissue. • The inhibitory effects of EA on these cytokines were significantly reversed by pretreatment with AM630.	EA activation of CB_2 receptors reduces inflammatory pain and proinflammatory cytokine expression in inflamed tissue.	Su et al., 2012
Inflammatory pain/zymosan administration in the TMJ	10 Hz EA (3 mA, 30 min) at LI4, LI11, ST36 and ST34, 1 h before or 2 h after zymosan administration in the TMJ	• EA significantly inhibited zymosan-induced hypernociception. • EA antinociception was significantly reversed by AM251. • EA anti-inflammatory effects were reversed by AM630. Both AM251 and AM630 were given as single, IP injections 10 min prior to EA treatment. • *CB_1R* and *CB_2R* gene expression was upregulated 6 h after zymosan-induced arthritis in EA-treated rats. • In EA-treated rats, *CB_1R* gene expression was significantly increased at 6 h after zymosan administration and increased still further at 24 h, whereas *CB_2R* gene expression peaked at 6 h after zymosan administration and was downregulated at 24 h.	Antinociceptive and anti-inflammatory effects of EA appeared to be mediated through CB_1 and CB_2 receptor activation.	Gondim et al., 2012
Inflammatory pain/CFA injection	2/100 Hz EA (1.0, 2.0, 3.0 mA, 20 min) at ST36 and BL60, once every other day starting from the 4th day after CFA injection, for 4 sessions	• EA improved thermal hyperalgesia and significantly increased levels of CB_1 expression in rat striatum. • AM251 significantly attenuated EA-induced increases in CB_1 expression. Single, IP injections of AM251 were administered on study day 10. • EA upregulated dopamine D_1 and D_2 receptor mRNA expression in the corpus striatum, which was effectively blocked by AM251.	EA analgesia in inflammatory pain is associated with upregulation of dopamine and CB_1 receptors in the rat corpus striatum.	Shou et al., 2013

(Continued)

TABLE 1 | Continued

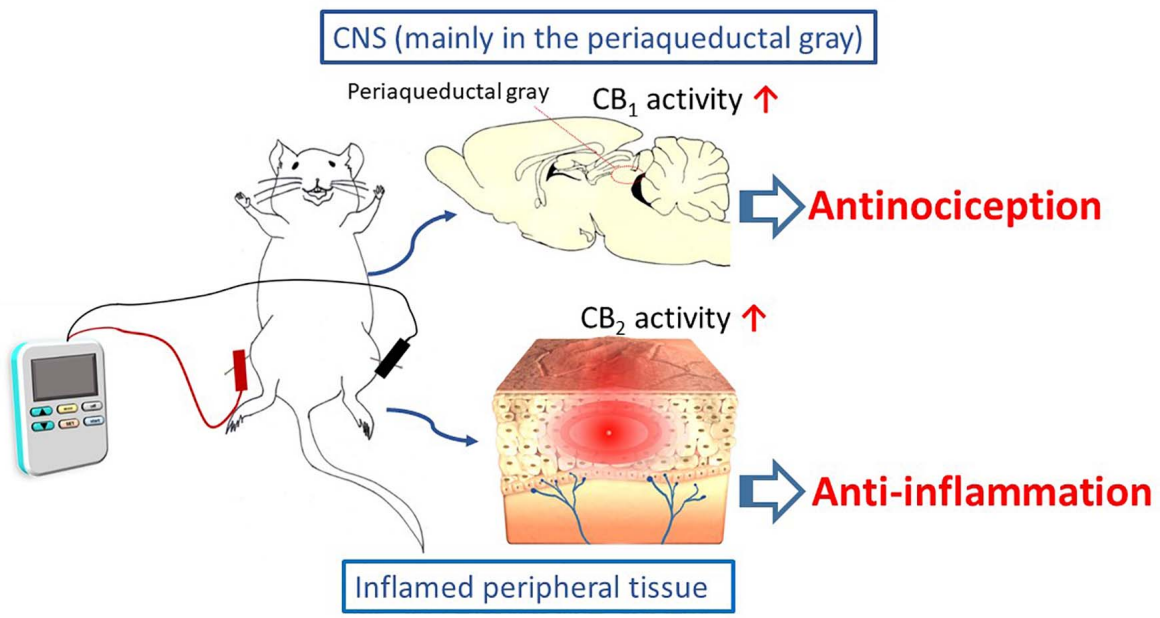
Pain model	Acupuncture treatment	Major findings	Conclusions	References
Migraine (induced by electrical stimulation of the trigeminal ganglion; TGES)	Ipsilateral 2/15 Hz EA (1 mA, 30 min) at GB20 and TE5 once daily for 5 days before TGES	• EA significantly attenuated TGES-induced increases in serum CGRP and PGE_2 levels, and inhibited the TGES-induced increase in neurogenic PPE. • EA significantly attenuated TGES-induced increases in COX2 and IL-1β protein levels in the trigeminal ganglion. • The effects of EA were reversed by CB_1R antagonism.	CB_1 receptors appeared to mediate EA anti-inflammatory effects in a rat model of migraine.	Zhang et al., 2016
Orofacial pain (induced by noxious heat applied to the face)	100 Hz EA (0.5 mA, 20 min) at ST36	• EA-induced antinociception was prolonged and intensified by pretreatment with an endocannabinoid metabolizing enzyme inhibitor (MAFP) and an anandamide reuptake inhibitor (VDM11). • EA-induced antinociception was blocked by pretreatment with AM251, but not by AM630. All study drugs were injected as single, IP doses, 10 min prior to EA treatment.	EA orofacial antinociception appeared to involve activation of CB_1 receptors.	Almeida et al., 2016
Inflammatory pain/MIA injection	*Knee OA model*: 2, 15, or 100 Hz EA (1 mA, 30 min) at Ex-LE4 and ST35, starting from 2 days after IA-injected MIA, once every other day for 4 weeks	• Chronic pain-induced reductions in levels of CB_1Rs and 2-AG expression in the midbrain were reversed by EA treatment. • Microinjection of AM251 into the vlPAG reversed the effects of EA on pain hypersensitivity and DNIC function. • In *GABA-CB1*$^{-/-}$ mice subjected to knee OA induction, the reduced thermal latencies and tactile thresholds were not significantly affected by EA. Similarly, EA had no effect on the reduction in 5-HT levels in the medulla following the induction of knee OA in *GABA-CB1*$^{-/-}$ mice.	It appears that the 2-AG-CB_1R-GABA-5-HT signaling pathway underlies the effects of EA on descending inhibitory control of 5-HT, improving DNIC function and inhibiting chronic pain. CB_1Rs on GABAergic neurons were involved in the effects of EA on DNIC function and descending inhibitory control of 5-HT in the medulla.	Yuan et al., 2018b
Inflammatory pain/MIA injection	*Knee OA model*: 2 Hz EA (1 mA, 30 min) at Ex-LE4 and ST35, starting from 2 days after IA-injected MIA, once every other day for 4 weeks	• EA significantly increased levels of CB_2R expression in fibroblasts and significantly reduced IL-1β-positive cells in the knee meniscus. • Knockout of the CB_2R blocked EA analgesia.	EA reduced levels of IL-1β expression by activating CB_2 receptors, which effectively reduced chronic pain in mice with knee OA.	Yuan et al., 2018a
Heat hyperalgesia and neuropathic pain	*Hot-plate test*: 2 Hz EA-PC6 (2 mA, for 20 min) *CCI model*: EA-PC6, non-MNS, or sham-PC6 were applied on postoperative day 8	• EA-PC6 reduced acute thermal nociceptive responses and neuropathy-induced mechanical allodynia; these effects were prevented by systemic or intra-vlPAG injection of an antagonist of OX_1Rs or CB_1Rs, but not by opioid receptor antagonists. • EA-PC6 increased the number of c-Fos-immunoreactive hypothalamic orexin neurons, and led to higher orexin A and lower GABA levels in the vlPAG. • EA-PC6-induced nociception was prevented by intra-vlPAG inhibition of 2-AG synthesis and was attenuated in *Cnr1*$^{-/-}$ mice.	EA-PC6 induces the release of an endogenous neuropeptide (orexin) from the hypothalamus to inhibit pain responses in mice through a CB_1R-dependent cascade that reduces inhibitory GABAergic control in the vlPAG.	Chen et al., 2018
Inflammatory pain/CFA injection	QD 2 Hz EA (1 mA, 30 min) at GB30 and GB34 on days 2, 4, and 6 after CFA injections	• EA significantly reduced CFA-induced thermal hyperalgesia and mechanical allodynia and attenuated CFA-induced activation of the NLRP3 inflammasome in inflamed skin tissues. • *In vitro* studies in a rat alveolar macrophage cell line revealed that activation of CB_2 receptors inhibited NLRP3 inflammasome activation.	EA appears to relieve inflammatory pain by inhibiting NLRP3 inflammasome activation in inflamed skin tissues through CB_2 receptors.	Gao et al., 2018
Inflammatory and neuropathic pain	*CCI model:* QD 2 Hz EA (1 mA, 30 min) at GB30 and GB34, starting from the 8th postoperative day and ending on the 14th postoperative day			

(Continued)

TABLE 1 | Continued

Pain model	Acupuncture treatment	Major findings	Conclusions	References
	Knee OA model: QD 2 Hz EA (1 mA, 30 min) at Ex-LE4 and ST35, starting from the 15th day after IA-injected MIA and ending on the 21st day *Sham:* Acupuncture needles were inserted into the study acupoints without electrical stimulation or manual manipulation	• Chemogenetic inhibition of GABAergic neurons in the vlPAG mimicked the effects of EA. • The combination of chemogenetic activation of GABAergic neurons and chemogenetic inhibition of glutamatergic neurons in the vlPAG was needed to reverse the effects of EA. • Specifically knocking out CB_1Rs on GABAergic neurons in the vlPAG abolished the EA effect on pain hypersensitivity.	EA synchronously inhibits GABAergic neurons and activates glutamatergic neurons in the vlPAG through CB_1Rs to produce EA-induced analgesia. The CB_1Rs on GABAergic neurons localized in the vlPAG was the basis of the EA effect on pain hypersensitivity.	Zhu et al., 2019

2-AG, 2-arachidonoylglycerol; 5-HT, 5-hydroxytryptamine or serotonin; AM1421, CB_2 receptor agonist; AM251, CB_1 receptor antagonist; AM630, CB_2 receptor antagonist; BID, twice daily; BL60, Kunlun; CB_1R, cannabinoid 1 receptor; CCI, chronic constriction nerve injury; CFA, complete Freund's adjuvant; CGRP, calcitonin gene-related peptide; DNIC, diffuse noxious inhibitory control; EA, electroacupuncture; ECS, endocannabinoid system; Ex-LE4, Neixiyan; GB30, Huantiao; GABA, γ-aminobutyric acid; GB20, Fengchi; GB34, Yanglingquan; IA, intra-articular; IP, intraperitoneal; IT, intrathecal; LI4, Hegu; LI11, Quchi; MIA, monosodium iodoacetate; MNS, median nerve stimulation; non-MNS, the same electrical stimulation as in the MNS group via acupuncture needles inserted in the middle of the lateral deltoid muscle (a non-median nerve-innervated location); OA, osteoarthritis; OX_1R, orexin 1 receptor; PC6, Neiguan; PPE, plasma protein extravasation; QD, once daily; p-ERK1/2, phosphorylated-extracellular signal-regulated kinase 1/2; PGE_2, prostaglandin E_2; sham-PC6, bilateral needle insertion at the PC6 acupoint without electrical stimulation; ST34, Liangqiu; ST35, Dubi; ST36, Zusanli; TE5, Waiguan; TGES, electrical stimulation of the trigeminal ganglion; vlPAG, ventrolateral periaqueductal gray.



SCHEME 1 | The cannabinoid pathway, including the CB_1 and CB_2 receptors, mediates the analgesic and anti-inflammatory effects of electroacupuncture.

endogenous endocannabinoids from the brain during a placebo response to pain, when non-opioid drugs were used in the preconditioning phase (Benedetti, 2012). For instance, the specific CB_1 receptor antagonist rimonabant can effectively block placebo analgesia elicited by non-opioid pharmacological conditioning with non-steroidal anti-inflammatory drugs (NSAIDs) in healthy volunteers (Benedetti et al., 2011). More animal and human studies are needed to determine how CB_1 receptors contribute to placebo and acupuncture analgesia.

Publications from 2009 onward supporting the involvement of endocannabinoids in EA analgesia are summarized in **Table 1**.

Functions of the Acupoints Selected in the Reviewed Studies

The acupoints reviewed in this article and listed in **Table 1** are primarily found in the upper and lower limbs. GB30 (*Huantiao*) is located on the sciatic nerve path and is used in TCM as a basic point in low back pain (Shao et al., 2015) and for motor function treatment (Yeo et al., 2014). GB34 (*Yanglingquan*) is found on the common peroneal nerve and is used as an additional point to GB30 when low back pain is associated with lower extremity numbness and pain (Shao et al., 2015). Stimulation

of GB34 activates the prefrontal cortex, the precentral gyrus and putamen in patients with Parkinson's disease; areas of the brain that exhibit dysfunction due to nigral dopamine depletion (Yeo et al., 2014). LI4 (*Hegu*) is located on the dorsum of the hand and LI11 (*Quchi*) at the elbow; both are suggested to be particularly useful for improving neck-shoulder-arm disorders (Shiro et al., 2014), with clinical evidence describing the relief of stress, facial pain, headache, toothache, neck, and shoulder pain (LI4) (He et al., 2004; Shen et al., 2009; Pavão et al., 2010; Grillo et al., 2014; Wang et al., 2015), pain-related conditions, and common fever (LI11) (Choi et al., 2018). Acupoints ST34 (*Liangqiu*, above the laterosuperior border of the patella), ST35 (*Dubi*, at the lower border of the patellar) and ST36 (*Zusanli*, on the anterior of the leg lateral to the edge of the tibia, below ST35) are effective for postoperative pain control (Liu et al., 2015); stimulation of ST36 is frequently used to treat dyskinesia and facilitate motor recovery after stroke, to treat pain, hypertension, and other physiological dysfunctions (Sun et al., 2019) such as migraine (Zhao et al., 2012). The acupoint combination of GB20 (*Fengchi*, situated below the base of the skull) and TE5 (*Waiguan*, near the dorsal wrist crease between the radius and ulna) is frequently used for treating migraine (Zhao et al., 2012), while EX-LE4 (*Neixiyan*) and ST35 (both located on the knee) are frequently paired for pain relief, such as in knee osteoarthritis (Ng et al., 2003). PC6 (*Neiguan*, on the palm side of the wrist and located on the median nerve path) is a classical acupoint that is used to treat cardiovascular disorders (Li et al., 2012), for providing postoperative analgesia (Xie et al., 2014), and for preventing nausea and vomiting (Cheong et al., 2013), while BL60 (*Kunlun*, on the posterior aspect of the knee) is frequently used in acupoint combinations for the treatment of low back pain (Lee et al., 2013).

CONCLUSION

No single mechanism can explain EA analgesia. Early preclinical investigations demonstrated that naloxone antagonizes EA analgesia, suggesting that the analgesic action is related to the release of a morphine-like factor in the CNS, supporting the endorphin hypothesis of acupuncture analgesia (Pomeranz and Chiu, 1976). Subsequent research revealed elevated levels of β-endorphin in human cerebrospinal fluid after acupuncture for recurrent pain (Clement-Jones et al., 1980) and in plasma met-enkephalin after EA in patients with chronic pain (Kiser et al., 1983). Different frequencies of EA have been found to elucidate different opiate secretions; low-frequency EA (2 Hz) is associated with analgesia involving met-enkephalin, β-endorphin, and endomorphin, which activate the μ- and δ-opioid receptors, while high-frequency EA (100 Hz) stimulates dynorphin, which activates the κ-opioid receptor (Han, 2004). Evidence on EA analgesia also implicates the midbrain monoamines serotonin and norepinephrine, and anti-inflammatory mechanisms mediated by opioid and non-opioid receptors in the periphery (Zhang et al., 2014).

The discovery of the cannabinoid receptors and endocannabinoids has inspired many investigations that have targeted ECS proteins, the cannabinoid receptors, and the enzymes responsible for the biosynthesis and degradation of the endogenous cannabinoid receptor ligands, in the hope of discovering therapeutic targets that can be treated with novel drugs for a wide range of diseases, including pain (Stasiulewicz et al., 2020). However, these investigations have been complicated by adverse effects arising from the multidirectional nature of the ECS and its inter-relationships with other pharmacological systems and biochemical pathways (Stasiulewicz et al., 2020).

EA interventions are potentially appropriate for activating the ECS. The evidence discussed in this review suggests that EA inhibits inflammatory and neuropathic pain, and that these effects may be associated with modulations of cannabinoid signaling within the ECS. This signaling is illustrated in **Scheme 1**. It appears that the cannabinoid pathway mediates the analgesic and anti-inflammatory effects of acupuncture, via the CB_1 and CB_2 receptors, respectively. However, the current results are all generated from animal studies. Evidence from animal studies remains fragmentary and clinical evidence is lacking. In contrast, evidence for the endorphins theory is supported by not only animal studies, but also human investigations.

Clearly, EA increases different types of endogenous opioids in humans, while naloxone, an opioid receptor antagonist, appears to attenuate acupuncture analgesia in humans. To fully inform the ECS theory of EA analgesia, further human studies are called for. As with the evidence in support of the endorphin hypothesis, biochemical evidence such as measurements of cerebrospinal fluid (CSF) concentrations of endocannabinoids are needed to show how the ECS is influenced by acupuncture interventions. For instance, one study has identified significantly lower CSF concentrations of AEA in patients with chronic migraine and those with probable chronic migraine and probable analgesic-overuse headache compared with non-migraine controls (Sarchielli et al., 2007). Interestingly, CB_1 receptor antagonism is a promising strategy in the treatment of obesity. However, CNS side effects associated with rimonabant and other CB_1 receptor antagonists emphasized the need for new classes of peripherally acting CB_1 receptor antagonists that do not affect the intricate balance between central and peripheral physiological signaling (Sharma et al., 2015; Lv et al., 2019). Ongoing efforts to develop safer selective peripherally acting CB_1 receptor antagonists, potent novel CB_1 receptor antagonists or inverse agonists may eventually result in therapeutics that target CB_1 receptors with reduced CNS side effects (Sharma et al., 2015; Yadav and Murumkar, 2018; Khan N. et al., 2019; Micale et al., 2019). At that point, it would be interesting to determine whether or not CB_1 receptor antagonists attenuate EA analgesia in humans. We would then expect to have much more clarity around the biological basis underlying the mechanisms of EA-induced antinociception involving CB_1 and CB_2 receptors in the ECS.

AUTHOR CONTRIBUTIONS

Y-HC conceived and advised on the manuscript. IM wrote the first draft of the manuscript. Both authors revised the manuscript and agreed with the published version of the manuscript.

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$BNST_{AV}^{GABA}$-PVN^{CRF} Circuit Regulates Visceral Hypersensitivity Induced by Maternal Separation in Vgat-Cre Mice

*Si-Ting Huang[1†], Zhi-Jing Song[1,2†], Yu Liu[1†], Wen-Chen Luo[1], Qian Yin[1] and Yong-Mei Zhang[1]**

[1]*Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, China,* [2]*Department of Anesthesiology, Xuzhou Municipal Hospital Affiliated with Xuzhou Medical University, Xuzhou, China*

Visceral hypersensitivity as a common clinical manifestation of irritable bowel syndrome (IBS) may contribute to the development of chronic visceral pain. Our prior studies authenticated that the activation of the corticotropin-releasing factor (CRF) neurons in paraventricular nucleus (PVN) contributed to visceral hypersensitivity in mice, but puzzles still remain with respect to the underlying hyperactivation of corticotropin-releasing factor neurons. Herein, we employed maternal separation (MS) to establish mouse model of visceral hypersensitivity. The neuronal circuits associated with nociceptive hypersensitivity involved paraventricular nucleus CRF neurons by means of techniques such as behavioral test, pharmacology, molecular biology, retrograde neuronal circuit tracers, electrophysiology, chemogenetics and optogenetics. MS could predispose the elevated firing frequency of CRF neurons in PVN in murine adulthood, which could be annulled via the injection of exogenous GABA (0.3mM, 0.2μl) into PVN. The PVN-projecting GABAergic neurons were mainly distributed in the anterior ventral (AV) region in the bed nucleus of stria terminalis (BNST), wherein the excitability of these GABAergic neurons was reduced. Casp3 virus was utilized to induce apoptosis of GABA neurons in BNST-AV region, resulting in the activation of CRF neurons in PVN and visceral hyperalgesia. In parallel, chemogenetic and optogenetic approaches to activate GABAergic $BNST_{AV}$-PVN circuit in MS mice abated the spontaneous firing frequency of PVN CRF neurons and prevented the development of visceral hypersensitivity. A priori, PVN^{CRF}-projecting GABAergic neurons in BNST-AV region participated in the occurrence of visceral hypersensitivity induced by MS. Our research may provide a new insight into the neural circuit mechanism of chronic visceral pain.

Keywords: visceral hypersensitivity, hypothalamic paraventricular nucleus, bed nucleus of stria terminalis, GABA neurons, corticotropin-releasing factor, maternal separation

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Hokkaido University, Japan
Marsida Kallupi,
University of California, United States

****Correspondence:***
Yong-Mei Zhang
zhangym700@xzhmu.edu.cn

†These authors have contributed equally to this work

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INTRODUCTION

The irritable bowel syndrome (IBS) is a common functional disease characterized by chronic abdominal pain and abnormality of bowel movement (Thompson et al., 1999; Farmer and Aziz, 2013), with a morbidity of approximately between 7 and 21% worldwide (Lovell and Ford, 2012; Vich Vila et al., 2018). The pathogenesis of IBS involves visceral hypersensitivity, abnormal gastrointestinal motility, disordered brain-gut-microbiota axis activity, psychological

comorbidities, etc. (Moloney et al., 2016; Melchior et al., 2018). Notably, visceral hypersensitivity is identified in 20–90% of patients with IBS (Azpiroz et al., 2007). However, the essential mechanisms underpinning visceral hypersensitivity in IBS still await further illumination.

Early life stress (ELS) is an adverse stressful event in the early stage of life, which predisposes multiple diseases in adulthood including IBS (De Kloet et al., 2005). Maternal separation (MS) is commonly adopted as an ELS model to investigate the underlying mechanisms of functional gastrointestinal and psychiatric disorders (O'Mahony et al., 2009). ELS is known to affect the development of synaptic plasticity and neural circuits (Sun et al., 2019). Our previous studies identified that corticotrophin-releasing factor (CRF) neurons in the paraventricular nucleus (PVN) and hypothalamic-pituitary-adrenal (HPA) axis were involved in the development of MS-induced visceral hypersensitivity (Zhang et al., 2016; Tang et al., 2017). The activation of PVN CRF neurons and HPA axis is regulated by a variety of mechanisms, such as glucocorticoid feedback (Kloet, 2013), excitatory activity of glutamate neurons (Gunn et al., 2013) and the inhibitory action of γ-aminobutyric acid (GABA) neurons (Cullinan et al., 2008). PVN is governed dominantly by GABAergic neurotransmission (Decavel and Van den Pol, 1990). Importantly, the GABAergic afferents onto PVN can reportedly play crucial roles in visceral hypersensitivity. Moreover, there is morphological evidence that the GABA synapses in medial parvocellular paraventricular hypothalamus (PVNmp) account for approximately half of all synapses (Decavel and Van den Pol, 1990), wherein PVNmp cells receive GABAergic inhibitory inputs mainly from the marginal structures, including the medial preoptic area, the bed nucleus of the stria terminalis (BNST), and the medial hypothalamic nucleus (Ulrich-Lai and Herman, 2009). Therefore, the revelation of the upstream inhibitory nucleus projecting to PVN CRF neurons is of significance.

BNST is recognized as "extended amygdala, "which connects the forebrain to the hypothalamus and brainstem regions and is associated with autonomic and neuroendocrine functions due to its remarkable effects on mood and emotion (Carlos et al., 2013; Lawrence, 2018). During the early postnatal period, BNST also undergoes developmental maturation like PVN CRF neurons, ELS renders it vulnerable to visceral hypersensitivity, anxiety-like and social behaviors, and so on (Emmons et al., 2021). BNST per se is an area with high heterogeneity in structure and function (Prewitt and Herman, 1998), with a vast majority of PVN CRF neurons receiving signals from its distinctive subregions. BNST is divided into the anterior and posterior regions, with the anterior area further divided into anterior middle (AM), anterior lateral (AL) and anterior ventral (AV) regions (Goodson and Kabelik, 2009; Davis et al., 2010). The vast majority of neurons in BNST-AV was GABAergic (Sun and Cassell, 1993), and most of GABAergic BNST-PVN projections are derived from BNST-AV GABAergic neurons to PVNmp, especially CRF neurons (Dong and Swanson, 2004; Dong and Swanson, 2005). MS can reportedly inhibit the activation of BNST neurons accompanied by decreased c-Fos expression (Banihashemi et al., 2011), whereas temporary MS suffices to evoke Fos expression within BNST (Fenoglio et al., 2006). In this respect, further exploration of the potential roles of BNST targeting visceral hypersensitivity instigated by MS would be of significance.

We hypothesized that inhibition of PVN-projecting GABAergic neurons in BNST-AV region could lead to the activation of PVN CRF neurons and the consequent visceral hypersensitivity in MS mice. To specifically validate the implication of GABAergic neurons in $BNST_{AV}$-PVN circuit, vesicular GABA transporter (Vgat)-Cre transgenic mice were recruited in this study.

MATERIALS AND METHODS

Animals

Vgat-ires-Cre knock-in mice (JAX number: 028862) aged 8–10 weeks were provided by the Jackson Laboratory in the United States. Vgat-Cre mice have Cre recombinase expression directed to inhibitory GABAergic neuron cell bodies for studying regulation of function or mapping the GABAergic neurons. Neonatal mice which were bred from the adult mice (one male with two females) until weaning at postnatal 21st day were randomly allotted in a standard triangular Plexiglas cage. All mice were housed under a 12h/12h light/dark cycle with food and water available ad libitum. All experiments were fully compliant with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978), and were approved by the Ethics Committee on Experimental Animal of Xuzhou Medical University.

Animal Experimental Groups

The neonatal mice were divided into non-maternal separation (NMS) group and maternal separation (MS) group according to Miranda et al. (Armando et al., 2013). Owing to the difficulty of gender identification, all the pups in MS group were separated from the dams for 6h every day as of the 2nd to 15th day after birth (9:00–12:00 a.m. and 15:00–18:00 p.m.). Albeit female animals could serve to mimic the common clinical symptoms in female IBS-patients, the variations of estrogen and progesterone in response to CRD may affect the pain perception and underlying pain circuitry during the estrus cycle. In addition, BNST is a sexually dimorphic structure rich in distinctive neuronal subpopulations (Zhang et al., 2018; Liu et al., 2019). Accordingly, we only recruited neonatal male mice, which were reared to the postnatal 8^{th} week for experimentation.

Reagents

Sheep polyclonal anti-Corticotropin Releasing Factor Antibody (NB110-81721, Novus, USA); rabbit anti-c-Fos mAb (2250s, Cell Signaling Technology, MA, USA); γ-aminobutyric acid (A2129-25G, Sigma-Aldrich LC, USA); muscimol hydrobromide (G019, Sigma-Aldrich); mouse monoclonal Anti-GAD 67 (MAB5406, Millipore, USA); rabbit polyclonal anti-GAD 67 (10408-1-AP, Proteintech, USA); mouse polyclonal anti-GAD 65 (20746-1-AP, Proteintech, USA); mouse anti-β-actin mAb (sc-47778, Santa Cruz, CA, USA); rabbit anti-GAPDH pAb (AC001, Abclonal, MA, USA); Alexa Fluor 488 donkey anti-Rabbit IgG (H + L) and

Alexa Fluor 594 donkey anti-Sheep IgG (H + L) (Life Technologies, CA, USA); DAPI staining solution (C1005, Beyotime, China); alkaline phosphatase horse anti-mouse IgG (ZB-2310, Zsbio, China); HRP-labeled goat anti-mouse IgG (H + L) (A0216, Beyotime, China); BCA protein assay kit (P0012 Beyotime, China); RIPA lysis buffer (P00138, Beyotime, China); phenyl-methanesulfonyl fluoride (PMSF) (ST506, Beyotime, China); sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (P0015, Beyotime, China); BCIP/NBT alkaline phosphatase color development kit (C3206, Beyotime, China); BeyoECL Moon kit (P0018, Beyotime, China).

Adeno-Associated Virus (AAV) Tools

PVN-microinjection: rAAV-CRF-EYFP-WPRE-pA (2.04×10^{12} vg/ml, Customized, BrainVTA, China) ; rAAV-Ef1α-DIO-mCherry-WPRE-pA (retro) (2.06×10^{12} vg/ml, BrainVTA, China).

BNST-microinjection: rAAV-Ef1α-DIO-mCherry-WPRE-pA (2.06×10^{12} vg/mL, BrainVTA, China); rAAV-flex-taCasp3-TEVp-WPRE-pA, AAV 2/9 (2.06×10^{12} vg/mL, BrainVTA, China). rAAV-Ef1α-DIO-hM4D(Gi)-mCherry-WPRE-pA (2.06×10^{12} vg/ml, BrainVTA, China); rAAV-Ef1α-DIO-hM3D(Gq)-mCherry-WPRE-pA (2.06×10^{12} vg/ml, BrainVTA, China); rAAV-Ef1α-DIO-hChR2(H134R)-mCherry-WPRE-pA (2.06×10^{12}vg/ml, BrainVTA, China); rAAV-Ef1α-DIO-eNpHR3.0-mCherry-WPRE-pA (2.06×10^{12} vg/ml, BrainVTA, China).

A duration of two or three weeks was adequate for the AAV expression. The microinjection volume was 200nl unilaterally at a rate of 60nl/min.

Detection of visceral Pain Threshold and Abdominal Withdrawal Reflex (AWR) Scores

For detection of visceral pain threshold as per the description by Julie et al. (Christianson and Gebhart, 2007), mice in both MS and NMS groups were anesthetized under sevoflurane, followed by insertion of an uninflated balloon coated with the paraffin oil into the colorectal tract, with the end of the balloon maintained 0.5cm away from the anal margin. After 15min of acclimatization, graded distension was performed by rapid inflation of the balloon to a pressure as specified values (20, 40, 60, 80mmHg) for 20s, followed by a 4min break. According to the response of colorectal walls of the mice to the expansion pressure, it is divided into the following four grades, i.e., AWR score: 0 point, no significant behavioral change; 1 point, motionless or only simple head movement; 2 points, contraction of abdominal wall muscles and contact with the table; 3 points, contraction of abdominal wall muscles and without contact with the table; 4 points, arching of abdominal wall with or without the arching of the body and pelvis. The pain threshold was determined as per the AWR scoring criteria, i.e., the minimum pressure value to induce significant contraction of the abdominal wall or lifting off the desktop (AWR score ≥3 points) recorded. Mice measurements were in triplicate and averaged for further analysis.

High Plus Maze

The elevated cross maze experiment was mainly adopted to observe the anxiety state of animals. In brief, the elevated cross maze is 50cm high from the ground, consisting of a vertically crossed open arm (60cm × 5cm, no border) and a closed arm (60cm × 5cm, high border 25cm) composed of two arms, with a central cross area of 5cm × 5cm in the middle. Each mouse was allowed a duration of 5min for measurement. Caution should be taken to avoid noise and light stimulation. The murine subjects were placed in the middle of the platform, simultaneously ensuring that the head of the mouse was positioned toward the closed arm. An entry was defined as placement of the four paws within boundaries of the arm. The test process was recorded by a camera connected to the computer throughout the experiment, with the times of entry into the open arm recorded.

Open Field Test

The device for the open field experiment was a box (50cm × 50cm × 45cm) with a white bottom evenly divided into 9 squares. The middle square was named as the central area and the ambient 8 squares outer area. Briefly, at the commencement of each experiment, the mice were gently placed in the central area. With the camera connected to the computer, the system was subsequently clicked on to start recording of mouse behavior within 5min. The observation index was: the percentage of the duration of mice in the central area.

Sucrose Preference Test

The sucrose preference assay can reflect the euphoria of mice. The decreased consumption of sucrose indicated dysphoria in mice, which was one of the key symptoms of depression. The mice were deprived of water for 24h and subsequently underwent sucrose preference assay. During the experiment, each mouse was housed separately and provided with two drinking bottles containing 1% sucrose solution and pure water. Both bottles of liquid were weighed and recorded in advance. During the test, the positions of two bottles were exchanged. After 24h, the two bottles were retrieved for weighing to calculate the ratio of sucrose consumption of each mouse within 24h according to the following formula: sucrose consumption percentage = sucrose consumption/(sucrose consumption + pure water consumption) × 100%.

Forced Swimming Test

Mice were gently placed in a glass tank (30cm in height, 15cm in diameter, 15cm in water depth), with the water temperature maintained at 25–30°C. Afterwards, the latency in which the mice struggled desperately to escape was recorded, and the duration in which the mice presented a typical "floating state" was recorded within 5 min, wherein "floating state" was defined as the motionless state of the mouse other than some necessary movement to keep the head above water. At the end of each test, fresh water was replaced to prevent the next mouse from being affected by the odor of the previous one and from infection from suffocation in water.

Catheterization and Stereotactic Administration via Cannula in PVN

Adult male Vgat-Cre mice, 23–28g in weight, were anesthetized with 1% pentobarbital sodium (60mg/kg, i.p.). According to the Atlas of Mouse Brain by Keith BJ Franklin and George Paxinos, the third edition, coordinates for localization were: PVN (A/P: −0.05–0.15 mm; L/R: ± 0.25 mm; D/V: −5.0–5.1mm), BNST (A/P: 0.9–1.0 mm; L/R: ± 0.8 mm; D/V: −4.7–4.75mm). Each mouse was injected at a constant rate of 60nl/min, with the needle remaining in site for 10min thereafter to prevent the drug reflux. The experiment would be conducted in the case of the virus expression for at least 14 or 21 days.

PVN catheterization was performed as follows: with the mouse fixed on the brain stereotaxic instrument as described above, the 5.0mm ferruled cannula should be vertically fixed on the holder of the locator with the length of the inserted core needle slightly longer than that of the cannula. The dental cement was mixed around the sleeve and the metal cap was screwed onto the cannula. After 1 week, mice were anesthetized with inhaled sevoflurane of low concentration, the metal cap of the cannula was unscrewed, and the catheter inserted into the cannula, followed by gentle administration of the drug via a micro-syringe pump. The catheterization of optical fiber was identical to the procedures described above.

Immunofluorescence Analyses

After deep anesthesia, the mice were transcardially perfused with 20ml of 0.9% saline, followed by infusion of 20ml 4% paraformaldehyde. The mouse brain was carefully isolated and further stored in 4% in polyoxymethylene at 4°C overnight for fixation before transference to 30% sucrose. The brain tissue was sectioned into 30μm-thick slices, and collected in 0.01M PBS. Slices were rinsed with PBS in triplicate (5min each), and blocked with 10% donkey serum for 2h prior to incubation with the c-Fos antibody (1: 1000) and CRF antibody (1: 200) diluted in PBS containing 0.3% Triton X-100 at 4°C for 24h. After PBS lavage, the corresponding sections were incubated with anti-mouse Alexa Fluro 488 or anti-rabbit Alexa Fluor 594 in dark at room temperature (r/t) for 2h. The tissue sections were counterstained with DAPI (4,6-diamino-2-phenylindole) before mounting with 90% glycerol and visualized with a confocal laser microscope (FV1000, Olympus, Tokyo, Japan).

Western Blot Analysis

Western blot analysis was adopted to characterize the protein expression in PVN and BNST-AV. Mice in each group were quickly decapitated to isolate brains and obtain target tissues. The brain tissues were placed into a 2ml pre-chilled Eppendorf tube containing RIPA lysis buffer with PMSF. The specimens were centrifuged at 12000rpm for 15min at 4°C, followed by collection of supernatants. 15μg of protein lyses was separated with 10% separation polyacrylamide gel before transference onto the PVDF membrane. With the addition of 5% skim milk and storage on a shaker for 2h for blockage, the membranes were incubated with primary antibody at 4°C in a shaker overnight. The primary antibodies included: mouse anti-CRF primary antibody (1: 1000), mouse anti-GAD65 and GAD67 primary antibodies (1: 1000), mouse anti-GAPDH primary antibody (1: 1000) or mouse anti-β-actin (1: 1000). After triplicated lavage with washing buffer for 5min on the next day, the membranes were incubated with the corresponding secondary antibody at r/t for 40min on a shaker. After rinse in triplicate with washing buffer 10min, the protein band were visualized by ProPlus image analysis system (NIH, Bethesda, MD, United States) and analyzed by Image J software.

In vitro Electrophysiology

Cell-attached recording is extensively adopted to study the spontaneous firing of mammalian neurons. PVN or BNST-AV neurons were recorded in brain slices via cell-attached electrophysiology. After anesthetization, each mouse was transcardially perfused, followed by isolation of mouse brain, which were to be sectioned into 280μm-thick slices. The sections were incubated in high-sugar cerebrospinal fluid at 34°C for 1h, and at r/t for another 30min. Then the brain slices were transferred to the perfusion tank on the electrophysiological table with a special pipette for recording. The brain slices were perfused with artificial cerebrospinal fluid (ACSF), which was fully oxygenated with a mixture of 95% O_2 and 5% CO_2. The composition of high-sugar cerebrospinal fluid was (mM): 3.5 KCl, 0.5 $CaCl_2$, 4.5 $MgSO_4$, 80 NaCl, 90 sucrose, 10 glucose, 1.25 NaH_2PO_4, 25 $NaHCO_3$ (295–305 mOsm, pH = 7.35). The composition of ACSF was (mM): 2.5 KCl, 126 NaCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 2.4 $CaCl_2$ and 10 glucose (295–305 mOsm, pH 7.35). Microelectrode was immerged in the electrode solution (Pippette solution) and the electrode resistance was set to 6–10MΩ. The composition of the electrode solution was (mM): 10 HEPES, 5 EGTA, 135K gluconate, 2 $MgCl_2$, 3 ATP-Mg, 0.5 $CaCl_2$, 0.2 GTP-Na (280–290 mOsm, pH = 7.25). The sealing resistance was greater than 70MΩ for recording the spontaneous discharge. The parameter was set at the current clamp with I = 0, the high wave filtering was 300kHz, and the low wave filtering was 0.5kHz, the signal acquisition frequency was 10kHz, and the recording commenced after the onset of a signal with a regular discharge frequency.

In adult mice with MS-induced chronic visceral pain, rAAV-Ef1α-DIO-hChR2(H134R)-mCherry-WPRE-pA was bilaterally injected into the BNST-AV area, and rAAV- CRF-EYFP-WPRE-pA virus was bilaterally injected into the PVN. After 21 days, the blue light (473nm) was employed to activate the BNST-AV GABAergic neuron terminals extending to PVN. The depolarization current of the GABA neurons in the BNST-AV area was recorded. The discharge frequencies during light-on period and before and after blue light excitation were counted separately. In pharmacological experiments, the firing frequencies before and after exogenous GABA perfusion were separately calculated.

Chemogenetics

rAAV-Ef1α-DIO-hM4D(Gi)-mCherry-WPRE-pA and rAAV-Ef1α-DIO-hM3D(Gq)-mCherry-WPRE-pA were bilaterally injected into the BNST-AV region. With respect to clozapine-N-oxide (CNO) administration in PVN, a trocar was implanted 0.2mm above the third ventricle adjacent to PVN (A/P:

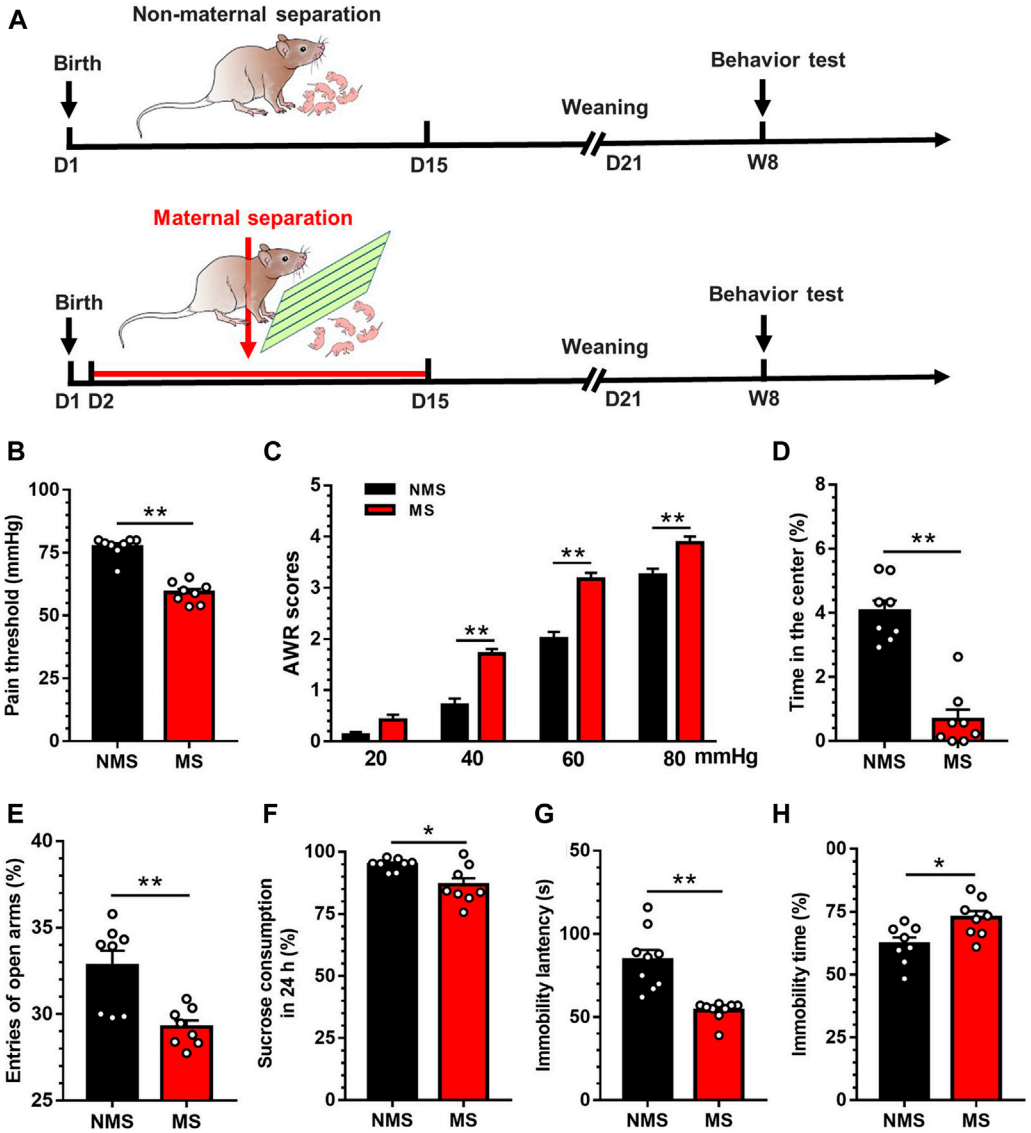

FIGURE 1 | Chronic visceral pain, anxiety- and depression-like behaviors were induced by MS in mice **(A)** The establishment of maternal separation model **(B)** Visceral pain threshold in MS group was decreased compared with NMS group ($n = 8$) **(C)** AWR scores in MS group were increased compared with NMS group ($n = 8$) **(D)** Duration in the center was significantly decreased in MS group compared with NMS group ($n = 8$) **(E)** The times of entry into open arms was significantly decreased in MS group compared with NMS group ($n = 8$) **(F)** The sucrose consumption in MS group was significantly decreased compared with NMS group in 24h ($n = 8$) **(G)** The latency of immobility in MS group was significantly decreased in comparison with NMS group in forced swimming test ($n = 8$) **(H)** The immobility duration in MS group was significantly increased compared with NMS group in forced swimming test ($n = 8$). Data are presented as the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with NMS group.

-0.05–0.15mm, L/R: ±0.25mm, D/V: -4.80mm from the bregma). Mice received PVN infusion of vehicle or CNO to stimulate the Gi/Gq-coupled designer receptor exclusively activated by designer drugs (DREADD). Behavioral experiments were performed at least 3 weeks thereafter to allow for sufficient viral expression.

Optogenetic Experiment

rAAV-Ef1α-DIO-hChR2(H134R)-mCherry-WPRE-pA and rAAV-Ef1α-DIO-eNpHR3.0-mCherry-WPRE-pA were bilaterally injected into the BNST-AV region. For optogenetic manipulation, the optical fiber was implanted above the third ventricle adjacent to PVN (A/P: −0.05–0.15 mm, L/R: ± 0.25mm, D/V: −4.80mm from the bregma). The optical power was 1.8mW for the blue laser (473nm) and 3.5mW for the yellow laser (589nm), as measured at the tip of the optic fiber. The photogenetic electrophysiology of isolated brain slices revealed the frequency of 10Hz and the wave width of 10ms, and blue laser with a wave length of 473nm could cause ChR2-mCherry + neurons to produce depolarizing current. Meanwhile, we also utilized a blue light with a frequency of 10Hz and a wave width of 10ms to activate the $BNST_{AV}$-PVN GABAergic neuron terminals,

aiming to investigate the specific circuit effects on the visceral pain threshold. Mice underwent constant photostimulation for 10min, with the visceral pain threshold detected 5min after illumination of blue light pulses. The average value was calculated in triplicate for further analysis.

Statistical Analysis

Data are expressed as mean ± S.E.M. Independent samples *t*-test was used between two groups; and one-way ANOVA was used among multiple groups. The time courses of pain thresholds were analyzed with a two-way repeated measure ANOVA. For statistical differences, post hoc Bonferroni or SNK was used for pairwise comparison. The test level was set as $\alpha = 0.05$, and p value less than 0.05 was considered statistically significant.

RESULTS

Maternal Separation in Neonatal Mice Induced Visceral Hypersensitivity, Anxiety- and Depression-like Behavior in Adulthood

The timeline of model establishment and behavioral detection is depicted in **Figure 1A**. Neonatal mice were separated from the maternal mouse for 6h per day as of the 2nd–15th day postnatally. To minimize the confounders in measurements in mice, each group ($n = 8$ mice) only underwent a single item of the behavior experiments performed by individual technicians. In this respect, the time point of each behavior experiments could not have resulted in the bias which might have occurred in mice undergoing all the behavior experiments. In parallel, mice with NMS underwent all the procedures other than MS. Consequently, visceral pain threshold in the MS group was significantly lower than that in the NMS group[$t(14) = 8.75$, $p < 0.01$; **Figure 1B**]. Likewise, the elevated AWR scores further authenticated the development of visceral hypersensitivity in MS mice rather than NMS group (**Figure 1C**). A two-way repeated measures ANOVA revealed that a significant difference in group [$F(1, 14) = 58.79$, $p < 0.01$], treatment [$F(3, 42) = 375.9$, $p < 0.01$] and group × treatment interaction [$F(3, 42) = 6.80$, $p < 0.01$]. Post hoc Bonferroni multiple comparisons indicated a significant increase in the AWR scores at 40, 60 and 80mmHg in MS mice as compared with NMS group ($p < 0.01$). As for the open field test, the duration in the central area was significantly lower in the MS group than in the NMS group [$t(14) = 7.38$, $p < 0.01$; **Figure 1D**]. The times of entry into open arms in the MS group was significantly lower than that in the NMS group in the elevated maze test [$t(14) = 3.71$, $p < 0.01$; **Figure 1E**], suggesting that MS could induce anxiety-like behavior in adult mice. The sucrose preference assays showed that sucrose consumption was significantly reduced at 24h in the MS group compared with the NMS group [$t(14) = 2.89$, $p < 0.05$; **Figure 1F**)] Forced swimming test results presented shortened immobility latency and prolonged immobility duration in the MS group vs. the NMS group [$t(16) = 4.81$, $p < 0.01$; **Figure 1G** $t(14) = 2.74$, $p < 0.05$; **Figure 1H**], indicating that MS induced depression in adult mice. These results demonstrated that neonatal MS mice were susceptible to the visceral hypersensitivity and anxiety- and depression-like behaviors in adulthood.

Involvement of Activation of CRF Neurons in PVN in the MS-Induced Visceral Hypersensitivity

To explore the mechanism that may underlie the development of visceral hypersensitivity, we first evaluated whether MS could induce the activation of PVN CRF neurons. The experimental flow is shown in **Figure 2A**. Western blot revealed that the expression of CRF protein in the PVN was significantly upregulated in the MS group as compared to the NMS group [$t(10) = 2.52$, $p < 0.05$; **Figure 2B**]. Afterwards, electrophysiological patch clamp technique was utilized to record the firing frequency of CRF neurons in PVN. For the specific recognition of CRF neurons, CRF-specific promoter AAV virus (rAAV-CRF-EYFP-WPRE-pA) was injected into PVN 21 days preceding the visceral pain threshold measurement and electrophysiological recording. **Figure 2C** illustrated the schema of CRF labeling neurons. The schematic diagram of firing frequency is shown in **Figure 2D**. One-way ANOVA showed a significant difference in discharge frequency of CRF neurons in PVN [$F(3,64) = 10.00$, $p < 0.01$; **Figure 2E**)]. Post hoc Bonferroni multiple comparisons showed that the firing frequency of CRF neurons was significantly increased compared with other three groups. In our previous study (Song et al., 2020), GABA has been reportedly implicated in the pathogenesis of visceral hypersensitivity, and we referred to the PVN administration of exogenous GABA (0.3mM, 0.2μL). The visceral hypersensitivity was altered after GABA treatment in NMS mice (**Figure 2F**). A two-way repeated measures ANOVA showed that a significant difference in group [$F(1, 14) = 11.82$, $p < 0.01$], time [$F(3, 42) = 13.59$, $p < 0.01$] and group × time interaction [$F(3, 42) = 12.41$, $p < 0.01$]. Post hoc Bonferroni multiple comparisons showed that the visceral pain threshold was significantly increased 30 and 60min after injection of exogenous GABA compared with saline injection ($p < 0.01$). Similarly, the visceral hypersensitivity was relieved after GABA treatment in MS mice (**Figure 2G**). A two-way repeated measures ANOVA showed that a significant difference in group [$F(1, 14) = 27.91$, $p < 0.01$], time [$F(3, 42) = 2.86$, $p = 0.048$] and group × time interaction [$F(3, 42) = 45.25$, $p < 0.01$]. Post hoc Bonferroni multiple comparisons showed that the visceral pain threshold was significantly increased 30 and 60min after injection of exogenous GABA compared with saline injection ($p < 0.01$). Further, electrophysiological recording was adopted to assess the spontaneous firing frequency of CRF neurons in PVN in mice with chronic visceral pain induced by neonatal MS, wherein GABA (3μM) was dissolved with artificial cerebrospinal fluid (ACSF). The schematic diagram of firing frequency is shown in **Figure 2H**. Exogenous GABA inhibited the discharge frequency of CRF neurons in PVN in mice with neonatal MS. One-way ANOVA showed a significant difference [$F(2,33) = 26.71$, $p < 0.01$; **Figure 2I**]. Post hoc Bonferroni multiple comparisons showed that the firing frequency of CRF neurons was significantly decreased with GABA perfusion compared with

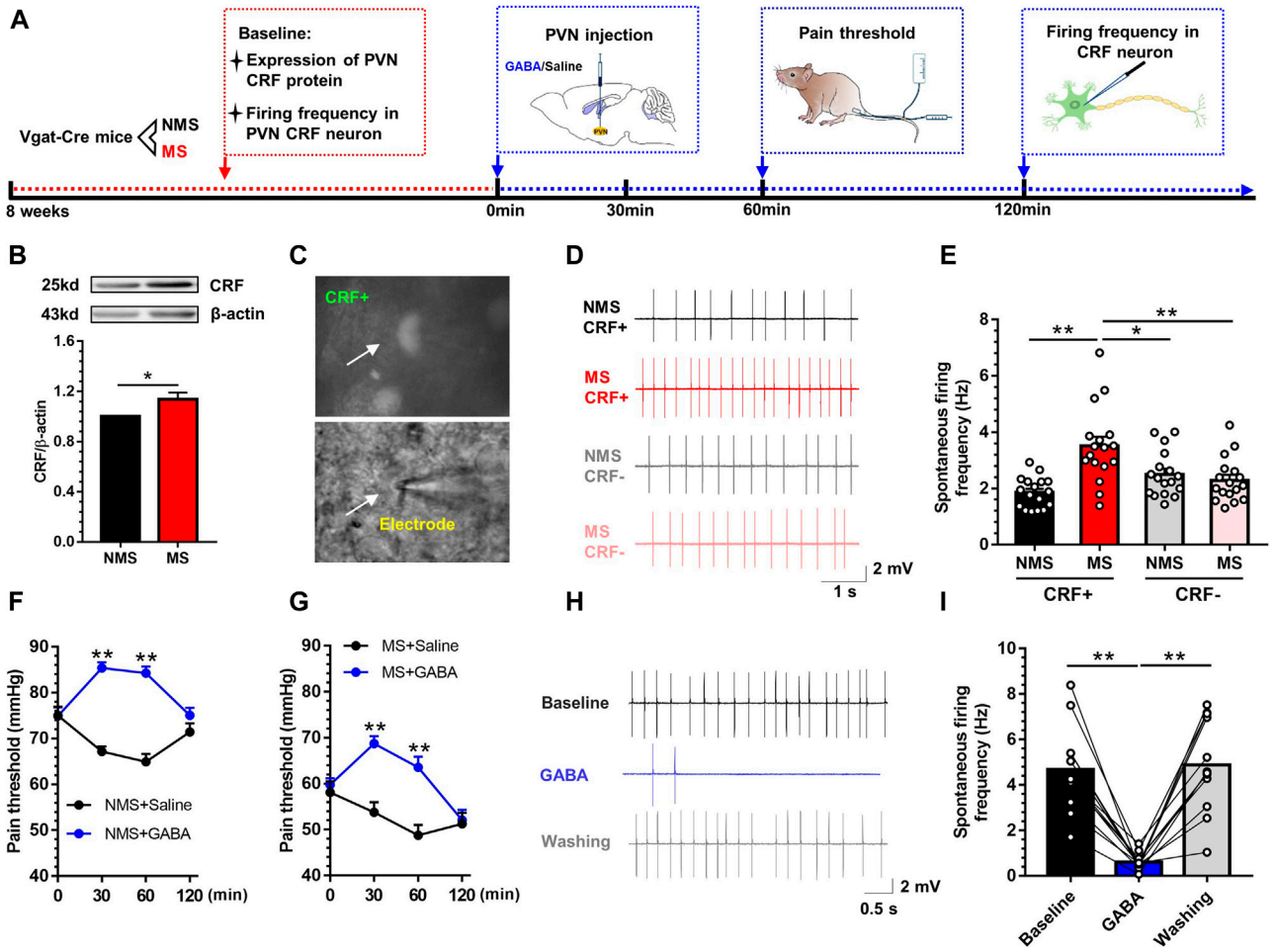

FIGURE 2 | MS induced the activation of CRF neurons in PVN, while microinjection of exogenous GABA into PVN could alleviate the visceral pain **(A)** The experiment flow and timeline for the activity of PVN CRF neurons **(B)** Western blot showed that the expression of CRF protein was significantly increased in MS group vs. NMS group ($n = 6$) **(C)** The diagram of specific CRF-neurons **(D)** Schema of the typical firing frequency **(E)** The spontaneous firing frequency of CRF neurons in MS group was significantly increased vs. NMS group ($n = 17$, six mice per group) **(F)** Injection of exogenous GABA in PVN increased the visceral pain threshold in NMS mice vs. saline group ($n = 8$) **(G)** Injection of exogenous GABA in PVN increased the visceral pain threshold in MS mice vs. saline group ($n = 8$) **(H)** Schema of the typical firing frequency **(I)** Perfusion of exogenous GABA throughout brain slices could decrease the CRF neurons firing frequency in PVN of mice vs. baseline and post-perfusion profiles ($n = 12$, six mice per group). Data are presented as the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. indicated group.

baseline and post-administration lavage. These results demonstrated that the PVN CRF neurons were activated in mice experiencing MS.

Distribution of GABAergic Neurons Projecting to PVN in the Anterior Ventral Area of BNST

Accumulative studies have confirmed that PVN receives inhibitory neuronal projections mostly from BNST. In order to further identify and map the specific areas of neuronal distribution, we first traced the upstream nuclei projecting to PVN via PVN-injection of Lumafluor/Red retrobeads. We observed the red beads were absorbed by PVN neurons and directly transmitted to mPFC, BNST-AV, BNST-AL, BNST-AM and DR (**Figure 3A**). The red beads of BNST could be identified in BNST-AL, BNST-AM and BNST-AV, and were mainly concentrated in BNST-AV. After calculation, the proportion of red beads retrograde to BNST-AV region accounts for 72.7% of the total projection area in BNST. Thereafter, GABA synthetases GAD65 and GAD67 were employed to detect the expression of GABA in the BNST-AV region. GAD65 and GAD67 are glutamic acid decarboxylase necessary for the synthesis of GABA neurons, with GAD65 mainly distributed in the synaptic cleft, whereas GAD67 mainly identified in neurons. As a result, the expression of GAD65 protein in the BNST-AV area was significantly decreased in the MS group compared with the NMS group [$t(10) = 3.34$, $p < 0.01$; **Figure 3B**], as well as the expression of GAD67 [$t(10) = 3.41$, $p < 0.01$; **Figure 3C**]. Afterward, Cre-dependent retrograde virus rAAV-Ef1α -DIO-mCherry-WPRE-pA was injected into PVN, and the mCherry fluorescence was detected in BNST-AV 21 days thereafter (**Figure 3D**). **Figure 3E**

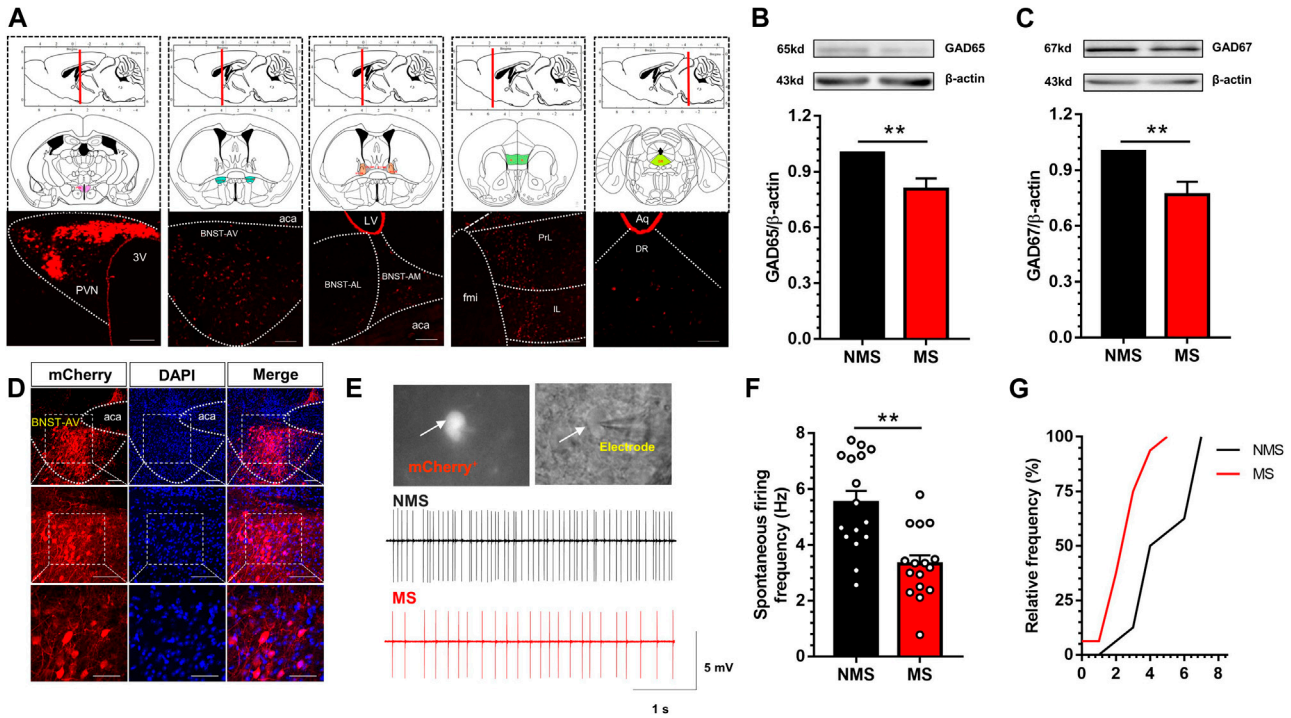

FIGURE 3 | Excitation of GABA neurons in BNST-AV region was decreased in mice with MS-induced chronic visceral pain **(A)** Luma was absorbed by PVN neurons and directly transmitted to mPFC, BNST-AV, BNST-AL, BNST-AM and DR (3V, third ventricle; fmi, forceps minor of corpus callosum; PrL: prelimbic cortex; IL: infralimbic cortex; aca, anterior limb of ac; LV, lateral ventricle; Aq, mesencephalic aqueduct; DR: dorsal raphe nucleus). scale bar = 100μm **(B–C)** Western blot analysis of GAD65 and GAD67 protein in the BNST-AV demonstrated that MS mice presented significant decrease in the expression of GAD65 protein (B, $n = 6$; $^{**}p < 0.01$) and GAD67 (D, $n = 6$; $^{**}p < 0.01$) compared with NMS group **(D)** The localization of GABAergic $BNST_{AV}$-PVN neurons via PVN-injected retrograde rAAV-Ef1α-DIO-mCherry-WPRE-pA in Vgat-Cre mice. Scale bar = 100μm except for the bottom marked by 50μm **(E)** The schema of spontaneous firing frequency in GABAergic neurons in BNST-AV regions **(F)** The spontaneous firing frequency of GABA neurons (mCherry+) in MS group was significantly decreased vs. NMS group ($n = 16$, six mice per group; $^{**}p < 0.01$) **(G)** The cumulative frequency distribution curves in MS groups were evidently shifted leftward.

described the schema of GABA neurons and discharge diagram of GABA neurons. Consequently, the spontaneous discharge frequency of GABA neurons was lower in the BNST-AV region in the MS group than in the NMS group [t (30) = 4.17, $p < 0.01$; **Figure 3F**], with the evidently leftward distribution curve of cumulative frequencies (**Figure 3G**). These data authenticated the exact existence of direct GABAergic $BNST_{AV}$-PVN projection, and the attenuated excitability of GABA neurons in BNST-AV area projecting to PVN in the case of MS-induced visceral hypersensitivity.

Ablation of BNST-AV GABAergic Neurons Induced the Activation of CRF Neurons in PVN and Visceral Pain

Subsequently, a specific pro-apoptotic virus was injected in BNST-AV region to observe whether GABA neurons in BNST-AV region were involved in the regulation of visceral pain in normal Vgat-Cre mice. The virus can specifically damage the GABAergic BNST-AV neurons in Vgat-Cre transgenic mice by means of BNST-AV injection of rAAV-flex-taCasp3-TEVp-WPRE-pA, and the morphology of GABAergic neurons in BNST-AV were observed via PVN injection of retrograde rAAV-Ef1α -DIO-mCherry-WPRE-pA (hereinafter referred to as Casp3 group), whereas mice in the control group were treated with retrograde rAAV-Ef1 alpha -DIO-mCherry-WPRE-pA (control group). The time flow of apoptotic experiment is shown in **Figure 4A**. The apoptotic effect of GABA neurons in the BNST-AV area could be verified by loss of original cellular morphology (**Figure 4B**). Moreover, the visceral hyperalgesia developed in the Casp3 group compared with the control group (**Figure 4C**). A two-way repeated measures ANOVA showed that a significant difference in group [($F(1, 14) = 43.41$, $p < 0.01$], time [$F(1, 14) = 32.77$, $p < 0.01$] and group × time interaction [$F(1, 14) = 34.14$, $p < 0.01$]. Post hoc Bonferroni multiple comparisons showed that the visceral pain threshold in Casp3 group was significantly decreased three weeks after microinjection ($p < 0.01$). Destruction of these regions will cause irreversible "neuronal death". Moreover, the visceral pain threshold in Casp3 group was still decreased 2 months after microinjection, and we speculated this effect will persist in the absence of a compensatory mechanism. Further, the activity of CRF neurons was detected to explore the regulatory role of GABAergic neurons in $BNST_{AV}$-PVN region. The colocalization of c-Fos and CRF in PVN was delineated in **Figure 4D**, and the population of c-Fos-labeled CRF neurons significantly increased after Casp3 virus treatment [t (8) = 6.19, $p < 0.01$; **Figure 4E**]. Likewise, the firing frequency of CRF neurons in PVN was

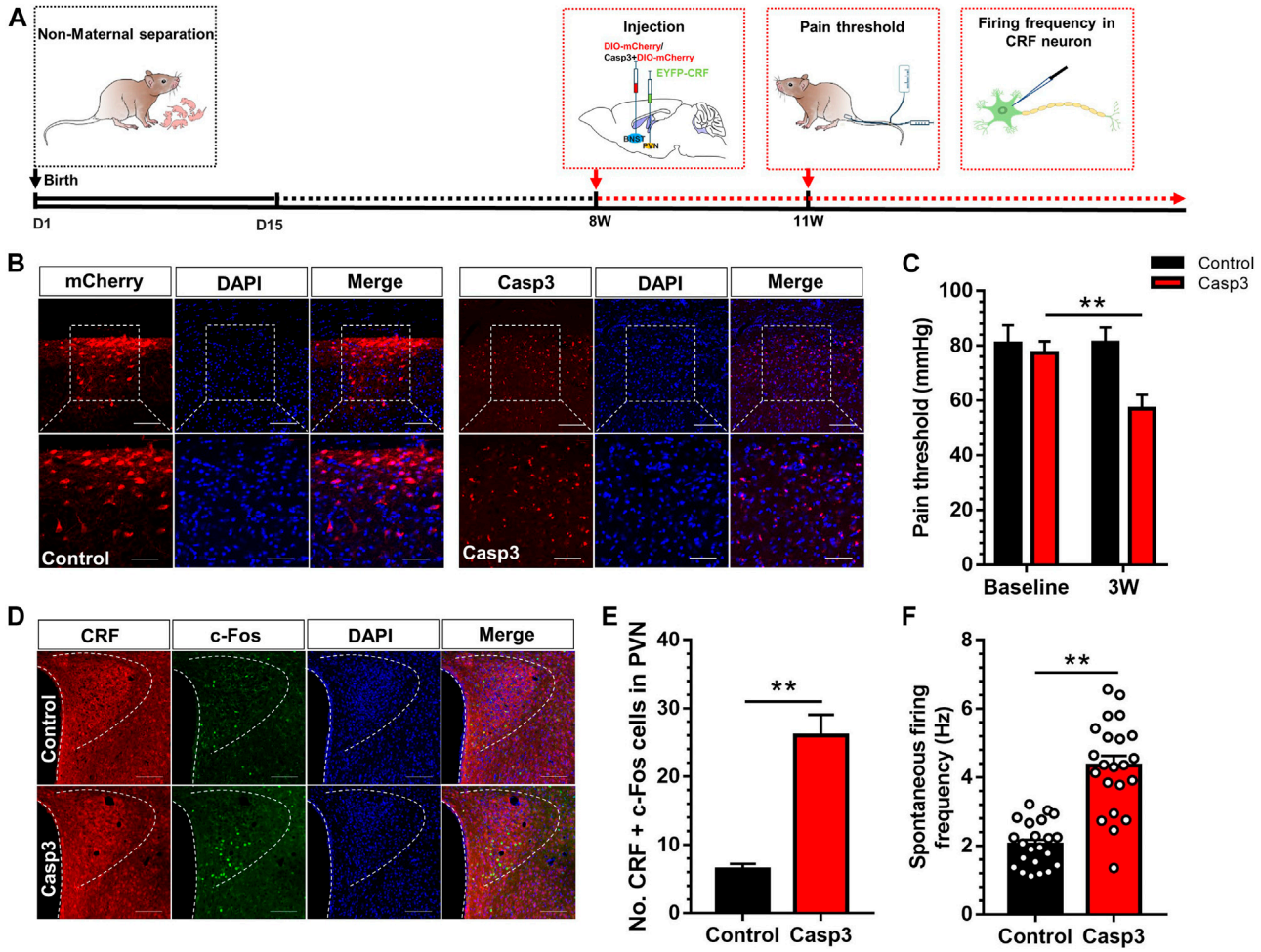

FIGURE 4 | Destruction of GABA neurons in BNST-AV region resulted in the activation of CRF neurons in PVN and the reduction of visceral pain threshold **(A)** The timeline for virus injection and behavioral detection **(B)** Coronal section was monitored for Vgat-mCherry fluorescence and stained with DAPI 3 weeks after BNST-AV injection of Casp3, and the GABA neurons in the BNST-AV region of Casp3 mice exhibited alterations of cell morphology vs. mCherry group. The upper Scale bar = 100μm. The bottom Scale bar = 50μm **(C)** The visceral pain threshold was significantly decreased 3 weeks after Casp3 treatment vs. its baseline ($n = 8$, $^{**}p < 0.01$) **(D)** Fluorescence image of anti-CRF and anti-c-Fos protein **(E)** The count of CRF neurons co-labeled with c-Fos was significantly increased in the PVN of Casp3 group vs. control group ($n = 3$, $^{**}p < 0.01$) **(F)** The spontaneous firing frequency of CRF neurons (EYFP+) in Casp3 group was significantly increased vs. control group ($n = 22$, six mice per group; $^{**}p < 0.01$).

significantly increased in Casp3 group compared with the control group (**Figure 4F**). These findings indicated that destruction of GABAergic BNST-AV neurons facilitated the activation of CRF neurons in PVN and the development of visceral hypersensitivity.

Inhibition of GABAergic $BNST_{AV}$-PVN Neurons Facilitated the Visceral Pain

Next, we adopted chemogenetic manipulation to silence the PVN-projecting BNST-AV GABAergic neurons. **Figure 5A** illustrated the experimental schema of bilateral infusion of AAV-DIO-mCherry or AAV-DIO-hM4Di-mCherry into the BNST-AV and incannulation above PVN in normal Vgat-Cre mice. Confocal image of hM4Di and GAD67 in BNST-AV area was shown in **Figure 5B**. 99.1% hM4Di-tagged Vgat neurons were GAD67-inmmue-positive (**Figure 5C**). In Vgat-Cre mice, intra-PVN administration of CNO facilitated the visceral hypersensitivity (**Figure 5D**). One-way ANOVA showed a significant difference [$F(3,20) = 14.89$, $p < 0.01$; **Figure 2E**]. Post hoc Bonferroni multiple comparisons showed a significant decrease in the visceral pain threshold compared with other three groups ($p < 0.01$). Furthermore, optogenetic technique was employed to validate the effects on the PVN-projecting BNST-AV GABAergic neurons. The virus strategy was shown in **Figure 5E**. Consistent with above results, optogenetic inhibition (593nm) of terminals of neurons projecting to GABAergic $BNST_{AV}$-PVN decreased the visceral pain threshold and facilitated pain (**Figure 5F**). One-way ANOVA showed a significant difference [$F(2,15) = 50.54$, $p < 0.01$; **Figure 5F**]. Post hoc Bonferroni multiple comparisons showed a significant decrease in the visceral pain threshold of eNpHR group compared with vehicle group ($p < 0.01$). These findings

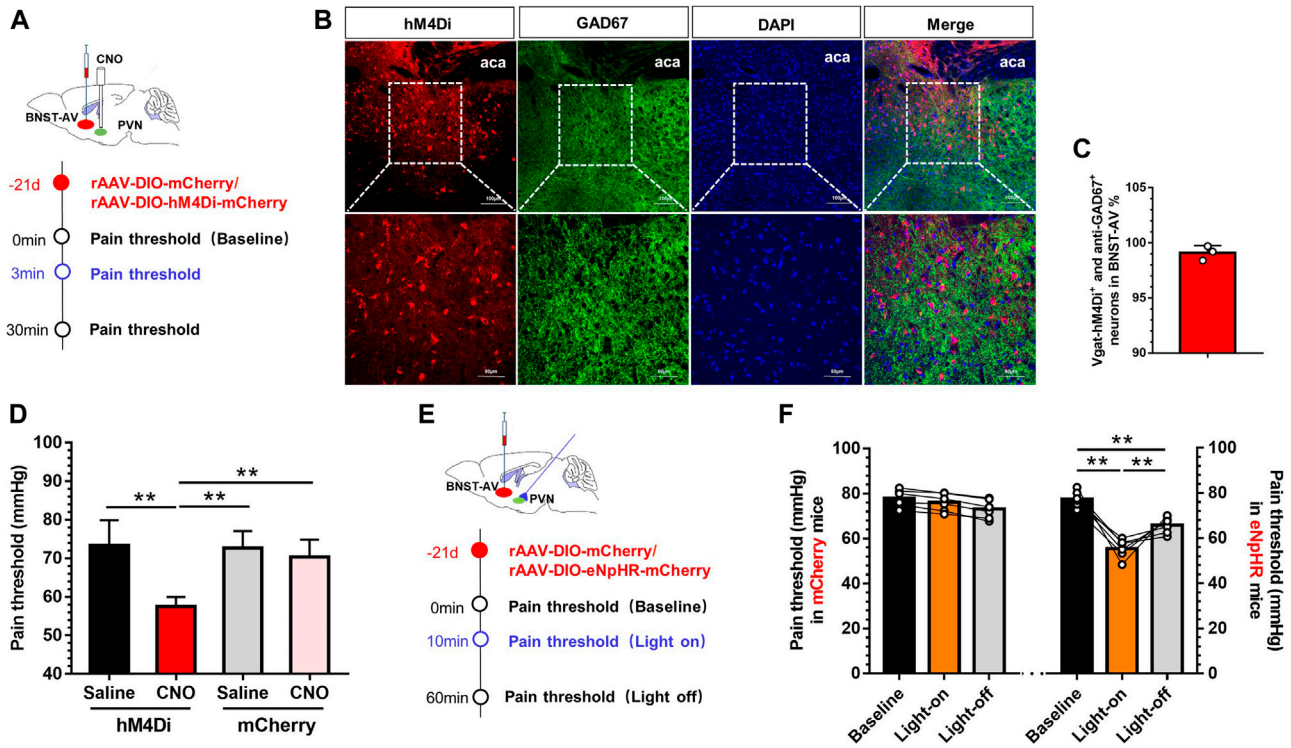

FIGURE 5 | Inhibition of GABAergic $BNST_{AV}$-PVN neurons induced the visceral pain in normal Vgat-Cre mice **(A)** Schematic diagram of AAV-DIO-hM4Di-mCherry injection into the BNST-AV and trocar implantation into the PVN in Vgat-Cre mice **(B)** The Vgat-hM4Di-mCherry neurons were stained with antibody to GAD67 to verify effects of virus-labeled GABAergic neurons in the BNST-AV **(C)** The co-standard rate of labeled GABAergic neurons and anti-GAD67 protein were 99.1% **(D)** The visceral pain threshold was significantly decreased post-CNO PVN injection ($n = 6$, $^{**}p < 0.01$) **(E)** Schematic diagram of AAV-DIO-eNpHR-mCherry injection into the BNST-AV and fiber-optic incannulation into the PVN in Vgat-Cre mice **(F)** The visceral pain threshold was significantly decreased after photoactivation of the terminal of PVN-projecting eNpHR-mCherry-positive neurons from BNST-AV area ($n = 6$, $^{**}p < 0.01$).

implied the important role of PVN-projecting GABAergic neurons in BNST-AV region in the modulation of visceral hypersensitivity.

Activation of GABAergic $BNST_{AV}$-PVN Neurons Inhibited the Activation of CRF Neurons in PVN and Alleviated Visceral Pain

Ultimately, we adopted identical chemogenetic and optogenetic approaches to manipulate GABAergic $BNST_{AV}$-PVN neurons in a cohort of Vgat-Cre mice with a history of MS. Mice received AAV-DIO-hM3Dq-mCherry injection as did hM4Di (**Figure 6A**). MS mice exhibited significantly attenuated visceral pain after PVN-injection of CNO compared with the vehicle group (**Figure 6B**), suggesting that MS induced long-term GABAergic $BNST_{AV}$-PVN inhibition. One-way ANOVA showed a significant difference [$F(3,20) = 31.99$, $p < 0.01$; **Figure 6B**]. Post hoc Bonferroni multiple comparisons showed a significant increase in the visceral pain threshold compared with other three groups ($p < 0.01$). Thereafter, we expressed excitatory Cre-dependent channelrhodopsin-2 (rAAV-Ef1-DIO-hChR2(H134R)-mCherry-WPRE-Pa) in the BNST-AV GABAergic neurons and monitored the visceral pain threshold during photo-stimulation of synaptic terminals. The experimental flow of virus injection was shown in **Figure 6C**. The visceral pain threshold was increased and pain was relieved under light-on condition in ChR2 group compared with vehicle group [$F(2,21) = 20.89$, $p < 0.01$; **Figure 6D**]. As illustrated in **Figure 6E**, the double immunofluorescence of ChR2 virus and anti-GAD67 protein. 98.4% ChR2-tagged Vgat neurons were GAD67-inmmue-positive (**Figure 6F**). **Figure 6G** depicted the combined technique with specific electrophysiological recording of CRF neurons in PVN. The schema of discharge frequency was presented in **Figure 6H**. The discharge frequency of CRF neurons in PVN was decreased compared with the profiles before and after light switching-on [$F(2,45) = 4.94$, $p < 0.01$; **Figure 6I**]. These findings further authenticated the pivotal role of PVN-projecting GABAergic neurons in BNST-AV region in the modulation of visceral hypersensitivity.

DISCUSSION

In this study, we characterized an MS model generated in Vgat-Cre mice. The results supported the hypothesis that MS can produce striking visceral hypersensitivity with concomitant activation of CRF neurons in PVN. Presynaptic innervation of PVN CRF neurons, as determined by lumafluor retrobeads and

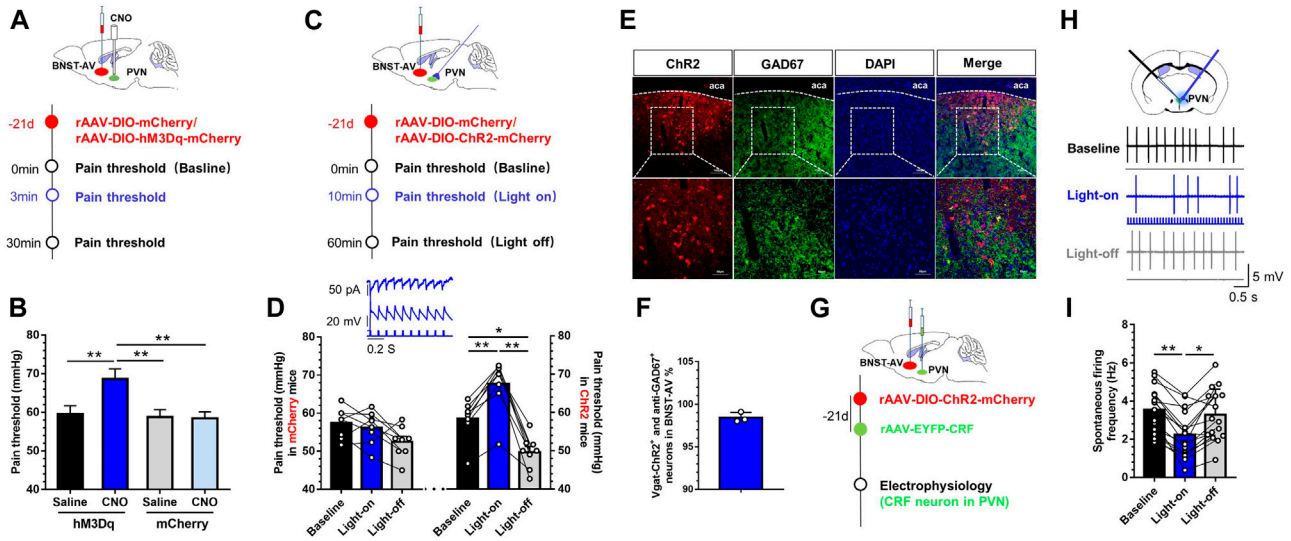

FIGURE 6 | Activation of GABAergic $BNST_{AV}$-PVN neurons alleviated the visceral pain in mice experiencing MS **(A)** Schema for injection of AAV-DIO-hM3Dq-mCherry into the BNST-AV for rescue of inhibitory neurons in Vgat-Cre mice experiencing MS **(B)** The visceral pain threshold significantly increased post-CNO PVN-injection ($n = 6$, $^{**}p < 0.01$) **(C)** Schematic diagram of AAV-DIO-ChR2-mCherry injection into the BNST-AV and fiber-optic incannulation into the PVN of Vgat-Cre mice **(D)** The visceral pain threshold was significantly increased after photoactivation of the terminal of PVN-projecting ChR2-mCherry-positive neurons from BNST-AV area ($n = 6$, $^{**}p < 0.01$), and optogenetic stimulation to GABA neurons in BNST-AV region led to laser-dependent hyperpolarization current (473nm laser, wave width: 10ms, frequency: 10Hz) **(E)** The Vgat-ChR2-mCherry neurons were stained with antibody to GAD67 to verify effects of virus-labeled GABAergic neurons in the BNST-AV **(F)** The co-standard rate of labeled GABAergic neurons and anti-GAD67 protein was 98.4% **(G)** *In vitro* electrophysiological recordings were conducted in ChR2-injected mice **(H)** The schema of spontaneous firing frequency in CRF neurons of PVN **(I)** Photoactivation of the terminal of PVN-projecting eNpHR-mCherry-positive neurons from BNST-AV area decreased the firing rate of CRF neurons in PVN from brain slice from MS mice ($n = 18$, six mice per group; $^{**}p < 0.01$).

retrograde virus, was markedly localized in BNST-AV region. Further, ablation/inhibition of GABAergic $BNST_{AV}$-PVN neurons via AAV-Casp3/AAV-hM4Di/AAV-eNpHR precipitated the activation of PVN CRF neurons and visceral hypersensitivity. Moreover, stimulation of GABAergic $BNST_{AV}$-PVN neurons by means of AAV-hM3Dq/AAV-ChR2 decreased the activities of PVN CRF neurons and alleviated the visceral hypersensitivity. These results implied that inhibition of GABA neurons in the BNST-AV region projecting to CRF neurons in PVN facilitated the development of visceral hypersensitivity. This study may yield a novel insight into the understanding of the circuit mechanism of visceral hypersensitivity as manifested in patients with IBS and provide a potential therapeutic target for IBS treatment.

In the present study, animal model of visceral hypersensitivity was established via neonatal MS in Vgat-Cre mice. Stress or an adverse event in the early life can potentially precipitate the visceral hypersensitivity and pain. Functional magnetic resonance imaging also demonstrates that perception of increased visceral hypersensitivity in IBS patients may benefit central management of pain (Flak et al., 2009). Consequently, visceral hypersensitivity is a key contributor of both brain and gut psychopathology as well as their underlying reciprocity (Senba and Ueyama, 1997). Further, ELS experience may contribute to persistent modifications of neurocircuitry, neuronal plasticity, functionality, and increase susceptibility to the development of both pain-relevant behavior profiles and psychiatric morbidities in patients with IBS (O'Mahony et al., 2017; Rincel et al., 2019; Zhu et al., 2017). Thus, MS in rodents is a well-validated model of ELS in mimicking IBS in humans (Gunn et al., 2013). A large body of literature has established mechanisms by which MS can impact the development of brain and the stress systems of the body, including visceral hypersensitivity, hyper-responsiveness of the HPA axis, peripheral motility abnormalities, altered intestinal permeability, etc. (Oines et al., 2012; Rincel and Darnaudery, 2020).

In line with previous reports (Fuentes and Christianson, 2018; Tang et al., 2017), we confirmed that MS Vgat-Cre mice presented pronounced visceral hypersensitivity and anxiety- and depression-like behaviors in adulthood, coupled with upregulation of CRF protein expression and activation of CRF neurons in PVN (**Figures 1**, **2**). The PVN has the densest distribution of CRF expression, and the CRF cell density in PVN was larger than 20000 cells/mm^3 (Peng et al., 2017). CRF + neurons account for 33.12% of PVN neurosecretory neurons (Simmons and Swanson 2009; Russell 2018). These CRF neurons are thought to be primarily glutamatergic but also partially GABAergic (Dabrowska et al., 2013). They receive inputs from various brain regions and send projections to the median eminence. The presence of GABA with certain CRF-containing neurons of the PVN projecting to the median eminence may terminate the CRF action (Meister et al., 1988). Moreover, GABA is reported to be a dominant inhibitory neurotransmitter in the PVN. PVN administration of exogenous GABA (0.3mM, 0.2μl) alleviated the visceral hypersensitivity in mice. As per Schmidt M's report (Schmidt

et al., 2001), we designated 3μM as the standard GABA concentration in the electrophysiology. Exogenous GABA inhibited the discharge frequency of CRF neurons in PVN in mice with neonatal MS. CRF neurons integrate both external and visceral stress-responsive information, hence regulating neuroendocrine, autonomic and cognitive and emotional outcomes (Jiang et al., 2019), etc. Besides the local influences on the stress responses, GABA in the PVN decreases sympathetic outflow and blood pressure, and inhibits the adipose afferent reflex that promotes lipolysis and energy expenditure (Ding et al., 2015). These GABAergic mechanisms in the PVN are important for the physiological integration. The activation of PVN CRF neurons might have been mediated by a variety of mechanisms, including the reduction of DNA methylation in epigenetics, early exposure to high concentrations of glucocorticoid and glucocorticoid receptor in the brain, multiple inflammatory factors, the modifications of the nervous system and neuroendocrine variations in the CNS (Heim et al., 1997), etc. Therein, BNST is known to receive direct limbic or cortical input and to intimately innervate the PVN. BNST exhibits sexual dimorphism due to its neuroanatomical connectivity and neurochemical property, the consensus regarding the characteristics of IBS. These findings premise our interest with respect to the neurocircuitry between the BNST and PVN neurons.

Here, we designated the Vgat-Cre mice on the grounds that the Cre-Lox system has provided the optimal target of genetically defined neurons via virus combination. Tracing of Lumafluor retrobeads showed that PVN received projection from BNST-AV, BNST-AL, BNST-AM, mPFC, DR, etc. Moreover, the red fluorescence was detected mainly in BNST-AV region 21 days after PVN microinjection of retrograde DIO-mCherry, which might be attributed to the optimal anatomical proximity and tight functional connections. Furthermore, MS mice presented downregulated expression of GAD65 and GAD67 proteins in BNST-AV region and decreased discharge frequency of GABA neurons (**Figure 3**). As is well acknowledged, BNST-AV is composed of over 90% GABAergic neurons, and reduced GABAergic inhibition would result in the activation of PVN-projecting neurons. Furthermore, the subnucleus interconnections of BNST are complicated by their asymmetry or reciprocity (Gungor et al., 2018). However, BNST-AL and BNST-AM gradually become smaller and fused to the ventral region at the anterior commissure. Accordingly, the anterior ventral region of BNST has been postulated to integrate the internal information and post-output to PVN (Walker et al., 2003).

Subsequently, in order to elucidate whether GABAergic $BNST_{AV}$-PVN neurons mediate visceral hypersensitivity in Vgat-Cre mice, we manipulated these neurons by means of apoptotic virus, chemogenetic and optogenetic approaches. Ablation of BNST-AV GABAergic neurons by means of Casp3-apoptotic virus induced the activation of CRF neurons in PVN and exacerbated the visceral pain in normal mice (**Figure 4**). Likewise, Cre-dependent viral infection of hM4Di-mCherry or eNpHR-mCherry in BNST-AV (PVN CNO-administration or light-stimulation) induced the decreased visceral pain threshold and visceral hypersensitivity precipitated by inhibition of GABAergic $BNST_{AV}$-PVN circuit in normal mice (**Figure 5**). By consensus, selective ablation of the posterior BNST or GABAergic anterior BNST neurons leads to stress-induced HPA axis activation and increased expression of c-Fos in PVN, suggesting that GABAergic neurons in BNST plays a vital role in suppressing the HPA axis in stress (Choi et al., 2008; Radley et al., 2009). However, Choi et al. reported that the anteroventral BNST lesions are involved in the inhibition of the HPA axis (Choi et al., 2007). Albeit the majority of neurons projecting to PVN are GABAergic, sporadic glutamatergic neurons of approximately 1–3% in BNST-AV region might contribute to the inhibitory effect (Csáki et al., 2000). Paradoxically, photoexcitation of PVN-innervating, ChR2-expressing GABAergic terminals from BNST-AV did enhance the visceral pain threshold and decrease the spontaneous discharge frequency of CRF neurons in PVN in MS mice, as well as the hM3Dq-expressing GABA terminals by chemogenetics (**Figure 6**). These findings are compatible with the hypothesis that dysfunction of GABAergic $BNST_{AV}$-PVN circuit might participate in the visceral hypersensitivity induced by neonatal colorectal distension in SD rats (Song et al., 2020).

In summary, our study demonstrated that Vgat-Cre mice subjected to MS may render the persistent visceral hypersensitivity-related dysfunction of GABAergic $BNST_{AV}$-PVN circuit, ultimately resulting in the activation of CRF neurons in PVN. Our findings provide circuit-based approaches to higher precision in defining the subregions and cell typology in visceral pain, highlighting the therapeutic orientation towards which manipulation of the GABAergic $BNST_{AV}$-PVN pathway could be a perspective for visceral pain.

CONCLUSIONS

In conclusion, we provided evidence that excitation of BNST-AV neurons could modulate the activity of CRF neurons in PVN in mice susceptible to visceral hypersensitivity. Dysfunction of GABAergic $BNST_{AV}$-PVN circuit predisposed the mice with neonatal MS to the disinhibition of PVN CRF neurons and the development of visceral hypersensitivity, thus validating the involvement of $BNST_{AV}{}^{GABA}$-PVN^{CRF} circuit in the regulation of visceral hypersensitivity. A priori, our present findings provide a potential neurocircuitry basis for therapeutic interventions in chronic visceral pain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Xuzhou Medical University.

AUTHOR CONTRIBUTIONS

Conceptualization, YZ; methodology, SH and ZS; formal analysis, YL; investigation, WL and QY; data curation, ZS; writing-original draft preparation, SH and YL; writing-review and editing, YZ; supervision, SH; project administration, YZ; funding acquisition, YZ and SH. All authors have read and agreed to the published version of the manuscript.

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Sirt2 in the Spinal Cord Regulates Chronic Neuropathic Pain Through Nrf2-Mediated Oxidative Stress Pathway in Rats

*Mengnan Zhao, Xiaojiao Zhang, Xueshu Tao, Bohan Zhang, Cong Sun, Pinying Wang and Tao Song**

Department of Pain Medicine, The First Hospital of China Medical University, Shenyang, China

Reduction in Nrf2-mediated antioxidant response in the central nervous system plays an important role in the development and maintenance of neuropathic pain (NP). However, the mechanisms regulating Nrf2 activity in NP remain unclear. A recent *in vitro* study revealed that Sirt2, a member of the sirtuin family of proteins, affects antioxidant capacity by modulating Nrf2 activity. Here we examined whether central Sirt2 regulates NP through Nrf2-mediated oxidative stress pathway. In a rat model of spared nerve injury (SNI)-induced NP, mechanical allodynia and thermal hyperalgesia were observed on day 1 and up to day 14 post-SNI. The expression of Sirt2, Nrf2 and its target gene NQO1 in the spinal cord in SNI rats, compared with sham rats, was significantly decreased from day 7 and remained lower until the end of the experiment (day 14). The mechanical allodynia and thermal hyperalgesia in SNI rats were ameliorated by intrathecal injection of Nrf2 agonist tBHQ, which normalized expression of Nrf2 and NQO1 and reversed SNI-induced decrease in antioxidant enzyme superoxide dismutase (SOD) and increase in oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the spinal cord. Moreover, intrathecal injection of a recombinant adenovirus expressing Sirt2 (Ad-Sirt2) that upregulated expression of Sirt2, restored expression of Nrf2 and NQO1 and attenuated oxidative stress in the spinal cord, leading to improvement of thermal hyperalgesia and mechanical allodynia in SNI rats. These findings suggest that peripheral nerve injury downregulates Sirt2 expression in the spinal cord, which inhibits Nrf2 activity, leading to increased oxidative stress and the development of chronic NP.

Keywords: SIRT2, NRF2 activity, oxidative stress, the spinal cord, chronic neuropathic pain

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Paolo Tonin,
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Damiana Scuteri,
University of Calabria, Italy

****Correspondence:***
Tao Song
songtaocmu@163.com

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INTRODUCTION

Neuropathic pain (NP), which is redefined as a "pain caused by lesion or disease of the somatosensory system," is an underestimated socioeconomic health problem affecting millions of people worldwide (Carrasco et al., 2018). A systematic review of epidemiological studies has estimated that the prevalence of NP is 6.9–10% (St. John Smith, 2018). The clinical symptoms of NP are different, including spontaneous pain, hyperalgesia, allodynia and paresthesia (Nickel et al., 2012), and the most commonly prescribed analgesics generally are less effective for NP. NP can become a chronic and hardly bearable condition, leading to increased episodes of depression and

suicide in some case (Torrance et al., 2013). A better understanding of the molecular mechanism underlying NP may lead to improvements in pain relief and quality of life in patients with NP.

Oxidative stress has been suggested to play an important role in the development and maintenance of neuropathic pain (Carrasco et al., 2018; Shim et al., 2019). Excessive reactive oxygen species (ROS) has a deleterious effect on organelles, antioxidant defenses and other biomolecules, leading to mitochondrial dysfunction, glial activation and inflammatory response. This adverse environment is ultimately responsible for the typical painful symptoms of NP (Carrasco et al., 2018). Nuclear factor erythroid derived-2-related factor 2 (Nrf2) is a transcription factor and master regulator of many antioxidant/detoxification genes. Nrf2 pathway has been considered as a critical cellular defense mechanism against oxidative stress (Lee and Johnson, 2004; Kaspar et al., 2009; Catanzaro et al., 2017). Accumulating evidence shows that Nrf2 pathway is involved in the pathogenesis of NP (Zhou et al., 2020; Pol, 2021), but the mechanism that mediates Nrf2 pathway in NP is unclear.

The sirtuins (sirts) are a family of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases (HDACs) that play important roles in many cellular functions, including histone deacetylation, protein acylation, and deacetylation (Singh et al., 2018). In addition, sirtuins have protective properties, antioxidant-promoting actions and ROS-suppressive effects in mammalian cells (Singh et al., 2018). For example, overexpression of Sirt2, a member of the sirtuin family, decreases levels of ROS and increases the expression of antioxidant enzymes such as MnSOD, catalase, and glutathione peroxidase in the cells treated with lipopolysaccharides or hydrogen peroxide (Kim et al., 2013). A recent study showed that inhibition of Sirt2 with its inhibitor AGK2 attenuated the (NAD+) -induced increases in Nrf2 mRNA expression and nuclear Nrf2 levels, which were accompanied by reduced antioxidant capacity in PC12 cells (Zhang et al., 2019). Moreover, Sirt2 has ability to regulate nuclear Nrf2 levels and its downstream antioxidant gene expression by modulating AKT phosphorylation (Cao et al., 2016). In the present study, we examined whether Sirt2 regulates NP through Nrf2-mediated oxidative stress. For this purpose, we used a spared nerve injury (SNI)-induced NP model, which mimics human NP related to peripheral nerve injury.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 180–220 g were purchased from Liaoning Changsheng Biological Center, Shenyang, China. The rats were housed in separated cages at 23–25°C and 50–60% humidity under a 12/12-h light/dark cycle with free access to water and food. The animal experiments were performed according to the Guiding Principles for Research Involving Animal and Human Beings, and the experimental procedures were approved by the Animal Care and Use Committee of China Medical University (IACUC no. 2019028).

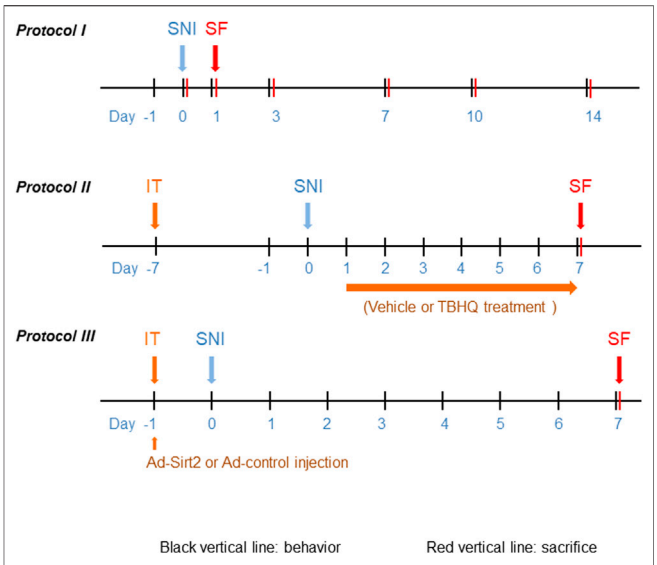

FIGURE 1 | Schematic illustration of the experimental protocols. SNI: spared nerve injury; IT: implantation of lumbar intrathecal catheter; SF: sacrifice.

Experimental Protocols

Protocol I

To examine the time course of changes in pain behaviors and expression of Nrf2, Sirt2 and NQO1 in the spinal cord, rats were assigned to sham (n = 5) and SNI (n = 30) groups. The SNI group underwent SNI surgery, while sham group received sham operation. Paw withdrawal threshold (PWT) to mechanical stimulation and paw withdrawal latency (PWL) to thermal stimulation were assessed at the ipsilateral hind paws 24 h prior to SNI and day 1, 3, 7, 10 and 14 post SNI. Five SNI rats were sacrificed after PWT and PWL measurement at each time point and sham rats were sacrificed at final time point. The protein was extracted from L4-6 spinal cord of injured side for molecular studies (**Figure 1A**).

Protocol II

To examine the role of Nrf2 in the spinal cord in regulation of NP, rats were divided into 5 experimental groups (n = 4 for each group): sham; SNI without treatment; SNI treated with DMSO (vehicle); SNI treated with Nrf2 agonist tBHQ at 1 μM; and SNI treated with tBHQ at 10 μM. Lumbar intrathecal catheter implantation was conducted 7 days prior to SNI. DMSO and tBHQ (dissolved in 10 μL DMSO) was intrathecally injected once a day for 7 days, starting from day 1 after SNI. PWT and PWL were measured daily and animals were sacrificed at the end of the protocol to collect spinal cord tissues for molecular studies. Additional SNI rats treated with DMSO and tBHQ at 10 μM (n = 3 for each group) were used for immunofluorescent study at the end of the protocol (**Figure 1B**). The doses of tBHQ used in this study for intrathecal injection were based on our preliminary

experiment showing that intrathecal injection of tBHQ at 1 μM significantly increased spinal Nrf2 expression and that intrathecal injection of tBHQ at 10 μM induced a greater increase in spinal Nrf2 expression in rats.

Protocol III

To determine the role of Sirt2 in regulation of Nrf2 pathway, rats were divided into 4 experimental groups (n = 5 for each group): sham; SNI without treatment; SNI treated with a recombinant adenovirus expressing control and GFP (Ad-control); and SNI treated with a recombinant adenovirus expressing Sirt2 and GFP (Ad-Sirt2, 1×10^8 PFU in 10 μL saline). Ad-control and Ad-Sirt2 were intrathecally injected 24 hours prior to SNI. PWT and PWL were measured daily and animals were sacrificed at the end of the protocol to collect spinal cord tissues for molecular studies. Additional SNI rats treated with Ad-control and Ad-Sirt2 (n = 3 for each group) were used for immunofluorescent study at the end of the protocol (**Figure 1C**).

Induction of Neuropathic Pain Model

The rat model of neuropathic pain was induced by unilateral SNI as previously described (Decosterd and Woolf, 2000; Guo et al., 2019). Briefly, rats were anesthetized by inhalation of 3% isoflurane, and a section was made directly through the biceps femoris muscle to expose the sciatic nerve and its three terminal branches: the common peroneal, tibial and sural nerves. The common peroneal and the tibial nerves were ligated tightly with 5.0 silk and transected distal to the ligation. Approximately 2–3 mm of the distal nerve stump was then excised. Muscle layers were closed using 4-0 chromic gut, and the skin incision was closed with wound staples. In sham surgery, the sciatic nerves were exposed but not ligated. Rats with sham surgery were used as control.

Implantation of Lumbar Intrathecal Catheter

Lumbar intrathecal catheter implantation was conducted as previously described (Størkson et al., 1996; Guo et al., 2019). Briefly, under 3% isoflurane anesthesia, an incision lateral to the midline was made and the polyethylene catheter was inserted into the subarachnoid space. The correct intrathecal localization was confirmed by a tail-flicking action and hind limb paralysis after administration of 2% lidocaine (10 μL) through the catheter in wakened animals.

Western Blot Analysis

Spinal cord (L4-6) was homogenized in ice-cold lysis buffer (Sigma-Aldrich, St. Louis, MO, United States) containing protease inhibitor cocktail. Supernatants were collected by centrifugation at 12,000 × g for 20 min at 4°C. The nuclear protein from spinal cord was prepared using a NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's instructions. Protein concentrations were determined with the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, United States). Equal amounts of protein were separated by 8% SDS-electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were placed in blocking buffer (5% milk in Tris-buffered saline with Tween-20) for one hour and then incubated over night at 4°C with primary antibodies to Sirt2 (1:500, Abcam, Cambridge, United Kingdom), Nrf2 (1:1,000, Abcam, Cambridge, United Kingdom), NQO1 (1:10,000, Abcam, Cambridge, United Kingdom), lamin B1 (1:1,000, Cell Signal Technology, MA, United Kingdom) and β-actin (1:1,000, Cell Signal Technology, Beverly, MA, United States). After incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, United States) for 1 h at room temperature, the labeled proteins were visualized by enhanced chemiluminescence detection system (GE Healthcare, Waukesha, WI, United States) and analyzed with ImageJ software (NIH, Bethesda, Maryland, United States). Results were normalized to β-actin or lamin B.

Measurements of Antioxidants and Oxidative Products

Spinal cord samples were collected and lyzed with RIPA buffer. The enzymatic activity of superoxide dismutase (SOD) was measured using the T-SOD assay kit (Jiancheng Bioengineering Institute, Nanjing, China) and levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determined using ELISA kits (Thermo Scientific, Rockford, IL, United States) according to the manufacturer's instructions.

Assessments of Mechanical Allodynia and Thermal Hyperalgesia

Mechanical allodynia was assessed by measuring the paw withdrawal threshold (PWT) in response to the stimulation of Von Frey filaments, as previously described (Chaplan et al., 1994; Guo et al., 2019). Briefly, rats were acclimatized in a plastic cage with a mesh bottom for 20 min prior to testing. PWT was assessed using a dynamic plantar esthesiometer (Ugo Basile, 37450, Italy), which consists of a force transduction fitted with a 0.5-mm diameter polypropylene rigid tip. A probe was applied perpendicularly to the mid-plantar surface of the hind paw with an increasing pressure. The cutoff pressure was set to be 50 g and the force that induced the withdrawal response was automatically recorded by the esthesiometer. For each animal, at least three measurements were performed with an interval of 5 min for the stimulation of each hind paw. Thermal hyperalgesia was determined by measuring the paw withdrawal latencies (PWL) in response to heat plate test. A hot plate (Ugo Basile Srl 7280, Gemonio, Italy) with a pre-set plate temperature of 52.5°C as recommended for rats (Hestehave et al., 2019) was used. As soon as the rat was placed onto the hot plate, the time between placement and licking, shaking or stepping of the hindpaws was recorded (Bannon and Malmberg, 2007). A cut-off-time was set at 30s to avoid tissue damage.

Immunofluorescent Study

The immunofluorescent study was performed as previously described (Wang et al., 2014), Briefly, rats were transcardially perfused with saline containing heparin (1 unit/ml) followed by 4% paraformaldehyde in 0.1 M PBS. Spinal cords were removed

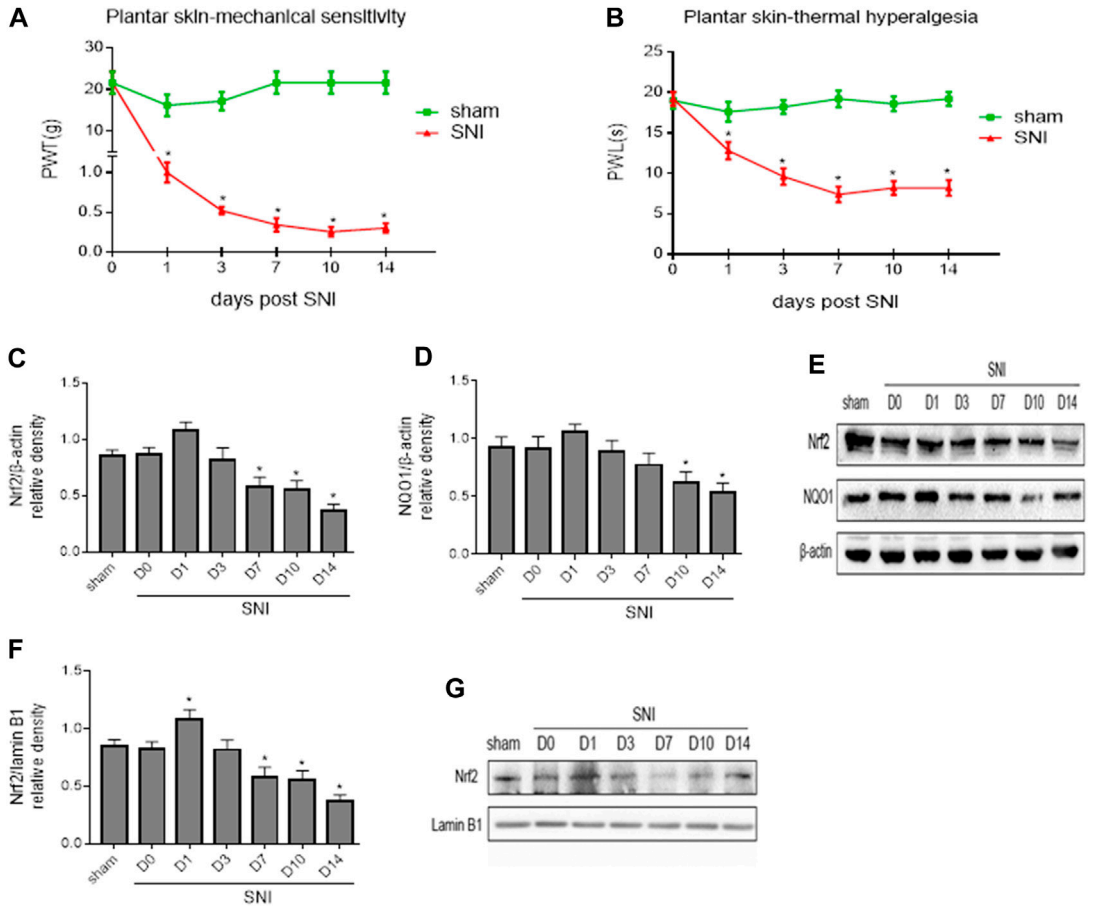

FIGURE 2 | (A,B): Paw withdrawal threshold (PWT) to mechanical stimulation and paw withdrawal latency (PWL) to thermal stimulation before (day 0) and 1, 3, 7, 10 and 14 days after spared nerve injury (SNI) or sham operation. **(C–E)**: The time course of changes in expression of Nrf2 and its downstream target NQO1 in the whole tissue lysates of the spinal cord in SNI rats. **(F,G)**: The time course of changes in expression of Nrf2 in the nuclear fractions of the spinal cord in SNI rats. Sham rats served as control. Values are expressed as mean ± SE (n = 5 for each group). *$p < 0.05$ vs sham or baseline (day 0).

and fixed overnight in 4% paraformaldehyde at 4°C and cryoprotected with 30% sucrose in 0.1 M PBS for 2 days. The spinal cords were sliced into 18-μm coronal sections. After being blocked with 5% goat serum in 0.3% Triton for 1 h at room temperature, the sections were incubated with primary to Sirt2 (1: 200, Abcam, Cambridge, United Kingdom or Nrf2 (1:200, Abcam, Cambridge, United Kingdom) overnight at 4 °C, followed by Alex Fluor 568 second antibody (1:200, Thermo Fisher Scientific, Waltham, MA, United States). DAPI was used for nuclear staining. Images were captured using a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss, Inc.).

Statistical Analysis

All data are presented as the mean ± SE. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc.). The differences between groups were analyzed by a one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests for multiple comparisons. Statistical significance was reached with P values below 0.05.

RESULTS

Time Course of Changes in Pain Behaviors and Expression of Nrf2 and its Downstream Target in the Spinal Cord Following SNI

Baseline measures of mechanical allodynia and thermal hyperalgesia were recorded 24 h prior to SNI to determine preinjury thresholds. NP behaviors were examined on 1, 3, 7, 10 and 14 days following SNI. As shown in **Figure 2**, there were no differences in mechanical allodynia (**Figure 2A**) and thermal hyperalgesia (**Figure 2B**) between groups at baseline. SNI induced significant mechanical allodynia as indicated by decreased PWT and thermal hyperalgesia as evidenced by reduced PWL within 1 day and lasting up to 14 days compared to sham controls and baseline. The maximal mechanical allodynia and thermal hyperalgesia in SNI group were observed at day 10 and day 7, respectively.

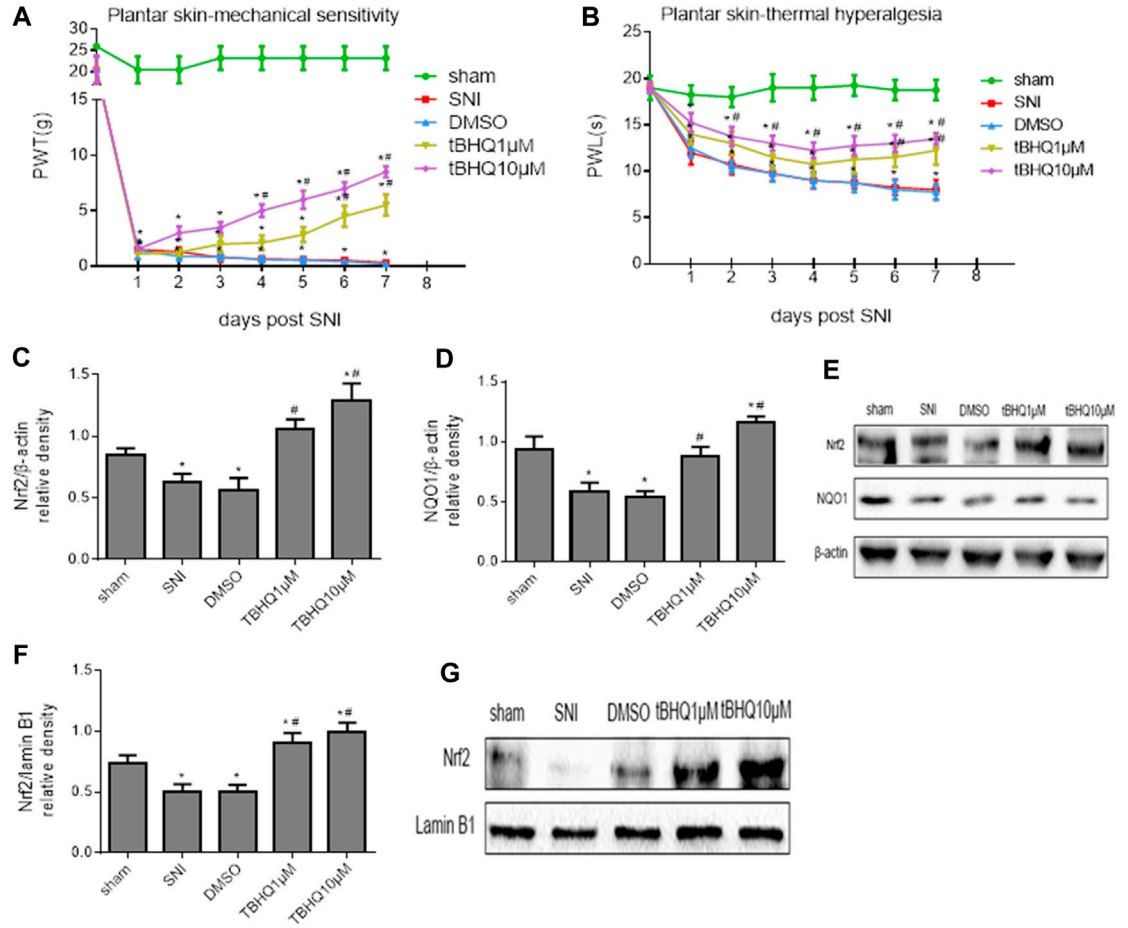

FIGURE 3 | (A,B): Effects of intrathecal injection of DMSO (vehicle) or different doses of Nrf2 agonist tBHQ on mechanical allodynia and thermal hyperalgesia in SNI rats. **(C–E)**: Effects of intrathecal injection of DMSO or different doses of Nrf2 agonist tBHQ on expression of Nrf2 and its downstream target NQO1 in the whole tissue lysates of the spinal cord in SNI rats. **(F,G)**: Effects of intrathecal injection of DMSO and different doses of Nrf2 agonist tBHQ on expression of Nrf2 in the nuclear fractions of the spinal cord in SNI rats. Sham rats without treatment served as control. Values are expressed as mean ± SE (n = 4 for each group). $^{*}p < 0.05$ vs sham or baseline (day 0); $^{\#}p < 0.05$ vs SNI or DMSO.

To explore the potential role of Nrf2 pathway in regulation of NP, we first examined the expression of Nrf2 and NQO1, a downstream target of Nrf2 that plays a protective role in various cells against oxidative stress, in the spinal cord of SNI rats. Western blot analysis revealed that the expression of Nrf2 (**Figures 2C,E**) and NQO1 (**Figures 2D,E**) in the whole tissue lysates of SNI group tended to be higher at day 1, but gradually decreased from day 7 following SNI, compared with baseline or sham group. Expression of Nrf2 in the nuclear fractions (**Figures 2F,G**) in SNI group was significantly increased at day 1, but then started to gradually decrease in the following day. Significant reduction in expression of Nrf2 in the nuclear fractions of SNI group was observed from day 7 as compared to baseline or sham group.

Activation of Nrf2 Pathway in the Spinal Cord Ameliorates NP

To examine whether activation of Nrf2 pathway in the spinal cord of SNI rats would ameliorate NP, SNI rats were treated with intrathecal injection of DMSO (vehicle) or Nrf2 agonist tBHQ at different doses. The mechanical allodynia (**Figure 3A**) and thermal hyperalgesia (**Figure 3B**) in SNI rats, compared with SNI rats without treatment, were attenuated by intrathecal tBHQ at both doses from day 4 for PWT and day 2 for PWL, respectively. Intrathecal injection of DMSO had no effects on mechanical allodynia and thermal hyperalgesia in SNI rats.

Western blots confirmed that expression of Nrf2 (**Figures 3C,E**) and NQO1 (**Figures 3D,E**) in the whole tissue lysates and expression of Nrf2 in the nuclear fractions (**Figures 3F,G**) in SNI rats without treatment were significantly decreased when compared with sham rats. Intrathecal tBHQ at either dose, but not DMSO, increased expression of Nrf2 and NQO1 in the whole tissue lysates and expression of Nrf2 in the nuclear fractions.

Immunofluorescent study showed that SNI rats treated with intrathecal tBHQ at a dose of 10 μM exhibited abundant Nrf2 immunoreactivity in the spinal cord, particularly in the nucleus, compared with SNI rats treated with intrathecal DMSO (**Figure 4**).

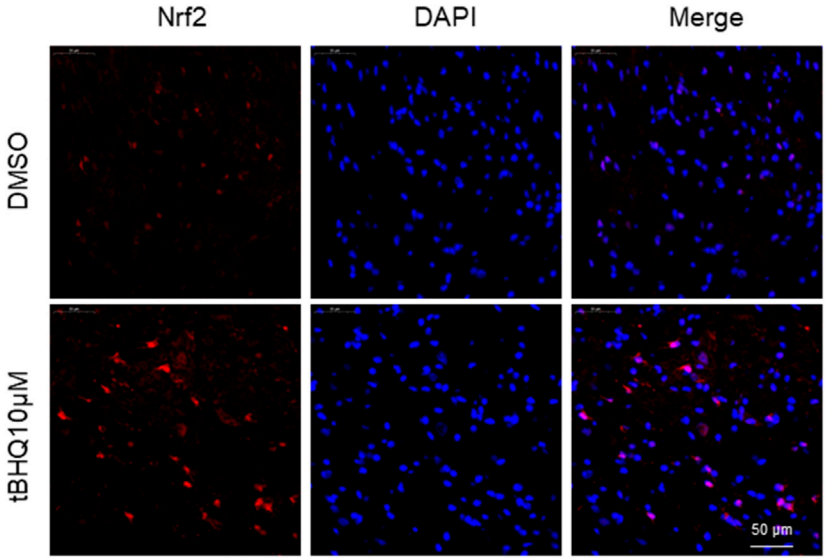

FIGURE 4 | Representative confocal images showing Nrf2 immunoreactivity (red) in the spinal cord in SNI rats treated with DMSO (vehicle) or Nrf2 agonist tBHQ at 10 μM. The nuclear staining by DAPI is shown in blue.

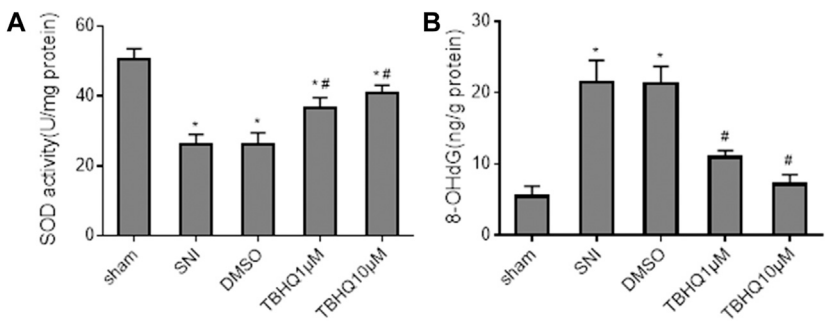

FIGURE 5 | Effects of intrathecal injection of DMSO or different doses of Nrf2 agonist tBHQ on levels of antioxidant enzyme superoxide dismutase (SOD, **(A)** and oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OHdG, **(B)** in the spinal cord of SNI rats. Sham rats without treatment served as control. Values are expressed as mean ± SE (n = 4 for each group). $^{*}p < 0.05$ vs sham; $^{\#}p < 0.05$ vs SNI or DMSO.

Because Nrf2 pathway mediates oxidative stress that has been implicated in the pathogenesis of NP, we also measured the levels of antioxidant enzyme SOD and oxidative stress marker 8-OHdG in the spinal cord. Consistent with expression of Nrf2 and NQO1, the levels of SOD were markedly decreased (**Figure 5A**), whereas the levels of 8-OHdG (**Figure 5B**) were increased in SNI rats without treatment. Intrathecal tBHQ at both doses completely reversed SNI-induced changes in SOD and 8-OHdG. Of note, intrathecal DMSO did not alter SNI-induced changes in SOD and 8-OHdG.

Time Course of Change in Expression of Sirt2 in the Spinal Cord Following SNI

New evidence reveals that Sirt2 regulates Nrf2 pathway in different tissues. We next examined the expression of Sirt2 in the spinal cord following SNI. As shown in **Figure 6**, the expression of Sirt2 in SNI rats was not altered at day 1 and day 3, but it was significantly decreased from day 7 and remained lower till the end of the experiment compared to baseline or sham rats.

Upregulation of Sirt2 in the Spinal Cord Alleviates NP, Which is Associated with Activation of Nrf2 Pathway and Decreased Oxidative Stress

To further determine whether upregulation of Sirt2 in the spinal cord could alleviate NP, we treated SNI rats with intrathecal injection of a recombinant adenovirus expressing Sirt2 and GFP (Ad-Sirt2) or control and GFP (Ad-control). As presented in **Figure 7**, SNI rats that received intrathecal Ad-Sirt2, but not Ad-control, had significantly reduced mechanical allodynia (**Figure 7A**) and thermal hyperalgesia (**Figure 7B**) as compared to SNI rats without treatment.

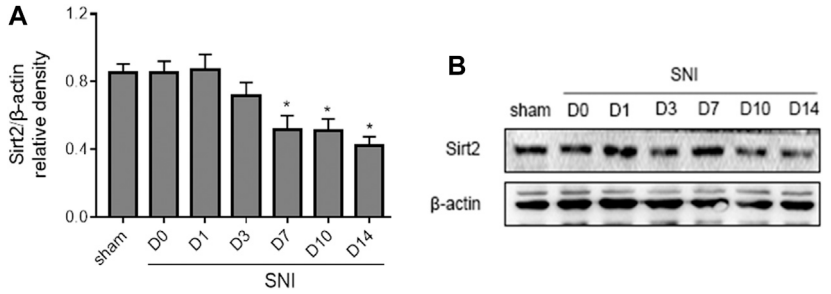

FIGURE 6 | The time course of changes in expression of Sirt2 in the spinal cord in SNI rats. Sham rats served as control. Values are expressed as mean ± SE (n = 5 for each group). *$p < 0.05$ vs sham or baseline (day 0).

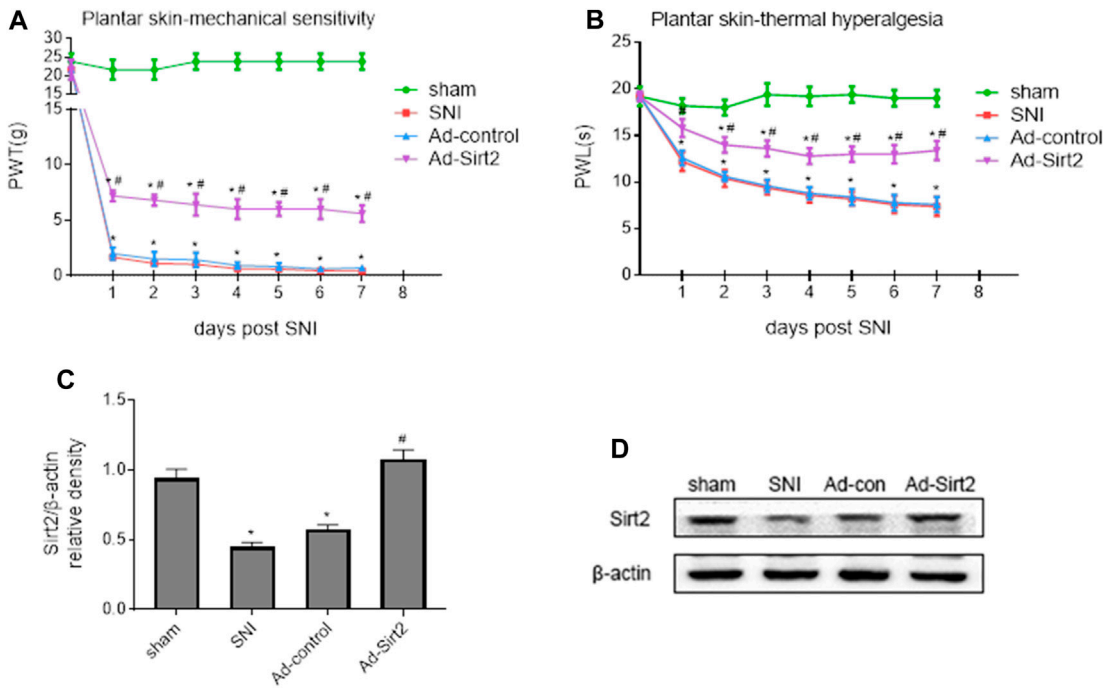

FIGURE 7 | (A,B): Effects of intrathecal injection of a recombinant adenovirus expressing Sirt2 and GFP (Ad-Sirt2) or control and GFP (Ad-control) on mechanical allodynia and thermal hyperalgesia in SNI rats. **(C,D)**: Effects of intrathecal injection of Ad-Sirt2 or Ad-control on expression of Sirt2 in the spinal cord of SNI rats. Sham rats without treatment served as control. Values are expressed as mean ± SE (n = 5 for each group). *$p < 0.05$ vs sham or baseline (day 0); $^{\#}p < 0.05$ vs SNI or Ad-control.

The expression of Sirt2 was significantly lower in the spinal cord of SNI rats without treatment compared with sham rats. Intrathecal Ad-Sirt2 restored expression of Sirt2 in the spinal cord of SNI rats to similar level as in sham group (**Figures 7C,D**).

Immunofluorescent study demonstrated GFP expression in the spinal cord in both SNI rats treated with intrathecal Ad-Sirt2 and SNI rats treated with intrathecal Ad-control. However, SNI rats treated with intrathecal Ad-Sirt2 had higher Sirt2 immunoreactivity than SNI rats treated with intrathecal Ad-control (**Figure 8**).

Importantly, we found that expression of Nrf2 (**Figures 9A,C**) and NQO1 (**Figures 9B,C**) in the whole tissue lysates and expression of Nrf2 in the nuclear fractions (**Figures 9D,E**) in SNI rats treated with intrathecal Ad-Sirt2 were normalized to those observed in sham rats. Moreover, intrathecal Ad-Sirt2 restored SNI-induced changes in SOD (**Figure 9F**) and 8-OHdG (**Figure 9G**) in the spinal cord. Intrathecal Ad-control had no effect on any of these measured parameters.

DISCUSSION

The main findings of the present study are: 1) Nrf2 activity and its downstream target NQO1 expression in the spinal cord are downregulated in rats with SNI-induced chronic NP; 2) upregulation of Nrf2 activity restores NQO1 expression and

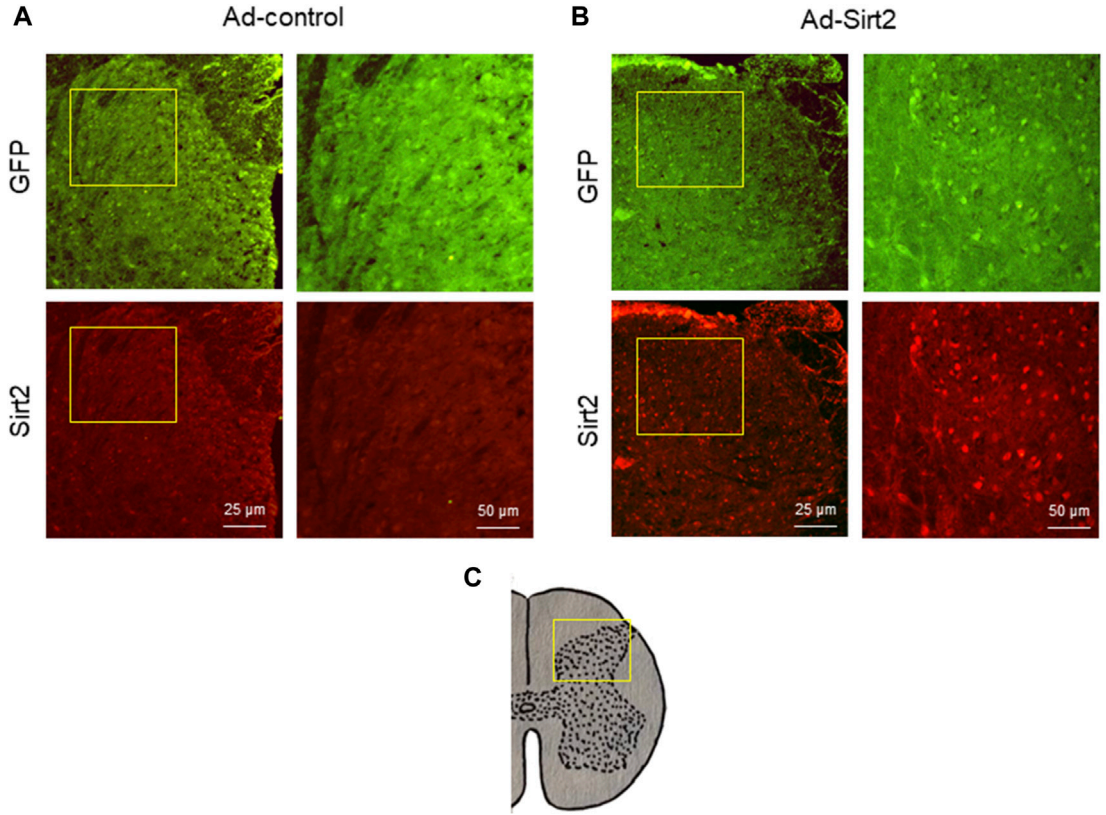

FIGURE 8 | (A,B): Representative confocal images showing GFP (green) and Sirt2 (red) immunoreactivity in the spinal cord of SNI rats treated with Ad-Sirt2 or Ad-control. Right column of images in Ad-control group or Ad-Sirt2 group: higher-power magnification of a section of the left images. **(C)**: Cross section of spinal cord showing where the left column of images in A and B were photographed.

prevents oxidative stress in the spinal cord, ameliorating mechanical allodynia and thermal hyperalgesia in rats with SNI-induced chronic NP; 3) Sirt2 expression in the spinal cord is reduced in rats with SNI-induced chronic NP; 4) overexpression of Sirt2 prevents reductions in Nrf2 activity and NQO1 expression as well as increase in oxidative stress in the spinal cord, leading to improvement of thermal hyperalgesia and mechanical allodynia.

Evidence supports that after peripheral or central nervous system injury, elevated extracellular glutamate levels activate multiple intracellular pathways including ROS formation (Carrasco et al., 2018). It is well known that increase in intracellular ROS plays a critical role in etiology of pain processes (Carrasco et al., 2018). For example, excessive ROS can promote hyperexcitability of dorsal root ganglia neurons through several mechanisms, including disrupted mitochondrial bioenergetics, which impairs energy production and ion homeostasis, resulting in spontaneous activity and degeneration of nociceptors (Grace et al., 2016). Excessive ROS may also indirectly cause neuronal hyperexcitability by promoting production of neuroinflammatory mediators (Grace et al., 2016; Ji et al., 2016). Interventions that attenuate oxidative stress have been shown to ameliorate NP in animals (Tiwari et al., 2012; Sharma et al., 2018). Nrf2 pathway plays an essential role in protecting cells and tissues from oxidative stress (Lee and Johnson, 2004; Kaspar et al., 2009; Catanzaro et al., 2017). Under physiological conditions, Nrf2 is sequestered in the cytosol by binding to its cytosolic inhibitor Keap1 and ubiquitinated for degradation. Under oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it binds to the antioxidant response element that initiates transcription of more than 200 antioxidant-related genes, leading to upregulation of a battery of antioxidants, including SOD, glutathione peroxidase and glutathione (Lee and Johnson, 2004; Kaspar et al., 2009; Catanzaro et al., 2017). Accumulating evidence shows that Nrf2 pathway is implicated in NP. For instance, activation of Nrf2 in the spinal cord with Nrf2 activator oltipraz attenuates oxidative stress and ameliorates mechanical allodynia in a rat model of paclitaxel-induced NP (Zhou et al., 2020). Electroacupuncture increases production of antioxidants and alleviates paclitaxel-induced NP by upregulating Nrf2 activity in the dorsal root ganglion (Zhao et al., 2019). Activation of Nrf2 activity can also improve mechanical allodynia and thermal hyperalgesia in diabetes-induced NP in mice (Xu et al., 2020). In the present study, we found that expression of Nrf2 and NQO1 in the whole tissue lysates of the spinal cord in SNI rats tended to be higher at day 1, but gradually decreased from day 7 following SNI. Expression of Nrf2 in the nuclear

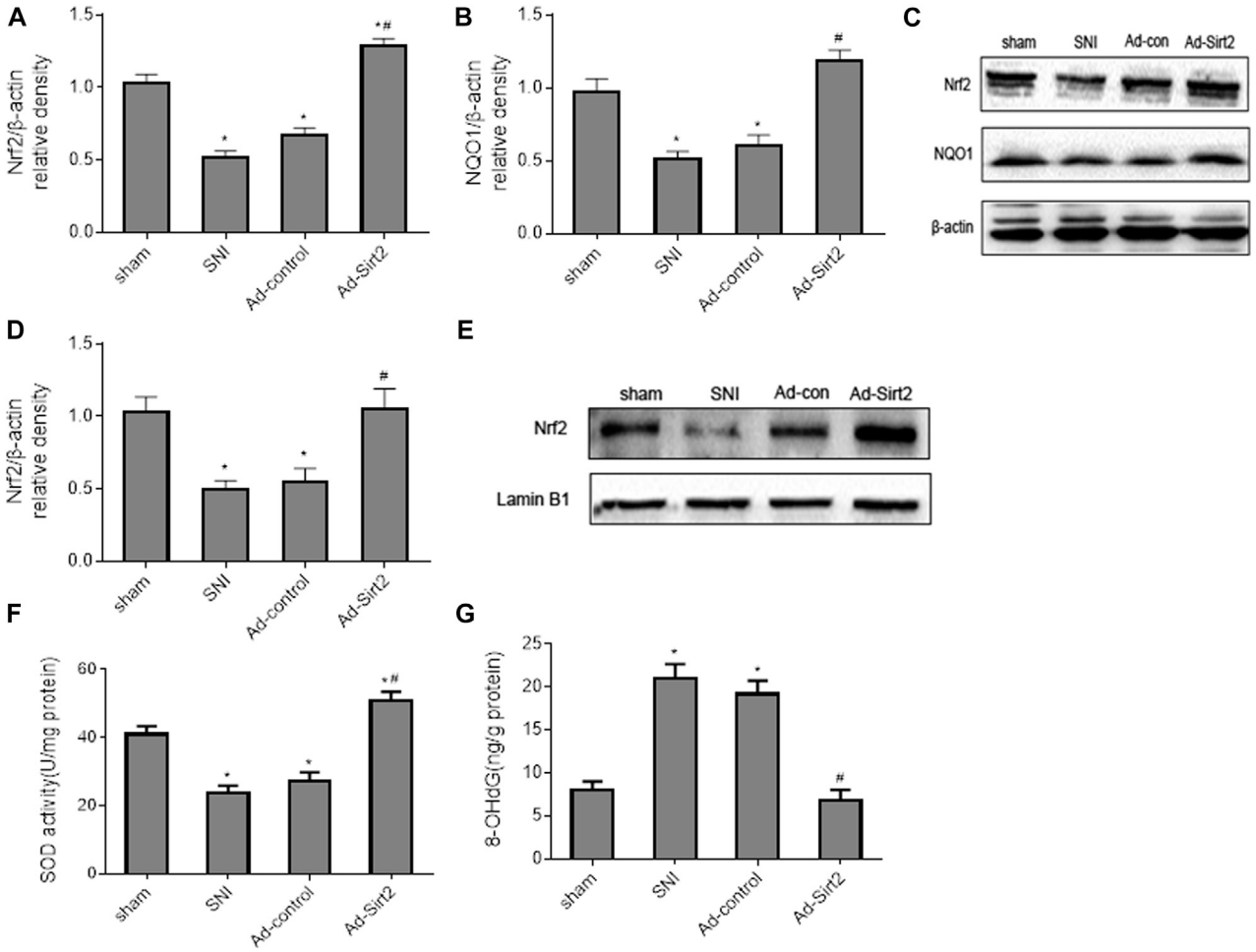

FIGURE 9 | (A–C): Effects of intrathecal injection of Ad-Sirt2 or Ad-control on expression of Nrf2 and its downstream target NQO1 in the whole tissue lysates of the spinal cord in SNI rats. **(D,E)**: Effects of intrathecal injection of Ad-Sirt2 or Ad-control on expression of Nrf2 in the nuclear fractions of the spinal cord in SNI rats. **(F,G)**: Effects of intrathecal injection of Ad-Sirt2 or Ad-control on levels of antioxidant enzyme superoxide dismutase (SOD) and oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the spinal cord of SNI rats. Sham rats without treatment served as control. Values are expressed as mean ± SE (n = 5 for each group). $^{*}p < 0.05$ vs sham; $^{\#}p < 0.05$ vs SNI or Ad-control.

fractions in SNI group was significantly increased at day 1, but then start to gradually decrease. These results suggest that expression of Nrf2 and translocation of Nrf2 into the nucleus are increased to protect cells from SNI-induced oxidative stress and cellular damage in the early stages of NP. However, both expression of Nrf2 and translocation of Nrf2 into the nucleus are downregulated over time in rats following SNI, leading to increased mechanical allodynia and thermal hyperalgesia. This finding is consistent with a recent study showing decreased Nrf2 expression in the spinal cord in rats following SNI. Our data also demonstrated that activation of Nrf2 pathway in the spinal cord with Nrf2 agonist tBHQ ameliorated NP, which was accompanied by increased Nrf2 downstream target NQO1 and antioxidant enzyme SOD and decreased oxidative stress marker 8-OHdG. These observations indicate that activation of Nrf2 pathway in the spinal cord is downregulated in rats following SNI, which enhances vulnerability of spinal cord tissues to oxidative insults and leads to increased oxidative stress, contributing to the development of chronic NP.

The sirtuins are a class of evolutionarily highly conserved nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases that play important roles in many cellular biological processes (Singh et al., 2018; Zhang et al., 2020). The sirtuin family of proteins consists of seven members (Sirt 1–7) (Singh et al., 2018; Zhang et al., 2020) and Sirt2 is an important member of the sirtuin family that plays a vital role in regulating oxidative stress, inflammation, and mitochondrial function (Zhang et al., 2020). Previous study reported that Sirt2 deacetylated FOXO3a in response to oxidative stress and reduced ROS production by upregulating expression of FOXO3a target genes in NIH3T3 cells (Wang et al., 2007). Additionally, Sirt2 has been shown to inhibit mitochondrial autophagy and protect annulus fibrosus cells from oxidative stress-induced apoptosis via regulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha

(Xu et al., 2019). A recent study reported that NADH-induced increase in the expression of nuclear Nrf2 in PC12 cells was prevented by both Sirt2 siRNA and Sirt2 inhibitor; Sirt2 siRNA also blocked the NADH-induced increases in the levels of intracellular antioxidant glutathione (Cao et al., 2016). This observation suggests that activation of Nrf2 can be regulated by Sirt2. Sirt2 is widely distributed in the peripheral and central nerve system tissues, including microglia (Wang et al., 2016), dopaminergic neurons (Szego et al., 2017), hippocampal neurons, spinal cord (Park et al., 2016) and dorsal root ganglion (Zhang and Chi, 2018). To explore the mechanism that regulates Nrf2 pathway in the development of chronic NP, we measured the expression of Sirt2 in the spinal cord. Our results showed that Sirt2 expression in the spinal cord was gradually decreased in rats following SNI. More importantly, we found that overexpression of Sirt2 in the spinal cord of SNI rats restored Nrf2 activity and NQO1 expression, reversed SNI-induced changes in levels of antioxidant enzyme SOD and oxidative stress marker 8-OHdG, and significantly improved thermal hyperalgesia and mechanical allodynia. These findings indicate that reduction in Nrf2 activity and NQO1 expression and increase in oxidative stress in the spinal cord in rats following SNI are mediated by Sirt2, which plays an important role in the development of chronic NP.

One limitation of this study should be acknowledged. The SNI animal model has been reported to exhibit persistent (>6 months) NP (Decosterd and Woolf, 2000). However, our current study only focused on the role of Nrf2 activity in the spinal cord in regulating NP at an early stage. Further studies are necessary to examine whether Nrf2 activity in the spinal cord also plays an important role in mediating NP at a later stage.

In conclusion, the present study demonstrates that peripheral nerve injury downregulates Sirt2 expression in the spinal cord, which inhibits Nrf2 activity, leading to increased oxidative stress and the development of chronic NP. Current medications used for neuropathic pain offer only symptomatic relief without treating the underlying pathophysiology. In addition, these medications are associated with various dose-limiting side effects (Fernandes et al., 2018, Bidve et al., 2020). A better understanding of cellular and molecular mechanisms of NP may provide the key to development of effective NP therapies. That have minimal side effects. This study provides new evidence that Nrf2 pathway in the spinal cord plays an important role in regulating chronic NP. Systemic or intrathecal delivery of Nrf2 activators to increase spinal Nrf2 levels or administration of small-molecule Nrf2-enhancing compounds to induce endogenous Nrf2 activity and expression in the spinal cord, may be therapeutic approaches for treatment of patients with chronic NP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University.

AUTHOR CONTRIBUTIONS

MZ designed the study, conducted experiments, acquired and analyzed data, and wrote the manuscript. XZ, XT, BZ, CS and PW conducted the experiments and acquired data. TS was responsible for the conception and supervision of the study and wrote the manuscript. All authors corrected drafts and approved the final version of the manuscript.

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