MONITORING ENDOGENOUS GPCRs : LESSONS FOR DRUG DESIGN

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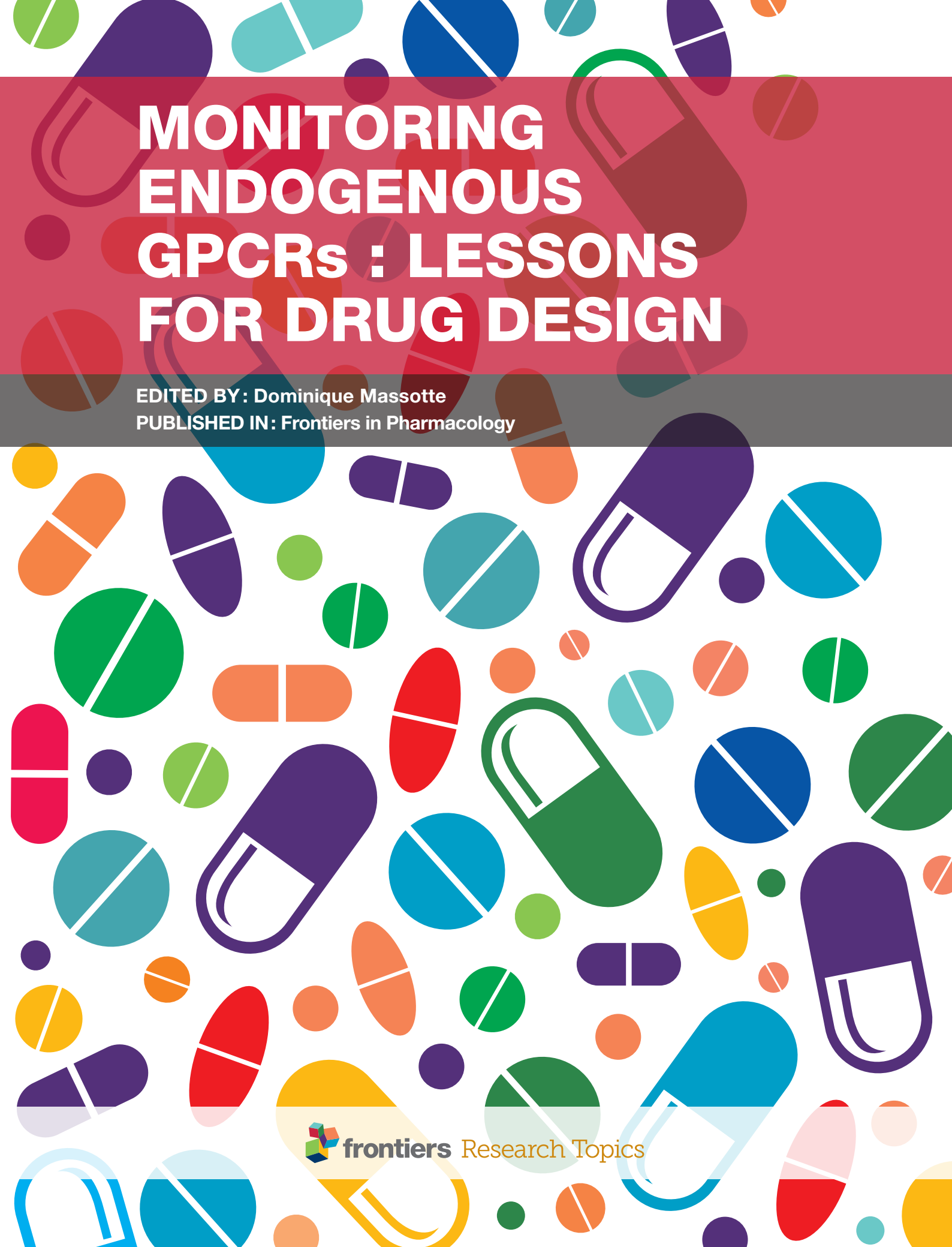

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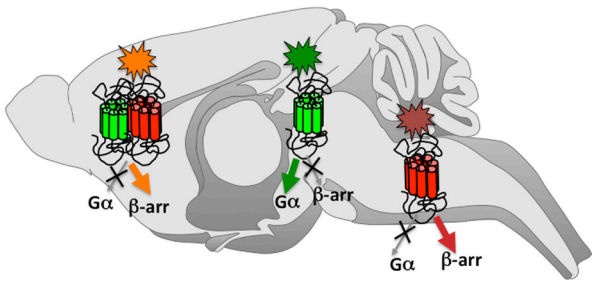
MONITORING ENDOGENOUS GPCRs : LESSONS FOR DRUG DESIGN

Topic Editor:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France



Increasing evidence indicate that GPCR signaling is dictated by the nature of the ligand. In vivo, this so-called ligand biased agonism can be achieved through binding of various endogenous ligands. In addition, receptor heteromers formed by physical association of two different GPCRs exhibit distinct ligand and signaling properties and significantly contribute to biased signaling. Both mechanisms concur to modulate GPCR activity and subsequent impact on cell physiology.

G protein-coupled receptors (GPCRs) are integral membrane proteins forming the fourth largest superfamily in the human genome. Many of these receptors play key physiological roles and several pathologies have been associated with receptor functional abnormalities. GPCRs therefore represent important goals for drug design in pharmaceutical companies since they constitute the target of about one third of the drugs currently on the market. However, endogenous GPCRs are most often difficult to study because of a lack of tools to target them specifically and single out their response to physiological or drug-elicited stimulations. Hence, studies mostly focused on recombinant receptors expressed in a variety of cellular models that do not always closely reflect the receptor natural environment and often deal with levels of expression exceeding by far physiological ranges. Recent technological developments combining for example genetically modified animals and advanced imaging approaches have improved our ability to visualize endogenous GPCRs. To date, trailing receptor activation, subsequent intracellular redistribution, changes in signaling cascade up to integrated response to a drug-elicited stimulation is at hand though the impact of a physiological challenge on receptor dynamics remains a major issue. Data however suggest that the receptor may embrace a different fate depending on the type of stimulation in particular if sustained or repeated. This suggests that current drugs may only partially mimic the genuine response of the receptor and may explain, at least in part, their secondary effects. Commonalities and specificities between physiological and drug-induced activation can thus represent valuable guidelines for the design of future drugs.

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EDITORIAL
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Editorial: Monitoring endogenous GPCRs: lessons for drug design

*Dominique Massotte**

Institut des Neurosciences Cellulaires et Intégratives, Strasbourg, France

Keywords: G protein-coupled receptors, biased signaling, heteromers, chronic pain, mood disorders

G protein-coupled receptors (GPCRs) are integral membrane proteins forming the fourth largest superfamily in the human genome. Many of these receptors play key physiological roles and several pathologies have been associated with receptor functional abnormalities. GPCRs therefore represent important goals for drug design in pharmaceutical companies since they constitute the target of about one third of the drugs currently on the market. However, endogenous GPCRs are most often difficult to study because of a lack of tools to target them specifically and single out their response to physiological or drug-elicited stimulations. To date, studies mostly focused on recombinant receptors expressed in a variety of cellular models that do not always closely reflect the receptor natural environment and often deal with levels of expression exceeding by far physiological ranges. Recent technological developments have improved our ability to visualize endogenous GPCRs and to address their signaling properties. Data suggest that the receptor may embrace a different fate depending on the ligand. This so-called biased signaling is getting growing importance in the GPCR field. Similarly, increasing attention is given to the concept of heteromerization that corresponds to the physical association of two receptor types resulting in new signaling properties. Investigating endogenous receptor activation and subsequent intracellular redistribution or addressing changes induced by drug-elicited stimulation from molecular and cellular events to integrated response is thus crucial for the development of new pharmacological tools and strategies. In this topic, timely overview as well as original reports present new tools, including genetically modified animals, and techniques available to track expression and signaling of endogenous GPCRs.

Brogi et al. (2014) review novel approaches in medicinal chemistry for class A GPCRs that all aim at more efficacy with less side effects. They are ranging from *in silico* studies for increased ligand selectivity and affinity to new orientations in ligand development including biased agonists that favor specific signaling cascades such as G protein or beta-arrestin dependent pathways, allosteric modulators or bivalent ligands that target heteromers. Thompson et al. (2014) illustrate the concept of biased agonism at the level of the endogenous somatostatin and opioid systems in the gut. In the case of opioid receptors, biaised agonism could be achieved through heteromer formation. In this context, Gonzalez-Maeso (2014) provides a brief overview of the techniques currently available to establish physical proximity between receptors *in vivo,* which represents the first criterion to postulate heteromer formation. In the same line, Gomes et al. (2014) introduce the generation of heteromer selective antibodies by substractive immunization strategy and discuss their use to get insight into class A heteromer-specific signaling *in vivo*. Moving to genetically modified animals, Ceredig and Massotte (2014) review the contribution of knock-in mice that express fluorescent proteins to neuroanatomy. The authors highlight the role of knock-in animals expressing fluorescent receptors for linking receptor trafficking, desensitization and behavioral output and for mapping receptor neuronal co-expression as a first hint toward *in vivo* heteromers. Knock-out animals on the opposite are deficient for a given receptor but proved powerful to decipher the specific role of a given GPCR in various physiopathological conditions. This is exemplified by Befort (2015) who reviews the relative contribution of opioid and cannabinoid receptors and their interactions in the context of reinforcing

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Nicholas Barnes, University of Birmingham, UK

***Correspondence:**
Dominique Massotte, d.massotte@unistra.fr

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behaviors and discusses the limitations of the approach. Genetically modified animals are also powerful tools to address GPCR signaling. As an example, GCaMP transgenic mice express engineered proteins containing Ca^{2+} binding motifs within a circularly permutated variant of the green fluorescent protein that undergo a conformational change upon elevation of intracellular Ca^{2+}. Partridge (2015) reviews the use of this Ca^{2+} sensor to monitor *in vivo* activation of Gq/11 coupled GPCRs in response to pharmacological stimulation. Alternatively, Bagley (2014) reports an original study that illustrates the utility of classical approaches such as electrophysiology as another powerful tool to identify the specific impact of a given receptor on neuronal activity. She addresses the identity of the Gi/o coupled receptor responsible for protein kinase A (PKA)-dependent increase of the GABA transporter GAT-1 in the periaqueductal gray, a phenomenon underlying increased GABAergic neuronal excitability and synaptic GABA release during opiate withdrawal. Combining perforated patch recording with selective pharmacological stimulation, Bagley clearly demonstrates that PKA dependent increase in GAT 1 is promoted by opioid receptor activation and not $GABA_B$ receptors possibly due to differential subcellular distribution of the two receptors within the neuron. Chen et al. (2014) also report a novel approach to monitor PKA activity in brain tissue by fluorescence lifetime imaging microscopy (FLIM) using two-photon microscopy using their newly developed PKA sensor FLIM-AKAR. FLIM-AKAR can be transfected or virally encoded for *in vivo* expression. The latter can be controlled by cre-dependent elements to target specific neuronal populations. This sensor reports the balance of PKA and phosphatase activity with less pH sensitivity and a broader dynamic range. Moreover, FLIM-AKAR being highly diffusible enables monitoring of PKA activity in dendritic spines. Finally, two reviews broach the functional role of endogenous opioid receptors. Cahill et al. (2014) expand our knowledge of the role of the kappa opioid receptor and its endogenous ligand dynorphin. The authors review evidence of the implication of the kappa-dynorphin system in the negative aspects related to pain, highlighting possible contribution in the high comorbidity of mood disorders associated with chronic neuropathic pain. Allouche et al. (2014) review the various mechanisms by which opioid receptors desensitize including aspects related to biased agonism and discuss their impact on the development of opiate tolerance.

Altogether, the topic covers various conceptual and technical approaches at the molecular, cellular or integrated level that can be generalized to challenge the functional role of endogenous class A GPCRs and to gather critical insight for novel therapeutic strategies.

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References

Allouche, S., Noble, F., and Marie, N. (2014). Opioid receptor desensitization: mechanisms and its link to tolerance. *Front. Pharmacol.* 5:280. doi: 10.3389/fphar.2014.00280

Bagley, E. E. (2014). Opioid and GABAB receptors differentially couple to an adenylyl cyclase/protein kinase a downstream effector after chronic morphine treatment. *Front. Pharmacol.* 5:148. doi: 10.3389/fphar.2014.00148

Befort, K. (2015). Interactions of the opioid and cannabinoid systems in reward: insights from knockout studies. *Front. Pharmacol.* 6:6. doi: 10.3389/fphar.2015.00006

Brogi, S., Tafi, A., Desaubry, L., and Nebigil, C. G. (2014). Discovery of GPCR ligands for probing signal transduction pathways. *Front. Pharmacol.* 5:255. doi: 10.3389/fphar.2014.00255

Cahill, C. M., Taylor, A. M., Cook, C., Ong, E., Moron, J. A., and Evans, C. J. (2014). Does the kappa opioid receptor system contribute to pain aversion? *Front. Pharmacol.* 5:253. doi: 10.3389/fphar.2014.00253

Ceredig, R. A., and Massotte, D. (2014). Fluorescent knock-in mice to decipher the physiopathological role of G protein-coupled receptors. *Front. Pharmacol.* 5:289. doi: 10.3389/fphar.2014.00289

Chen, Y., Saulnier, J. L., Yellen, G., and Sabatini, B. L. (2014). A PKA activity sensor for quantitative analysis of endogenous GPCR signaling via 2-photon FRET-FLIM imaging. *Front. Pharmacol.* 5:56. doi: 10.3389/fphar.2014.00056

Gomes, I., Gupta, A., Bushlin, I., and Devi, L. A. (2014). Antibodies to probe endogenous G protein-coupled receptor heteromer expression, regulation, and function. *Front. Pharmacol.* 5:268. doi: 10.3389/fphar.2014.00268

Gonzalez-Maeso, J. (2014). Family a GPCR heteromers in animal models. *Front. Pharmacol.* 5:226. doi: 10.3389/fphar.2014.00226

Partridge, J. G. (2015). Utilizing GCaMP transgenic mice to monitor endogenous Gq/11-coupled receptors. *Front. Pharmacol.* 6:42. doi: 10.3389/fphar.2015.00042

Thompson, G. L., Canals, M., and Poole, D. P. (2014). Biological redundancy of endogenous GPCR ligands in the gut and the potential for endogenous functional selectivity. *Front. Pharmacol.* 5:262. doi: 10.3389/fphar.2014.00262

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Discovery of GPCR ligands for probing signal transduction pathways

Simone Brogi[1,2], Andrea Tafi[2], Laurent Désaubry[3] and Canan G. Nebigil[4]*

[1] European Research Centre for Drug Discovery and Development (NatSynDrugs), University of Siena, Siena, Italy
[2] Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy
[3] Therapeutic Innovation Laboratory, UMR7200, CNRS/University of Strasbourg, Illkirch, France
[4] Receptor Signaling and Therapeutic Innovations, GPCR and Cardiovascular and Metabolic Regulations, Biotechnology and Cell Signaling Laboratory, UMR 7242, CNRS/University of Strasbourg – LabEx Medalis, Illkirch, France

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Andrzej Pilc, Polish Academy of Sciences, Poland
Sebastien Granier, Institut National de la Santé et de la Recherche Médicale, France
Helmut Schmidhammer, University of Innsbruck, Austria

***Correspondence:**
Canan G. Nebigil, Receptor Signaling and Therapeutic Innovations, GPCR and Cardiovascular and Metabolic Regulations, Biotechnology and Cell Signaling Laboratory, UMR 7242, CNRS/University of Strasbourg – LabEx Medalis, ESBS Pole API, Boulevard Sébastien Brandt, BP 10413 Illkirch, France
e-mail: nebigil@unistra.fr

G protein-coupled receptors (GPCRs) are seven integral transmembrane proteins that are the primary targets of almost 30% of approved drugs and continue to represent a major focus of pharmaceutical research. All of GPCR targeted medicines were discovered by classical medicinal chemistry approaches. After the first GPCR crystal structures were determined, the docking screens using these structures lead to discovery of more novel and potent ligands. There are over 360 pharmaceutically relevant GPCRs in the human genome and to date about only 30 of structures have been determined. For these reasons, computational techniques such as homology modeling and molecular dynamics simulations have proven their usefulness to explore the structure and function of GPCRs. Furthermore, structure-based drug design and *in silico* screening (High Throughput Docking) are still the most common computational procedures in GPCRs drug discovery. Moreover, ligand-based methods such as three-dimensional quantitative structure–selectivity relationships, are the ideal molecular modeling approaches to rationalize the activity of tested GPCR ligands and identify novel GPCR ligands. In this review, we discuss the most recent advances for the computational approaches to effectively guide selectivity and affinity of ligands. We also describe novel approaches in medicinal chemistry, such as the development of biased agonists, allosteric modulators, and bivalent ligands for class A GPCRs. Furthermore, we highlight some knockout mice models in discovering biased signaling selectivity.

Keywords: G protein-coupled receptors, GPCR, homology modeling, high throughput docking, biased agonists, biased signaling, allosteric modulators, bivalent ligands

INTRODUCTION

G protein-coupled receptors (GPCRs) use canonical (G protein-mediated) and non-canonical (G protein-independent, β-arrestin dependent) signaling pathways to assert their biological functions (Luttrell et al., 1999; Beaulieu et al., 2005; Lefkowitz and Shenoy, 2005; Abbas and Roth, 2008).

The ligands can bind to receptor either competitively (orthosterically) by interacting with the same receptor-binding site as the endogenous agonist or allosterically by exerting effects through a distinct binding site. Ligands binding at the orthosteric sites have been classified as agonists, antagonists, and/or inverse agonists based on their ability to mainly modulate G protein signaling. The ligands can directly stabilize the "active" receptor conformations via a non-standard binding site (known as allosteric agonism) or modulate the binding of orthosteric ligands (known as allosteric modulation). Those ligands acting outside the orthosteric hormone binding sites can selectively engage subsets of signaling responses as "functional selectivity" or "ligand-biased signaling" (Khoury et al., 2014).

Several studies have shown that multivalent ligands, but not a monovalent ligands bind to the extracellular domains of receptors and trigger intracellular signaling by ligand-promoted receptor clustering (Sigalov, 2012). Ligands can be monovalent or bivalent, targeting specific GPCR dimers that may provide drugs with enhanced potency, selectivity, and therapeutic index. Biased ligands at GPCRs preferentially stimulate one intracellular signaling pathway over another (Violin et al., 2014). This functional selectivity of the ligands is extremely useful for elucidating the signal transduction pathways for both the therapeutic actions and the side effects of drugs. There is growing interest in developing biased GPCR ligands to yield safer, better tolerated, and more effective drugs.

Here, we discuss the discovery of GPCR ligands including biased agonists, allosteric modulators, and bivalent ligands and biased signaling selectivity for the class A GPCRs focusing on structure-based drug design (SBDD) and *in silico* screening (High Throughput Docking), medicinal chemistry, and genetic loss-of-function strategies.

STRUCTURE-BASED DRUG DESIGN AND *IN SILICO* SCREENING (HIGH THROUGHPUT DOCKING) IN GPCRs DRUG DISCOVERY

Computational methods represent invaluable tools in medicinal chemistry, including drug discovery step. Concerning the ligand discovery in GPCRs field, different techniques have been applied for selecting potential and selective chemical derivatives

that bind to GPCRs (Andrews et al., 2014). Homology modeling and ligand screening, utilizing structure-, and/or ligand-based approaches represent the most common approaches to discover *in silico* novel ligands. Recently, fragment-based protocols have also been used. The impact of computational techniques in GPCR drug discovery has been relevant, due to the extreme difficulties for obtaining experimental high-resolution structural information on the active and inactive state of GPCRs. After the crystallization of the first mammalian GPCR (bovine rhodopsin; Palczewski et al., 2000; **Figure 1**), homology-modeling technique has been extensively adopted to predict structures and functions of different GPCRs and also to perform *in silico* screening.

In fact, sequence analysis suggested that family A GPCRs share the same arrangement, showing a high sequence similarity of the seven transmembrane helices, confirming the suitability of rhodopsin as a template (Li et al., 2010). During the last decade, we have seen a dramatic improvement in crystallization methods. Indeed, after about 7 years from the first solved structure of a mammalian GPCR, several three-dimensional structures have been published. The second crystallized GPCR was β_2-adrenergic receptor (β_2AR; Cherezov et al., 2007; Rasmussen et al., 2007) and then the β_1AR (Warne et al., 2008). Subsequently, an exponential growth of crystallized GPCR structures in the protein data bank was observed. Actually, the three dimensional structures available of class A GPCRs comprise: the adenosine A_{2A} receptor (Jaakola et al., 2008), the D_3 dopamine receptor (Chien et al., 2010), the chemokine receptors CXCR1 (Park et al., 2012), CXCR4 (Wu et al., 2010), and CCR5 (Tan et al., 2013) the histamine H_1 receptor (Shimamura et al., 2011), the sphingosine 1 phosphate receptor (Hanson et al., 2012), the M_2 and M_3 muscarinic receptors (Haga et al., 2012; Kruse et al., 2012), the μ, k, and δ opioid receptors (Manglik et al., 2012; Wu et al., 2012; Fenalti et al., 2014) as well as the nociceptin receptor (NOP; Thompson et al., 2012), bovine opsin receptors (Park et al., 2008; Scheerer et al., 2008), neurotensin receptor (White et al., 2012), the serotonin $5HT_{1B}$ and $5HT_{2B}$ receptors (Wacker et al., 2013; Wang et al., 2013a), protease-activated receptor 1 (PAR1; Zhang et al., 2012), the smoothened receptor (SMO; Wang et al., 2013b), and P2Y12 receptor (Zhang et al., 2014). Very recently, also a crystal structure of class B and C GPCRs such as glucagon receptor (Siu et al., 2013), corticotropin-releasing factor 1 (CRF_1) receptor (Hollenstein et al., 2013) and metabotropic glutamate receptor 5 (Dore et al., 2014) respectively, have been reported.

These achievements are largely attributable to the application of high-throughput methods for lipidic cubic phase (LCP) crystallography (Cherezov et al., 2004) and protein engineering with the generation of GPCR-T4 lysozyme (Rosenbaum et al., 2007) and GPCR–BRIL fusion proteins (Chun et al., 2012). Thermo stabilization (Serrano-Vega et al., 2008) methods represent another useful tool appropriate to GPCRs crystallization. Notably, these techniques can be generally applicable to structurally diverse GPCRs. Moreover, a relevant number of receptors have been solved with both bound antagonists and agonists.

The availability of numerous different GPCR templates offers diverse options in GPCR modeling. In particular, the application of multiple templates to the homology modeling protocols has been demonstrated to improve the reliability of the computational models including GPCRs (Fernandez-Fuentes et al., 2007; Mobarec et al., 2009; Sokkar et al., 2011; Cappelli et al., 2013; Gemma et al., 2014b).

In conclusion, the availability of a relevant number of crystal structures improves results of homology modeling procedures by using novel methodology such as multiple-templates based alignment for building the structure of GPCRs as well as the three-dimensional structure of any type of proteins (Cappelli et al., 2013; Gemma et al., 2014a; Giovani et al., 2014). Moreover, the

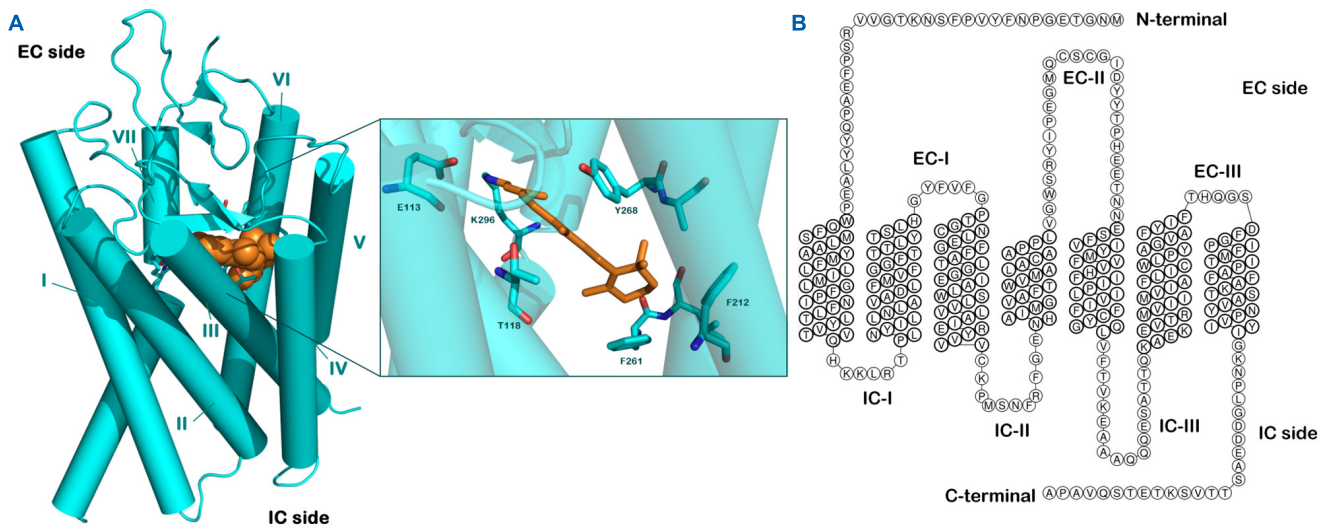

FIGURE 1 | Structure of rhodopsin. (A) Crystal structure of bovine rhodopsin covalently linked with retinal adapted from PDB file 1F88. **(B)** Snake-like diagram for the bovine rhodopsin highlighting extracellular (EC) and intracellular (IC) loops.

accessibility of GPCR crystal structures unlocked opportunities to use alternative methods for GPCR drug discovery, mainly SBDD. SBDD approaches are extensively used in drug discovery of novel compounds based on three dimensional protein structures using various computational methods. The impact of GPCR crystal structures on SBDD has been instantaneous and has led to the discovery of novel ligands for different GPCRs (Kooistra et al., 2013). Furthermore, as above mentioned the increase of GPCR determined structures assures a large number of potential available templates, guaranteeing an improvement of quality of GPCR homology models for virtual screening. Indeed, virtual screening has become a routine tool for selecting putative lead compounds and identifying potential drug candidates for a given target (Brogi et al., 2009, 2011, 2013; Castelli et al., 2012). Although ligand-based methods were found to be useful for structurally non-characterized targets, high throughput docking is clearly the most popular approach used in receptor based virtual screening using both experimental and theoretical sources (Abagyan and Totrov, 2001). In this review, we will present an overview of the most relevant structure-based approaches for identifying novel ligands, targeting allosteric, and/or orthosteric binding sites, for some of the class A GPCRs.

DRUG DESIGN AND DISCOVERY IN CARDIOVASCULAR DISEASES

β-adrenergic receptors

The selectivity of compounds for β_1- and β_2-(AR) is an important issue to take into account in current adrenoreceptor drug design. A structure-based design approach using protein–ligand crystal structures of the β_1AR is the first example of GPCR crystallography with ligands derived from fragment screening. In fact, the structures of the stabilized β_1AR in complex with two ligands were determined at resolutions of 2.8 and 2.7 Å, respectively (Christopher et al., 2013). A very elegant work has been recently carried out by Christopher et al. (2013) using biophysical fragment screening of a thermostabilized β_1AR. They also applied surface plasmon resonance (SPR) to identify moderate affinity, high ligand efficiency (LE) arylpiperazine hits. Subsequent hit to lead follow-up confirmed the activity of the chemotype. Vilar et al. (2010) evaluated the applicability of ligand-based and structure-based models to quantitative affinity predictions and virtual screening for ligands of the β_2AR.

The crystal structure of β_2AR has been used by Kolb et al. (2009) to investigate the advantages and limitations of the structure-based approach in ligand discovery. The authors docked about 1,000,000 commercially available compounds against the β_2AR structure. Twenty five hits have been selected and submitted to biological evaluation. Six compounds were active with binding affinities <4 μM, with the best molecule that showed a Ki of 9 nM. Moreover, five of these molecules have been found as inverse agonists (Kolb et al., 2009).

Sabio et al. (2008) and Topiol and Sabio (2008) performed a high-throughput docking with proprietary and commercial databases to investigate the usefulness of crystal structure for discovery of novel chemical classes acting as β_2AR inhibitors. These findings were further validated using X-ray structures of β_2AR/Timolol (Hanson et al., 2008), via *in silico* high-throughput docking of proprietary and commercial databases. This study resulted in the identification of ligands with relevant affinity for β_2AR (Sabio et al., 2008; Topiol and Sabio, 2008).

More recently, Weiss et al. (2013) reported a prospective, large library virtual screen of 3.4 million molecules, yielding four full agonists and two partial agonists. The exploration of features that confer selectivity to the designed compounds has also been investigated by Xing et al. (2009). The authors developed a selective pharmacophore model based on a series of selective β_2AR agonists, presenting the first study using a ligand-based computational approach to generate specific three-dimensional pharmacophore hypotheses for the β_2AR from its selective agonists. The best pharmacophore hypothesis consisted of five chemical features (one hydrogen-bond acceptor, one hydrogen-bond donor, two ring aromatic, and one positive ionizable feature). The result was in accordance with the reported interactions between the β_2AR and agonists. Interestingly, the pharmacophore hypothesis can perfectly differentiate β_2-agonists from β_1-agonists, providing a valuable tool for virtual screening to find selective compounds against β_2AR.

Endothelin receptors

In mammals, endothelins (ETs) are potent regulators of vessel functions involved in the pathophysiology of cancer, congestive heart failure, cardiovascular, proteinuria, and glomerulosclerosis. These peptides (ET_{1-3}) exert their biological effects via activation of four ET receptors, ET_A, ET_{B1}, ET_{B2}, and ET_C. Activation of the ET_A receptor is associated with pronounced vasoconstriction whereas ET_B receptor occupation is linked to vasodilation. In addition, other subtypes of the ET_B receptor exist, one mediating vasodilation (ET_{B1}) and the other eliciting constriction (ET_{B2}). An additional receptor subtype, ET_C, has been identified although its physiological significance is uncertain (Pollock et al., 1995)

Funk et al. (2004) applied a pharmacophore model of endothelin-A (ET_A) selective receptor antagonists for screening a chemical database and identified two structurally novel lead compounds with satisfactory affinity for ET_A receptor.

Angiotensin receptors

Angiotensins are oligopeptides that exert their biological actions through the binding to specific angiotensin receptors (AT_1, AT_2, AT_3, and AT_4 receptors). It has been demonstrated that these receptors could be targeted for developing novel effective drugs for the treatment of hypertension, cardiovascular disorders, diabetic nephropathy, atherosclerosis (Goodfriend et al., 1996).

A series of symmetrically bis-substituted imidazole analogs has been designed based on docking studies, utilizing for the first time an extra hydrophobic binding cleft of the modeled AT_1 receptor (Agelis et al., 2013). Four of the synthesized compounds showed high binding affinity to the AT_1 receptor and high antagonistic activity (potency) similar or even superior to that of Losartan.

In an attempt to identify new AT_1 receptor antagonists Pal and Paliwal (2012) developed a pharmacophore-based virtual screening protocol, which led to the identification of two active

AT_1 receptor antagonists with diverse structures (Pal and Paliwal, 2012).

DRUG DESIGN AND DISCOVERY IN NEUROLOGICAL DISORDERS AND PAIN

Dopamine receptors

Dopamine exerts its function via five different receptors (D_1, D_2, D_3, D_4, and D_5 receptors). This system plays a pivotal role in central nervous system and has been demonstrated to be involved in a series of neurological and psychiatric diseases such as Parkinson's disease, schizophrenia, bipolar disorder, drug addiction, and Huntington's disease (Pivonello et al., 2007; Beaulieu and Gainetdinov, 2011). The discovery of ligands able to modulate the dopaminergic system remains challenging and a lot of computational efforts were carried out for selecting potent and selective ligands.

In 2010, the crystal structure of D_3 receptor was solved, which definitely confirmed the utility of homology models in GPCRs drug discovery (Chien et al., 2010). Indeed, Carlsson et al. (2011) docked over 3.3 million molecules against a homology model, and 26 of the highest ranking were tested for binding. Six had affinities ranging from 0.2 to 3.1 μM. Subsequently, the crystal structure was used and the docking screen repeated. Of the 25 compounds selected, five showed affinities ranging from 0.3 to 3.0 μM. One of the new ligands from the homology model screen was optimized reaching an affinity to 81 nM. The paper clearly demonstrated the feasibility of high throughput docking using modeled GPCRs.

The solved crystal structure of D_3 receptor with a D_2/D_3 selective antagonist provides an opportunity to identify subtle structural differences between closely related GPCRs that can be exploited for novel drug design. In an elegant work Lane et al. (2013) performed virtual screening for orthosteric and putative allosteric ligands of D_3 receptor using two optimized crystal-structure-based models. The authors employed in the computational protocol a receptor with an empty binding pocket (D_3 receptor-APO), and a receptor in complex with dopamine (D_3 receptor-Dopa). Potential hits retrieved by using the two models were submitted to biological evaluation and functional characterization. Pharmacological studies showed 14 novel ligands with a binding affinity better than 10 μM in the D_3 receptor-APO candidate list (56% hit rate), and eight novel ligands in the D_3 receptor-Dopa list (32% hit rate). Most ligands in the D_3 receptor-APO model spanned both orthosteric and extended pockets and behaved as antagonists at D_3 receptor. Among the identified ligands, one compound showed the highest potency of dopamine inhibition ($IC_{50} = 7$ nM). In contrast, compounds identified by the D_3 receptor-Dopa model were predicted to bind an allosteric site at the extracellular extension of the pocket. Such compounds showed a variety of functional activity profiles. In fact, at least two compounds were non-competitive allosteric modulator of dopamine signaling in the extracellular signal-regulated kinase and β-arrestin recruitment assays. The high affinity and LE of the chemically diverse hits identified in this mentioned study evidently demonstrated the utility of structure-based screening in targeting allosteric sites of GPCRs (Lane et al., 2013).

Very recently, Vass et al. (2014) reported a prospective structure based virtual fragment screening on D_3 and the H_4 receptors. Representative receptor conformations for ensemble docking were obtained from molecular dynamics (MD) trajectories. Biological evaluation confirmed hit rates ranged from 16 to 32%. Hits had high LE values in the range of 0.31–0.74 and also acceptable lipophilic efficiency, demonstrating that the X-ray structure, the homology model, and structural ensembles were all found suitable for docking based virtual screening of fragments against these GPCRs.

Muscarinic receptors

The muscarinic acetylcholine receptors (M_1–M_5) are promising targets for the treatment of chronic obstructive pulmonary disease, urinary incontinence, and diabetes. Unfortunately, the lack of subtype specificity has remained a major obstacle to develop clinically useful muscarinic ligands. Very recently, Kruse et al. (2013) used the crystal structure of the M_2 and M_3 receptors as a template to identify, by means of structure-based docking, novel muscarinic ligands. Interestingly, one compound was a partial agonist at the M_3 receptor without measurable M_2 agonism that was able to stimulate insulin release from a mouse β-cell line (Kruse et al., 2013).

Cannabinoid (CB) receptors

The cannabinoid 1 receptor (CB1 receptor) and the cannabinoid 2 receptor (CB2 receptor) are members of the GPCR family (Matsuda et al., 1990). Agonists of both cannabinoid receptor subtypes produce strong antinociceptive effects in animal models of chronic, neuropathic, and inflammatory pain and are intensively investigated as potential new analgesic and antiinflammatory agents. CB1 antagonists are clinically established to be effective in treating obesity, obesity-related cardio-metabolic disorders, and substance abuse, but there are currently no marketed CB1 antagonists. The relevance of CB2-mediated therapeutics is well established in the treatment of pain, neurodegenerative, and gastrointestinal tract disorders (Di Marzo, 2008; Brogi et al., 2011; Pasquini et al., 2012).

Pandey et al. (2014) used homology model and high throughput docking to discover new chemical classes of CB1 antagonists that may serve as starting point for drug development. The authors developed and validated a homology model of CB1 based on a bovine rhodopsin template, which led to the discovery of seven compounds with an inhibitory potency >50% at 10 μM (Pandey et al., 2014). Wang et al. (2008) identified a novel class of azetidinones as CB1 antagonists by also using virtual screening methods. Meng et al. (2010) reported the identification of the benzhydrylpiperazine scaffold as a potential scaffold to develop novel CB1 receptor modulators using a privileged structure-based approach. The authors identified a highly potent and selective CB1 receptor inverse agonist that was able to reduce body weight in diet-induced obese Sprague–Dawley rats.

A recent work carried out by Renault et al. (2013) highlighted the importance related to crystallization of class-A GPCRs in a range of active states, identifying specific anchoring sites for CB2 agonists retrieved in an agonist-bound homology model of CB2 receptor. Docking-scoring enrichment tests of a high-throughput

virtual screening of 140 compounds led to 13 hits within the μM affinity range. Interestingly, a relevant number of selected hits behaved as CB2 agonists, among them two novel unrelated full agonists were identified. Notably, the exclusive discovery of agonists illustrated the reliability of this agonist-bound state model in the discovery of GPCR ligands with desired behavior (Renault et al., 2013).

Recently, some of us described a three-dimensional quantitative structure–selectivity relationships (3D-QSSR) study for selectivity of a series of structurally diverse ligands characterized by a wide range of selectivity index values for cannabinoid CB1 and CB2 receptors (Brogi et al., 2011). 3D-QSSR explorations were expected to provide design information for the design of selective CB2 ligands. The computational model proved to be predictive, with r^2 of 0.95 and Q^2 of 0.63. In order to get prospective experimental validation, the selectivity of an external data set of 39 compounds reported in the literature was predicted by means of 3D-QSSR model ($r^2 = 0.56$). Subsequently, a quinolone derivative predicted to be a selective CB2 ligand was synthesized and found to be an extremely selective CB2 ligand displaying high CB2 affinity ($Ki = 4.9$ nM), while being devoid of CB1 affinity ($Ki > 10{,}000$ nM). This finding confirmed that the ligand-based tool represent a valuable complementary approach to docking studies performed on homology models of GPCRs.

Opioid receptors

Opioids are key medications for the treatment of pain.The μ-opioid receptors (MORs), δ-opioid receptors (DORs), κ-opioid receptors (KORs), and nociceptin-opioid receptor (NOP) have been isolated and cloned. The receptors were found throughout the peripheral and central nervous system. Their important role in mediating pain, drug addiction, and depression has been established. Very recently crystal structures of all classes of opioid receptor have been solved (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012; Fenalti et al., 2014). Below is reported one of the first computational efforts using the crystal structure of the KOR.

Negri et al. (2013) applied a structure-based computational protocol using the crystal structure of KOR receptor, discovering a selective novel KOR agonist, exhibiting analgesic effects without activating reward pathways. Remarkably, the novel derivatives have been identified as novel pharmacological tools to study the involvement of KOR in the etiology of drug addiction, depression, and pain (Negri et al., 2013).

ALLOSTERIC MODULATORS AND BIVALENT LIGANDS

ALLOSTERIC MODULATORS

The binding site of the endogenous agonist is qualified as orthosteric. In general, antagonists, and inverse agonists typically occupy also this site, which is usually buried at the core of the receptor or located at its extracellular N-terminal end. In addition, exist allosteric sites that bind synthetic drugs or endogenous mineral cations, such as sodium, calcium, zinc, and magnesium, which can also modulate the activity of the receptor (Christopoulos, 2002). More specifically, allosteric ligands may promote or reduce the binding of orthosteric ligands. Their effects on receptor activation could be in a positive, negative, or neutral manner. Allosteric modulators offer several advantages over classical approaches. Allosteric modulator can modulate affinity via conformational coupling between the orthosteric and allosteric binding sites or modulate efficacity by altering the functional response of the receptor to orthosteric ligand binding. These mechanisms can be dominant for a particular allosteric drug candidate and have significant value in the drug development process. Allosteric modulators can have a chemical structure unrelated to that of competitive agonist or antagonist drugs, offering a novel class of small molecule drug candidates.

The orthosteric binding sites within A class GPCR family are highly conserved due to the evolutionary pressure to retain amino acid sequences necessary for binding of the endogenous ligand. In contrast, allosteric modulator binding sites have much greater structural diversity than endogenous ligand binding sites, displaying a very high selectivity for a receptor subtype (Mohr et al., 2013).

Negative allosteric modulators (NAMs) bind at the allosteric binding site to inhibit the efficacy or affinity to the orthosteric binding site of the agonists while they have no intrinsic agonist efficacy. Two mechanisms can be invoked: the NAMs may stabilize an inactive conformation of the receptor that lowers the affinity of the agonists, or alternatively they raise the energy barrier necessary to activate the receptor activation, which diminishes the intensity of the output response (**Figure 2**, Burford et al., 2011).

On the very opposite, the binding of positive allosteric modulators (PAMs) to their allosteric binding site promotes the binding of the agonists at the orthosteric site or lower the barrier of energy involved in the shift to the active conformation of the receptor (Burford et al., 2011). The major drawback with this class of drugs is that they do not display any pharmacological effect in the absence of the endogenous (or exogenous) orthosteric agonist. Hence, a PAM in combination with an orthosteric agonist can increase the efficacy of the orthosteric compound. The PAM can allow a decrease in the dose administered, thereby improving the overall side-effect profile (**Figure 2**).

Silent allosteric modulators (SAMs) are neutral allosteric ligands. They have no effect on orthosteric agonist affinity or efficacy. However, SAMs can act as competitive antagonists at the same allosteric site, blocking PAM or NAM activity. SAMs can be effective tools to show that presumed PAM or NAM effects are receptor-mediated (Burford et al., 2013).

Interestingly, minor structural modifications are sufficient to transform a NAM into a PAM. Such a subtle effect have not been reported yet with class A GPCRs, even though it is likely that it will be found in a close future, due to the growing importance of this field of research. A striking example of this phenomenon concerns allosteric ligands of metabotropic glutamate receptors (mGluR5), class C family of the GPCRs, (**Figure 2**). While compound 1 is a partial NAM that only partially block mGluR5 signaling, introduction of a mere methyl can convert this compound to a full NAM or PAM (Williams et al., 2010).

Already two NAMs and one PAM have been approved for clinical use: Maraviroc (Celsentry), plerixafor (Mozobil), and Cinacalcet (Mimpara; **Figure 3**). Maraviroc is a high-affinity

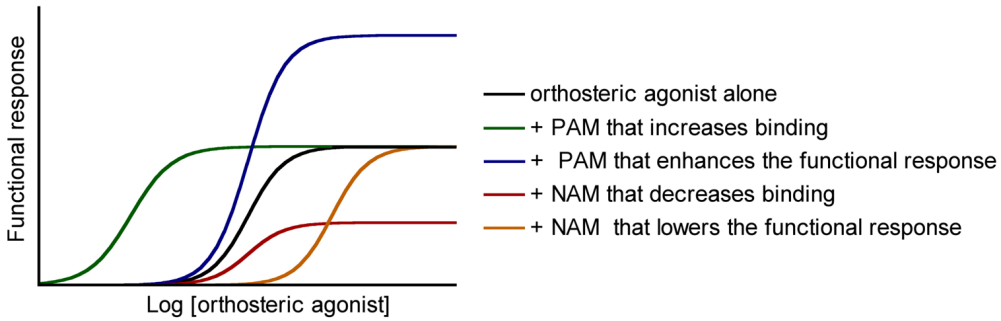

FIGURE 2 | Functional responses of allosteric modulators. Positive and negative allosteric modulators (positive allosteric modulators and negative allosteric modulators) may modulate the affinity and/or the efficacy of orthosteric agonists.

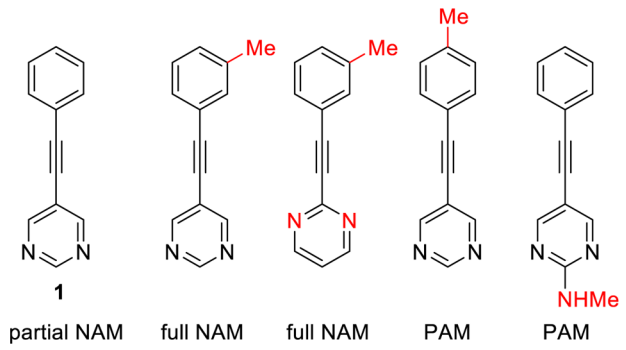

FIGURE 3 | Selected examples of mGluR5 allosteric ligands illustrating how a minimal structural variation can deeply affect the allosteric profile.

NAM of the CCR5 receptor that blocks the interaction of the HIV-glycoprotein 120 with this receptor (Fatkenheuer et al., 2005). It was approved in 2007 for the treatment of HIV in combination with antiretroviral agents. Plerixafor is a NAM of the chemokine receptor CXCR4. This medicine is used to promote the release stem cells into the bloodstream after autologous stem cell transplantation (Scholten et al., 2012).

Cinacalcet is a PAM of the calcium-sensing receptor (CaSR) of parathyroid hormone (PTH) producing cells. In a feedback mechanism, activation of CaSR by cinacalcet inhibits PTH release. This medicine was approved in 2004 for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease on dialysis, and hypercalcaemia in patients with parathyroid cancer (Torres, 2006).

MONOVALENT LIGANDS SPECIFIC FOR GPCR HETERODIMERS

It is now well established that GPCRs may form homodimers, heterodimers, or oligomers. Even though their physiological function is not fully apprehended, these dimerizations and oligomerizations have major repercussions on ligand binding, activation of signaling pathways and cellular trafficking. Therefore, targeting specific GPCR dimers may provide drugs with enhanced potency, selectivity, and therapeutic index. Two types of such drugs that are specific for a specific GPCR dimer have been described (**Figure 4**). The first type concerns monovalent drugs, such as 6′-guanidinonaltrindole (6′GNTI), NNTA or SKF83959, that bind to only one receptor at a time. The second one concerns bivalent drugs that bind to two receptors at the same time (**Figures 4** and **5**).

Waldhoer et al. (2005) found that 6′-GNTI behaves as an extremely potent agonist in cells expressing both DORs and MORs and established that this drug selectively activates the δOR–κOR heterodimer (Waldhoer et al., 2005). *In vivo*, 6′-GNTI induces a potent analgesia when administered intrathecally. This δOR–κOR heterodimer was found to be expressed in a tissue selective fashion suggesting that such a drug may induce less side effects than classical OR agonists. Similarly, NNTA selectively activates

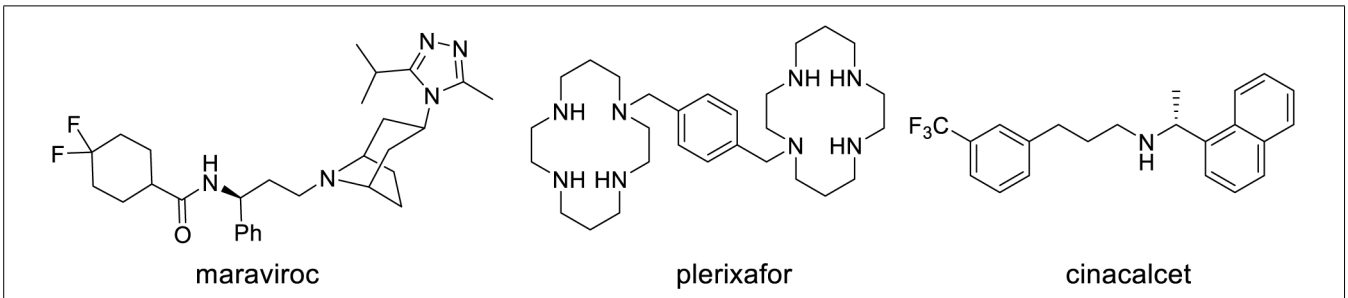

FIGURE 4 | Structure of approved allosteric modulators.

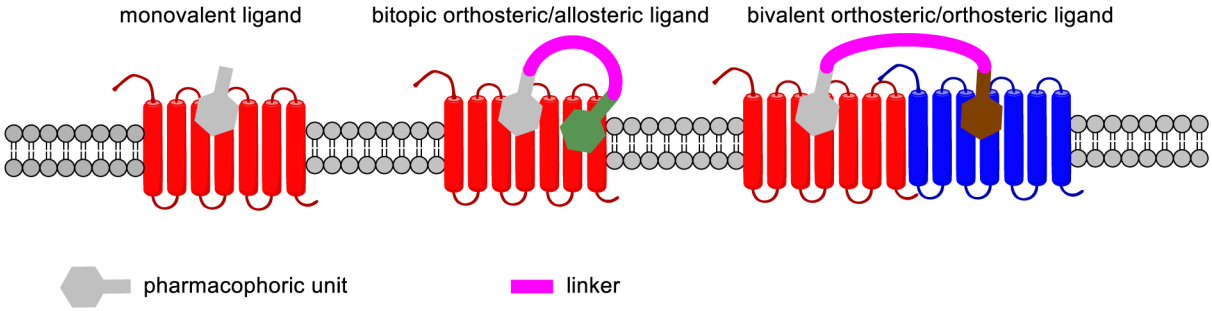

FIGURE 5 | Binding mode of monovalent, bitopic orthosteric/allosteric, and bivalent orthosteric/allosteric ligands of GPCRs.

the μOR–κOR heterodimer to induce a potent antinociceptive response devoid of physical dependence in mice (Yekkirala et al., 2011).

Another interesting example is provided by SKF83959 that selectively targets the D_1–D_2 dopaminergic heterodimer to increase intracellular calcium levels through activation of $G_{q/11}$ (Rashid et al., 2007). Interestingly, this drug does not activate adenylyl cyclase, which is normally induced by the signaling of D_1 or D_2 receptors.

BIVALENT LIGANDS

Different domains of GPCRs such as intracellular loops (ICL), extracellular loops (ECL), and transmembrane domains (TM) are known to participate in ligand recognition and receptor dimerization. Many GPCRs can form oligomers with conformational rearrangements of the receptors that impact their signaling (Percherancier et al., 2005).

Bivalent ligands are composed of two pharmacophoric units connected through a linker (while monovalent drugs encompass only one pharmacophoric unit). The pharmacophores may be identical (and in that case, the ligand is termed as homobivalent) or different in the case of heterobivalent ligands. These pharmacophoric units may either bind to the orthosteric site and an allosteric site within the same receptor or to two orthosteric binding sites located on two different receptors (**Figure 6**).

Often the large size and molecular weight of bivalent ligands severely reduce their bioavailability and hinder their use in *in vivo* studies. However, these limitations are not irretrievable and few bivalent ligands have shown interesting *in vivo* pharmacological activities, even though none of them entered a clinical trial (**Figure 7**).

Portoghese and colleagues have conjugated a μ agonist (oxymorphone) to a δ antagonist of opioids receptors (naltrindole) through a 21-atom linker (Daniels et al., 2005). The resulting compound, MDAN-21, was able to cross the blood brain barrier to induce antinociception. Impressively, this drug was 50 times more potent than morphine, and did not induce tolerance or physical dependence after chronic treatment.

In another example, Halazy and colleagues dimerized a 5-HT_{1B} agonist, sumatriptan, through a linker to obtain an orally active drug that induced a stronger hypothermia than sumatriptan itself (Perez et al., 1998). It is remarkable that such a drug could cross the blood–brain barrier in spite of its elevated molecular weight, polar surface area, and number of hydrogen-bond donors, suggesting that an active transport is probably involved.

SR141716 is another interesting bivalent drug that combines two units of a cannabinoid CB1 receptor antagonist/inverse agonist (Zhang et al., 2010). This compound was found to efficiently cross the blood–brain barrier to inhibit the antinociceptive effects of a cannabinoid agonist.

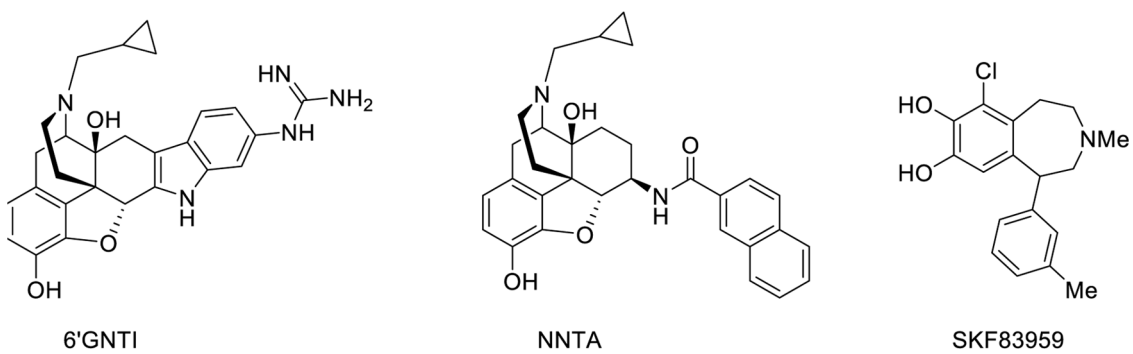

FIGURE 6 | Structure of monovalent drugs acting on GPCR dimers.

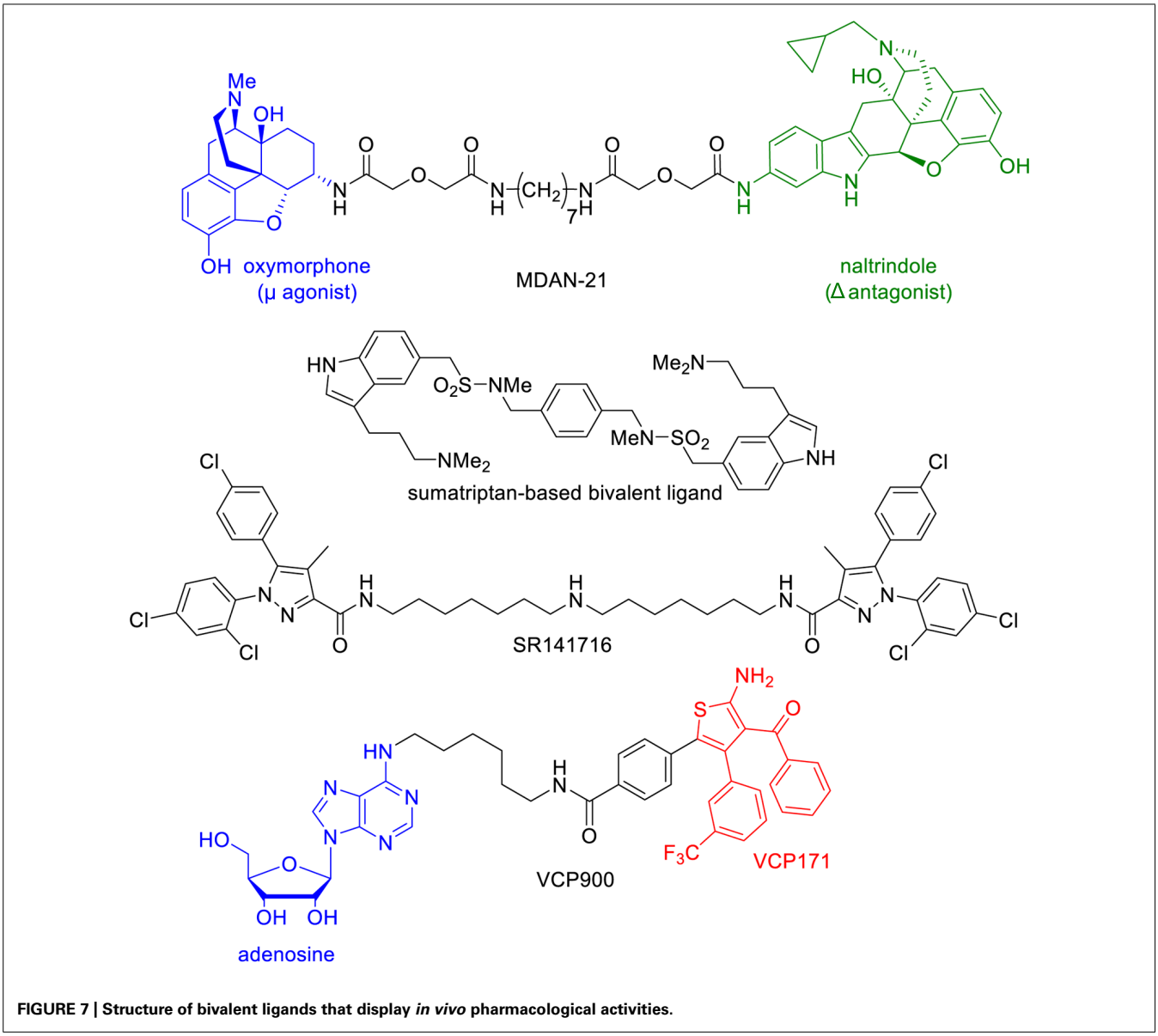

FIGURE 7 | Structure of bivalent ligands that display *in vivo* pharmacological activities.

Very recently, Christopoulos and colleagues conjugated adenosine to VCP171, a PAM of the adenosine A1 receptor ($A_{1A}R$; Valant et al., 2014). The obtained compound, called VCP746 binds to both the orthosteric and allosteric sites and behaves as a biased agonist (**Figure 7**). Importantly, it protects *in vitro* cardiomyoblasts and cardiomyocytes against simulated ischemia, but in contrast to classical $A_{1A}R$ agonists it does not perturb rat atrial heart rate *in vivo*.

BIASED-SIGNALING SELECTIVITY

G protein-coupled receptors ligands are described by their efficacy (agonist, antagonist, partial agonist, or inverse agonist) and target (receptor type and subtype). Recently, great attention has been devoted to functional selectivity of GPCR ligands for the development of better therapeutic drugs with potentially fewer off-target and/or side effects. Ligand bias has been described based on their functional selectivity that preferentially signal through either G protein- or β-arrestin-mediated pathways.

Allosteric ligands can induce biased G protein signaling, thus representing interesting opportunities for drug discovery. Moreover, biasing β-arrestin-dependent signaling has also been shown to be potentially beneficial in heart diseases.

To delineate the contributions of G proteins and β-arrestins to GPCR function several approaches have been used including targeted genetic deletion of GRKs or β-arrestins, RNA silencing of G protein and β-arrestin, and small-molecule inhibitors of specific signal transduction pathways (DeWire et al., 2007). An important approach to investigate whether GPCR ligands are G protein-biased or β-arrestin-biased agonists is the use of β-arrestin knockout mice (Rominger et al., 2014). Indeed, the recapitulation of improved pharmacology in β-arrestin KO mice by a ligand demonstrates that this ligand is a G-protein biased

ligand and may be particularly sensitive to the acute desensitization effects of β-arrestin. Inversely, minor pharmacological effects in β-arrestin KO mice indicate that β-arrestin is required for the specific intracellular signaling pathways of these β-arrestin-biased ligands.

Biased ligands that selectively activate β-arrestin signaling pathways over G_q G_i and G_s-coupled GPCRs have already been reported (Violin and Lefkowitz, 2007; Gesty-Palmer et al., 2009). Biased signaling can also results from mutation of receptors (Leach et al., 2012; Sbai et al., 2014; **Figure 8**).

Another advanced approach is receptor activated solely by synthetic ligand' (RASSLs). The chemical genetic approach involves the expression of a mutant form that can be activated by synthetic drugs but not by the endogenous ligands. For example a specifically mutated muscarinic receptor can be activated by clozapine-*N*-oxide (CNO), but not by acetylcholine (Armbruster et al., 2007). This approach has been utilized to determine GPCR signaling pathways important in β-islet function (Guettier et al., 2009), neuronal networks involved in neurological responses such as locomotion learning and memory (Garner et al., 2012), limbic seizures, and metabolism (Kong et al., 2012).

G PROTEIN-BIASED MORPHINE μ-OPIOID RECEPTOR (MOR) LIGANDS

Both MOR and DOR are involved in analgesic effect of opioids. Thermal nociception is primarily modulated by MORs while mechanical nociception is primarily mediated by DOR (Scherrer et al., 2009), suggesting that these receptors are expressed in distinct circuits. Opioids cause postoperative nausea and vomiting, constipation, and sedation, giving significant patient discomfort, and can prolong hospital stay (Anastassopoulos et al., 2011). The respiratory suppression also limits opioid dosing, leaving many patients in pain during recuperation (Dahan, 2007). The classical μ opioid morphine increases efficacy and duration of analgesic response with reduced gastrointestinal dysfunction, and less respiratory suppression in β-arrestin-2 knockout mice compared to wild-type mice. This data clearly suggested that G protein-biased MOR agonists would be more efficacious with reduced adverse than current opioids.

The G protein-biased MOR agonist TRV130 has robust G protein signaling, with less β-arrestin recruitment and receptor internalization. TRV130 increases analgesia with reduced CNS depression and reduced gastrointestinal dysfunction compared with morphine. Thus TRV130 may provide a marked improvement over current opioids in postoperative care. It also holds great promise for chronic pain management, where constipation is a severe and often dose-limiting adverse event. TRV130 has been currently evaluated in human clinical trials for the treatment of acute severe pain (Chen et al., 2013). TRV130 displays broad dose margins between MOR-mediated pharmacology and intolerance in healthy volunteers (Soergel et al., 2014).

Similarly, a β-arrestin–MAPK pathway mediates stress and aversion-associated effects of kappa opioid receptor agonists, suggesting that biased kappa opioid ligands could provide analgesia without the dysphoric effects associated with classic kappa opioid agonists (Bruchas et al., 2010).

β-ARRESTIN-BIASED DOPAMINE D_2 LIGANDS

Dopamine plays a major role in reward-motivated behavior and motor control. The physiological actions of dopamine are mediated by five distinct but closely related GPCRs that are divided into two major groups: the D_1 and D_2 classes of dopamine receptors (Vallone et al., 2000). This classification is generally based on the original biochemical observations showing that dopamine is able to modulate adenylyl cyclase (AC) activity. Non-canonical modes of dopamine D_2 receptor (D_2R) signaling via β-arrestin is important for the therapeutic actions of both antipsychotic and antimanic agents. Aripiprazole, a FDA-approved atypical antipsychotic drug, was one of the first functionally selective D_2R ligands identified (Urban et al., 2007; Mailman and Murthy, 2010). However, aripiprazole could behave as a full agonist, a partial agonist, or an antagonist at D_2R depending on the cell type (Shapiro et al., 2003; Urban et al., 2007).

It was found that the antipsychotic action of an aripiprazole analog, UNC9975, was attenuated in the β-arrestin-2 knockout mice. UNC9975 also represents unprecedented β-arrestin–biased ligands for a G_i-coupled GPCR. Significantly, UNC9975 is an

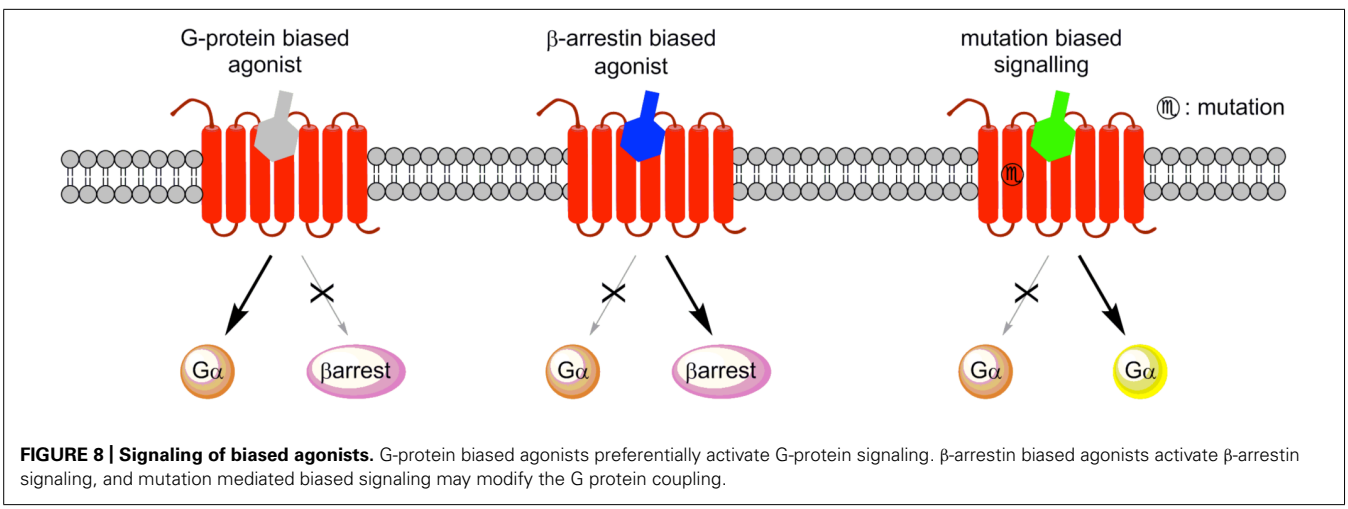

FIGURE 8 | Signaling of biased agonists. G-protein biased agonists preferentially activate G-protein signaling. β-arrestin biased agonists activate β-arrestin signaling, and mutation mediated biased signaling may modify the G protein coupling.

antagonist of G_i-regulated cAMP production and partial agonist for D_2R/β-arrestin-2 interactions. Importantly, UNC9975 displayed potent antipsychotic-like activity without inducing motoric side effects *in vivo* (Masri et al., 2008). This β-arrestin–biased ligand shows a potent ability to suppress both *d*-amphetamine and phencyclidine-induced hyper locomotion in mice, indicating that it possesses antipsychotic activities *in vivo*. β-arrestin–biased ligands induce a lack of internalization. Thus, we can assume that drugs that induce internalization would ultimately foster tachyphylaxis and receptor down-regulation (Allen and Roth, 2011).

MISSENSE MUTATION GPCR LEADING BIASED SIGNALING IN DISEASES

Many biased signaling are due to the ligand (Whalen et al., 2011), but few examples of biased signaling induced by a mutation of receptors have also been reported (Rajagopal et al., 2010).

A natural mutation leading to biased signaling has been identified in the thyroid stimulating hormone (TSH) receptor gene. The mutant TSH receptor still couples to G_s and activates cAMP but completely loses G_q-mediated inositol phosphate production. This mutation on TSH receptor causes euthyroid hyperthyrotropinemia with increased radioiodine uptake (Grasberger et al., 2007).

Another example is the natural mutations in the human calcium sensing receptor that activate both G_q-dependent production of inositol phosphate and the G_q- and $G_{i/o}$-dependent phosphorylation of ERK (Leach et al., 2012; Nygaard et al., 2013). It is generally assumed that biased signaling is an intrinsic property of a given ligand-GPCR complex, whereby a GPCR exists in several conformations, each of which is preferentially stabilized and activated by selective ligands (Nygaard et al., 2013). Likewise, the mutations leading to biased signaling are supposed to affect the equilibrium between the different receptor conformations.

The mutations in the GPCRs can lead to biased downstream signaling and may induce pathogenic and, in some cases, protective roles.

Prokineticins are anorexigenic and angiogenic hormones that couple to two GPCRs, PKR1, and PKR2 (Nebigil, 2009; Dormishian et al., 2013; Szatkowski et al., 2013). Mutations in the prokineticin receptor 2 (PKR2) have been found in 10% of patients with Kallmann syndrome that is characterized by hypogonadotropic hypogonadism. To date, 21 missense mutations of PKR2 have been identified in Kallmann syndrome patients. Some of these mutations are related with the G_q-dependent signaling pathway (Sinisi et al., 2008; Abreu et al., 2012; Sbai et al., 2014). However, certain mutations on this receptor affect β-arrestin recruitment (R80C) or the G_q and G_i signaling pathways (R164Q) with normal G_s signaling. The G_q-dependent signaling defect of the R164Q receptor makes this mutation most likely pathogenic. The mutation R268C affecting a residue in the third intracellular loop of the receptor selectively impairs $G_{i/o}$-dependent signaling of the receptor and is considered non-pathogenic (Sbai et al., 2014). It remains unclear whether the β-arrestin-dependent signaling defect for the R80C mutation on PKR2 has a pathogenic effect with respect to Kallmann syndrome.

BIASED LIGANDS IN DISEASES

Two GPCRs, the angiotensin II (AngII) type 1 receptor (AT_1R) and the β-ARs are targets of widely used cardiovascular drugs. They are now potential therapeutic targets for biased ligands (DeWire and Violin, 2011).

The peptide hormone angiotensin II (AngII) is a vasopressor that regulates salt and fluid homeostasis, modulating vasoconstriction, and aldosterone secretion, as well as thirst and inflammation (Benigni et al., 2010). Angiotensin-converting enzyme inhibitors that lower AngII levels and angiotensin receptor blockers are widely used in treating hypertension and other cardiovascular diseases. The AT_1R couples primarily to $G_{\alpha q}$ signaling, leading to phosphatidylinositol bisphosphate hydrolysis, generating diacylglycerol, mobilizing calcium, and activating signaling enzymes such as protein kinase C. AT_1R is also involved in β-arrestin–dependent signals, activation of epidermal growth factor receptor transactivation, Src, and JAK/STAT (Saito and Berk, 2001; Wei et al., 2003; Oliveira et al., 2007). One body of evidence for distinct AT_1R signaling came from receptor mutagenesis. AT_1R effects can be divided into distinct G-protein–dependent and G-protein–independent signals *in vivo*. Reduction or elimination of β-arrestin-1 or β-arrestin-2 expression with siRNA *in vitro* or genetic deletion *in vivo* showed that cardioprotective effect of AT_1R is mediated by β-arrestin-2 signaling. TRV120027, a selective and β-arrestin–biased AT_1R ligand blocks AngII-dependent hypertension while increasing cardiomyocyte contractility, promoting cytoprotective, or antiapoptotic signals and preserving kidney function to provide a great benefit in acute heart failure (Monasky et al., 2013). TRV120027 is now in clinical trials for the treatment of acute heart failure (Soergel et al., 2014).

Endothelins play a key role in vascular homeostasis. ET_A and AT_1 receptor antagonists both lower blood pressure in hypertensive patients. Accordingly, a dual ET_A and AT_1 receptor antagonist may be more efficacious antihypertensive drug than current medicines.

Epinephrine binds to cardiac β_1AR and stimulates inotropy through G-protein signals, resulting in increased heart rate, blood pressure, and metabolic stress, promoting cardiomyocyte apoptosis. Several studies demonstrated that β_1AR G-protein and β-arrestin pathways normally strike a balance between apoptosis associated with prolonged inotropy and counteracting cardioprotection. When this balance is disrupted in the absence of β-arrestin signaling, apoptosis increases and cardiac function decreases. Activation of β-arrestin scaffolded calcium/calmodulin-dependent kinase II by the β_1AR requires cAMP, thus the net effect of a β-arrestin–biased ligand is cardioprotective.

A biased ligand for β_1AR, carvedilol activates the cardioprotective β-arrestin–mediated epidermal growth factor receptor transactivation-signaling pathway. Carvedilol has shown potentially superior clinical efficacy over other β-blockers in terms of cardiovascular events after myocardial infarction (Kopecky, 2006) and perhaps mortality (Poole-Wilson et al., 2003). The contributions of function of GRK/β-arrestin to the clinical efficacy of carvedilol remain unclear.

Collectively, substantial data suggest that biased ligands will have distinct and perhaps more beneficial effects than unbiased agonists. Biased signaling is proposed to be useful in several diseases, including heart failure (β-ARs), hypertension (α-ARs), neuropsychiatric and/or neurodegenerative disorders (histamine H_1 receptors), schizophrenia, Parkinson's disease (dopamine receptors), psychosis and depression (serotonin receptors), hypothyroidism (TSH receptor), hyperlipidemia (nicotinic acid receptor), diabetes (GLP1). However, it is possible that biased signaling could be associated with undesirable side effects and even contribute to disease. For example, the bacterium *Neisseria meningitidis* interacts in a biased and allosteric manner with the β_2AR to initiate signaling cascades that facilitate meningeal colonization (Brissac et al., 2012).

CONCLUSION

A substantial increase in our understanding of GPCR pharmacology has provided an array of ligands that target both orthosteric and allosteric sites of GPCRs as well as ligands that have properties of bias stimuli. The recent identification of a PAM and NAM binding site, together with the synthesis of *in vivo* effective ligands, represents a novel, and likely more favorable, option for pharmacological manipulations of the GPCRs. Biased ligands offer safer, better-tolerated, and more efficacious drugs. However, in some cases a path to successful drug development for targets that have been abandoned because of on-target adverse pharmacology in the clinical proof-of-concept studies due to additional receptor signaling and regulatory mechanisms rather than β-arrestin pathway.

The complexity of GPCR signaling requires a synergistic role for experimental and computational methods in producing novel therapeutics with maximal clinical efficacy and lowest toxicity. Combining computational methods with sophisticated transgenic and chemical genetic animal models, the next generation of GPCR ligands will unquestionably employ rational design principles to deliver GPCR ligands with minimal side-effects.

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REFERENCES

Abagyan, R., and Totrov, M. (2001). High-throughput docking for lead generation. *Curr. Opin. Chem. Biol.* 5, 375–382. doi: 10.1016/S1367-5931(00)00217-9

Abbas, A., and Roth, B. L. (2008). Arresting serotonin. *Proc. Natl. Acad. Sci. U.S.A.* 105, 831–832. doi: 10.1073/pnas.0711335105

Abreu, A. P., Noel, S. D., Xu, S., Carroll, R. S., Latronico, A. C., and Kaiser, U. B. (2012). Evidence of the importance of the first intracellular loop of prokineticin receptor 2 in receptor function. *Mol. Endocrinol.* 26, 1417–1427. doi: 10.1210/me.2012-1102

Agelis, G., Resvani, A., Koukoulitsa, C., Tumova, T., Slaninova, J., Kalavrizioti, D., et al. (2013). Rational design, efficient syntheses and biological evaluation of *N,N'*-symmetrically bis-substituted butylimidazole analogs as a new class of potent Angiotensin II receptor blockers. *Eur. J. Med. Chem.* 62, 352–370. doi: 10.1016/j.ejmech.2012.12.044

Allen, J. A., and Roth, B. L. (2011). Strategies to discover unexpected targets for drugs active at G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* 51, 117–144. doi: 10.1146/annurev-pharmtox-010510-100553

Anastassopoulos, K. P., Chow, W., Ackerman, S. J., Tapia, C., Benson, C., and Kim, M. S. (2011). Oxycodone-related side effects: impact on degree of bother, adherence, pain relief, satisfaction, and quality of life. *J. Opioid Manag.* 7, 203–215. doi: 10.5055/jom.2010.0063

Andrews, S. P., Brown, G. A., and Christopher, J. A. (2014). Structure-based and fragment-based GPCR drug discovery. *ChemMedChem* 9, 256–275. doi: 10.1002/cmdc.201300382

Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S., and Roth, B. L. (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5163–5168. doi: 10.1073/pnas.0700293104

Beaulieu, J. M., and Gainetdinov, R. R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63, 182–217. doi: 10.1124/pr.110.002642

Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., and Caron, M. G. (2005). An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 122, 261–273. doi: 10.1016/j.cell.2005.05.012

Benigni, A., Cassis, P., and Remuzzi, G. (2010). Angiotensin II revisited: new roles in inflammation, immunology and aging. *EMBO Mol. Med.* 2, 247–257. doi: 10.1002/emmm.201000080

Brissac, T., Mikaty, G., Dumenil, G., Coureuil, M., and Nassif, X. (2012). The meningococcal minor pilin PilX is responsible for type IV pilus conformational changes associated with signaling to endothelial cells. *Infect. Immun.* 80, 3297–3306. doi: 10.1128/IAI.00369-12

Brogi, S., Corelli, F., Di Marzo, V., Ligresti, A., Mugnaini, C., Pasquini, S., et al. (2011). Three-dimensional quantitative structure-selectivity relationships analysis guided rational design of a highly selective ligand for the cannabinoid receptor 2. *Eur. J. Med. Chem.* 46, 547–555. doi: 10.1016/j.ejmech.2010.11.034

Brogi, S., Kladi, M., Vagias, C., Papazafiri, P., Roussis, V., and Tafi, A. (2009). Pharmacophore modeling for qualitative prediction of antiestrogenic activity. *J. Chem. Inf. Model.* 49, 2489–2497. doi: 10.1021/ci900254b

Brogi, S., Papazafiri, P., Roussis, V., and Tafi, A. (2013). 3D-QSAR using pharmacophore-based alignment and virtual screening for discovery of novel MCF-7 cell line inhibitors. *Eur. J. Med. Chem.* 67, 344–351. doi: 10.1016/j.ejmech.2013.06.048

Bruchas, M. R., Land, B. B., and Chavkin, C. (2010). The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res.* 1314, 44–55. doi: 10.1016/j.brainres.2009.08.062

Burford, N. T., Clark, M. J., Wehrman, T. S., Gerritz, S. W., Banks, M., O'Connell, J., et al. (2013). Discovery of positive allosteric modulators and silent allosteric modulators of Pharmacophore modeling for qualitative prediction of antiestrogenic activity-opioid receptor. *Proc. Natl. Acad. Sci. U.S.A.* 110, 10830–10835. doi: 10.1073/pnas.1300393110

Burford, N. T., Watson, J., Bertekap, R., and Alt, A. (2011). Strategies for the identification of allosteric modulators of G-protein-coupled receptors. *Biochem. Pharmacol.* 81, 691–702. doi: 10.1016/j.bcp.2010.12.012

Cappelli, A., Manini, M., Valenti, S., Castriconi, F., Giuliani, G., Anzini, M., et al. (2013). Synthesis and structure-activity relationship studies in serotonin 5-HT(1A) receptor agonists based on fused pyrrolidone scaffolds. *Eur. J. Med. Chem.* 63, 85–94. doi: 10.1016/j.ejmech.2013.01.044

Carlsson, J., Coleman, R. G., Setola, V., Irwin, J. J., Fan, H., Schlessinger, A., et al. (2011). Ligand discovery from a dopamine D_3 receptor homology model and crystal structure. *Nat. Chem. Biol.* 7, 769–778. doi: 10.1038/nchembio.662

Castelli, M. P., Casu, A., Casti, P., Lobina, C., Carai, M. A., Colombo, G., et al. (2012). Characterization of COR627 and COR628, two novel positive allosteric modulators of the GABA(B) receptor. *J. Pharmacol. Exp. Ther.* 340, 529–538. doi: 10.1124/jpet.111.186460

Chen, X. T., Pitis, P., Liu, G., Yuan, C., Gotchev, D., Cowan, C. L., et al. (2013). Structure-activity relationships and discovery of a G protein biased mu opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro-[4.5]decan-9-yl]ethyl})amine (TRV130), for the treatment of acute severe pain. *J. Med. Chem.* 56, 8019–8031. doi: 10.1021/jm4010829

Cherezov, V., Peddi, A., Muthusubramaniam, L., Zheng, Y. F., and Caffrey, M. (2004). A robotic system for crystallizing membrane and soluble proteins in lipidic mesophases. *Acta Crystallogr. D. Biol. Crystallogr.* 60, 1795–1807. doi: 10.1107/S0907444904019109

Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., et al. (2007). High-resolution crystal structure of an engineered

human beta2-adrenergic G protein-coupled receptor. *Science* 318, 1258–1265. doi: 10.1126/science.1150577

Chien, E. Y., Liu, W., Zhao, Q., Katritch, V., Han, G. W., Hanson, M. A., et al. (2010). Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science* 330, 1091–1095. doi: 10.1126/science.1197410

Christopher, J. A., Brown, J., Dore, A. S., Errey, J. C., Koglin, M., Marshall, F. H., et al. (2013). Biophysical fragment screening of the beta1-adrenergic receptor: identification of high affinity arylpiperazine leads using structure-based drug design. *J. Med. Chem.* 56, 3446–3455. doi: 10.1021/jm400140q

Christopoulos, A. (2002). Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat. Rev. Drug Discov.* 1, 198–210. doi: 10.1038/nrd746

Chun, E., Thompson, A. A., Liu, W., Roth, C. B., Griffith, M. T., Katritch, V., et al. (2012). Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. *Structure* 20, 967–976. doi: 10.1016/j.str.2012.04.010

Dahan, A. (2007). Respiratory depression with opioids. *J. Pain Palliat. Care Pharmacother.* 21, 63–66. doi: 10.1080/J354v21n01_15

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

DeWire, S. M., Ahn, S., Lefkowitz, R. J., and Shenoy, S. K. (2007). Beta-arrestins and cell signaling. *Annu. Rev. Physiol.* 69, 483–510. doi: 10.1146/annurev.ph.69.013107.100021

DeWire, S. M., and Violin, J. D. (2011). Biased ligands for better cardiovascular drugs: dissecting G-protein-coupled receptor pharmacology. *Circ. Res.* 109, 205–216. doi: 10.1161/CIRCRESAHA.110.231308

Di Marzo, V. (2008). Targeting the endocannabinoid system: to enhance or reduce? *Nat. Rev. Drug Discov.* 7, 438–455. doi: 10.1038/nrd2553

Dore, A. S., Okrasa, K., Patel, J. C., Serrano-Vega, M., Bennett, K., Cooke, R. M., et al. (2014). Structure of class C GPCR metabotropic glutamate receptor 5 transmembrane domain. *Nature* 511, 557–562. doi: 10.1038/nature13396

Dormishian, M., Turkeri, G., Urayama, K., Nguyen, T. L., Boulberdaa, M., Messaddeq, N., et al. (2013). Prokineticin receptor-1 is a new regulator of endothelial insulin uptake and capillary formation to control insulin sensitivity and cardiovascular and kidney functions. *J. Am. Heart Assoc.* 2, e000411. 10.1161/JAHA.113.000411

Fatkenheuer, G., Pozniak, A. L., Johnson, M. A., Plettenberg, A., Staszewski, S., Hoepelman, A. I., et al. (2005). Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. *Nat. Med.* 11, 1170–1172. doi: 10.1038/nm1319

Fenalti, G., Giguere, P. M., Katritch, V., Huang, X. P., Thompson, A. A., Cherezov, V., et al. (2014). Molecular control of delta-opioid receptor signaling. *Nature* 506, 191–196. doi: 10.1038/nature12944

Fernandez-Fuentes, N., Rai, B. K., Madrid-Aliste, C. J., Fajardo, J. E., and Fiser, A. (2007). Comparative protein structure modeling by combining multiple templates and optimizing sequence-to-structure alignments. *Bioinformatics* 23, 2558–2565. doi: 10.1093/bioinformatics/btm377

Funk, O. F., Kettmann, V., Drimal, J., and Langer, T. (2004). Chemical function based pharmacophore generation of endothelin-A selective receptor antagonists. *J. Med. Chem.* 47, 2750–2760. doi: 10.1021/jm031041j

Garner, A. R., Rowland, D. C., Hwang, S. Y., Baumgaertel, K., Roth, B. L., Kentros, C., et al. (2012). Generation of a synthetic memory trace. *Science* 335, 1513–1516. doi: 10.1126/science.1214985

Gemma, S., Brogi, S., Novellino, E., Campiani, G., Maga, G., Brindisi, M., et al. (2014a). HCV-targeted antivirals: current status and future challenges. *Curr. Pharm. Des.* 20, 3445–3464. doi: 10.2174/13816128113199990630

Gemma, S., Brogi, S., Patil, P. R., Giovani, S., Lamponi, S., Cappelli, A., et al. (2014b). From (+)-epigallocatechin gallate to a simplified synthetic analogue as a cytoadherence inhibitor for *P. falciparum*. *RSC Adv.* 4, 4769–4781. doi: 10.1039/c3ra45933k

Gesty-Palmer, D., Flannery, P., Yuan, L., Corsino, L., Spurney, R., Lefkowitz, R. J., et al. (2009). A beta-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation. *Sci. Transl. Med.* 1, 1ra1. doi: 10.1126/scitranslmed.3000071

Giovani, S., Penzo, M., Brogi, S., Brindisi, M., Gemma, S., Novellino, E., et al. (2014). Rational design of the first difluorostatone-based PfSUB1 inhibitors. *Bioorg. Med. Chem. Lett.* 24, 3582–3586. doi: 10.1016/j.bmcl.2014.05.044

Goodfriend, T. L., Elliott, M. E., and Catt, K. J. (1996). Angiotensin receptors and their antagonists. *N. Engl. J. Med.* 334, 1649–1654. doi: 10.1056/NEJM199606203342507

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

Grasberger, H., Van Sande, J., Hag-Dahood Mahameed, A., Tenenbaum-Rakover, Y., and Refetoff, S. (2007). A familial thyrotropin (TSH) receptor mutation provides in vivo evidence that the inositol phosphates/Ca2+ cascade mediates TSH action on thyroid hormone synthesis. *J. Clin. Endocrinol. Metab.* 92, 2816–2820. doi: 10.1210/jc.2007-0366

Guettier, J. M., Gautam, D., Scarselli, M., Ruiz De Azua, I., Li, J. H., Rosemond, E., et al. (2009). A chemical-genetic approach to study G protein regulation of beta cell function in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19197–19202. doi: 10.1073/pnas.0906593106

Haga, K., Kruse, A. C., Asada, H., Yurugi-Kobayashi, T., Shiroishi, M., Zhang, C., et al. (2012). Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* 482, 547–551. doi: 10.1038/nature10753

Hanson, M. A., Cherezov, V., Griffith, M. T., Roth, C. B., Jaakola, V. P., Chien, E. Y., et al. (2008). A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor. *Structure* 16, 897–905. doi: 10.1016/j.str.2008.05.001

Hanson, M. A., Roth, C. B., Jo, E., Griffith, M. T., Scott, F. L., Reinhart, G., et al. (2012). Crystal structure of a lipid G protein-coupled receptor. *Science* 335, 851–855. doi: 10.1126/science.1215904

Hollenstein, K., Kean, J., Bortolato, A., Cheng, R. K., Dore, A. S., Jazayeri, A., et al. (2013). Structure of class B GPCR corticotropin-releasing factor receptor 1. *Nature* 499, 438–443. doi: 10.1038/nature12357

Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. R., et al. (2008). The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322, 1211–1217. doi: 10.1126/science.1164772

Khoury, E., Clement, S., and Laporte, S. A. (2014). Allosteric and biased g protein-coupled receptor signaling regulation: potentials for new therapeutics. *Front. Endocrinol.* 5:68. doi: 10.3389/fendo.2014.00068

Kolb, P., Rosenbaum, D. M., Irwin, J. J., Fung, J. J., Kobilka, B. K., and Shoichet, B. K. (2009). Structure-based discovery of beta2-adrenergic receptor ligands. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6843–6848. doi: 10.1073/pnas.0812657106

Kong, D., Tong, Q., Ye, C., Koda, S., Fuller, P. M., Krashes, M. J., et al. (2012). GABAergic RIP-Cre neurons in the arcuate nucleus selectively regulate energy expenditure. *Cell* 151, 645–657. doi: 10.1016/j.cell.2012.09.020

Kooistra, A. J., Roumen, L., Leurs, R., De Esch, I. J., and De Graaf, C. (2013). From heptahelical bundle to hits from the Haystack: structure-based virtual screening for GPCR ligands. *Methods Enzymol.* 522, 279–336. doi: 10.1016/B978-0-12-407865-9.00015-7

Kopecky, S. L. (2006). Effect of beta blockers, particularly carvedilol, on reducing the risk of events after acute myocardial infarction. *Am. J. Cardiol.* 98, 1115–1119. doi: 10.1016/j.amjcard.2006.05.039

Kruse, A. C., Hu, J., Pan, A. C., Arlow, D. H., Rosenbaum, D. M., Rosemond, E., et al. (2012). Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* 482, 552–556. doi: 10.1038/nature10867

Kruse, A. C., Weiss, D. R., Rossi, M., Hu, J., Hu, K., Eitel, K., et al. (2013). Muscarinic receptors as model targets and antitargets for structure-based ligand discovery. *Mol. Pharmacol.* 84, 528–540. doi: 10.1124/mol.113.087551

Lane, J. R., Chubukov, P., Liu, W., Canals, M., Cherezov, V., Abagyan, R., et al. (2013). Structure-based ligand discovery targeting orthosteric and allosteric pockets of dopamine receptors. *Mol. Pharmacol.* 84, 794–807. doi: 10.1124/mol.113.088054

Leach, K., Wen, A., Davey, A. E., Sexton, P. M., Conigrave, A. D., and Christopoulos, A. (2012). Identification of molecular phenotypes and biased signaling induced by naturally occurring mutations of the human calcium-sensing receptor. *Endocrinology* 153, 4304–4316. doi: 10.1210/en.2012-1449

Lefkowitz, R. J., and Shenoy, S. K. (2005). Transduction of receptor signals by beta-arrestins. *Science* 308, 512–517. doi: 10.1126/science.1109237

Li, Y. Y., Hou, T. J., and Goddard, W. A. III. (2010). Computational modeling of structure-function of g protein-coupled receptors with applications for drug design. *Curr. Med. Chem.* 17, 1167–1180. doi: 10.2174/092986710790827807

Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., et al. (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 283, 655–661. doi: 10.1126/science.283.5402.655

Mailman, R. B., and Murthy, V. (2010). Third generation antipsychotic drugs: partial agonism or receptor functional selectivity? *Curr. Pharm. Des.* 16, 488–501. doi: 10.2174/138161210790361461

Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., et al. (2012). Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature* 485, 321–326. doi: 10.1038/nature10954

Masri, B., Salahpour, A., Didriksen, M., Ghisi, V., Beaulieu, J. M., Gainetdinov, R. R., et al. (2008). Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13656–13661. doi: 10.1073/pnas.0803522105

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561–564. doi: 10.1038/346561a0

Meng, T., Wang, J., Peng, H., Fang, G., Li, M., Xiong, B., et al. (2010). Discovery of benzhydrylpiperazine derivatives as CB1 receptor inverse agonists via privileged structure-based approach. *Eur. J. Med. Chem.* 45, 1133–1139. doi: 10.1016/j.ejmech.2009.12.018

Mobarec, J. C., Sanchez, R., and Filizola, M. (2009). Modern homology modeling of G-protein coupled receptors: which structural template to use? *J. Med. Chem.* 52, 5207–5216. doi: 10.1021/jm9005252

Mohr, K., Schmitz, J., Schrage, R., TrāNkle, C., and Holzgrabe, U. (2013). Molecular alliance - from orthosteric and allosteric ligands to dualsteric/bitopic agonists at G protein coupled receptors. *Angew. Chem. Int. Ed. Engl.* 52, 508–516.

Monasky, M. M., Taglieri, D. M., Henze, M., Warren, C. M., Utter, M. S., Soergel, D. G., et al. (2013). The beta-arrestin-biased ligand TRV120023 inhibits angiotensin II-induced cardiac hypertrophy while preserving enhanced myofilament response to calcium. *Am. J. Physiol. Heart Circ. Physiol.* 305, H856–H866. doi: 10.1152/ajpheart.00327.2013

Nebigil, C. G. (2009). Prokineticin receptors in cardiovascular function: foe or friend? *Trends Cardiovasc. Med.* 19, 55–60. doi: 10.1016/j.tcm.2009.04.007

Negri, A., Rives, M. L., Caspers, M. J., Prisinzano, T. E., Javitch, J. A., and Filizola, M. (2013). Discovery of a novel selective kappa-opioid receptor agonist using crystal structure-based virtual screening. *J. Chem. Inf. Model.* 53, 521–526. doi: 10.1021/ci400019t

Nygaard, R., Zou, Y., Dror, R. O., Mildorf, T. J., Arlow, D. H., Manglik, A., et al. (2013). The dynamic process of beta(2)-adrenergic receptor activation. *Cell* 152, 532–542. doi: 10.1016/j.cell.2013.01

Oliveira, L., Costa-Neto, C. M., Nakaie, C. R., Schreier, S., Shimuta, S. I., and Paiva, A. C. (2007). The angiotensin II AT1 receptor structure-activity correlations in the light of rhodopsin structure. *Physiol. Rev.* 87, 565–592. doi: 10.1152/physrev.00040.2005

Pal, M., and Paliwal, S. (2012). In silico identification of novel lead compounds with AT1 receptor antagonist activity: successful application of chemical database screening protocol. *Org. Med. Chem. Lett.* 2, 7. doi: 10.1186/2191-2858-2-7

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., et al. (2000). Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289, 739–745. doi: 10.1126/science.289.5480.739

Pandey, P., Roy, K. K., Liu, H., Elokely, K. M., Pettaway, S., Cutler, S. J., et al. (2014). Search for cannabinoid receptor 1 antagonists using structure-based virtual screening: identification of natural product hits. *Planta Med.* 80, PF11. doi: 10.1055/s-0034-1382589

Park, J. H., Scheerer, P., Hofmann, K. P., Choe, H. W., and Ernst, O. P. (2008). Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* 454, 183–187. doi: 10.1038/nature07063

Park, S. H., Das, B. B., Casagrande, F., Tian, Y., Nothnagel, H. J., Chu, M., et al. (2012). Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature* 491, 779–783. doi: 10.1038/nature11580

Pasquini, S., Mugnaini, C., Ligresti, A., Tafi, A., Brogi, S., Falciani, C., et al. (2012). Design, synthesis, and pharmacological characterization of indol-3-ylacetamides, indol-3-yloxoacetamides, and indol-3-ylcarboxamides: potent and selective CB2 cannabinoid receptor inverse agonists. *J. Med. Chem.* 55, 5391–5402. doi: 10.1021/jm3003334

Percherancier, Y., Berchiche, Y. A., Slight, I., Volkmer-Engert, R., Tamamura, H., Fujii, N., et al. (2005). Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *J. Biol. Chem.* 280, 9895–9903. doi: 10.1074/jbc.M411151200

Perez, M., Pauwels, P. J., Fourrier, C., Chopin, P., Valentin, J. P., John, G. W., et al. (1998). Dimerization of sumatriptan as an efficient way to design a potent, centrally and orally active 5-HT1B agonist. *Bioorg. Med. Chem. Lett.* 8, 675–680. doi: 10.1016/S0960-894X(98)00090-0

Pivonello, R., Ferone, D., Lombardi, G., Colao, A., Lamberts, S. W., and Hofland, L. J. (2007). Novel insights in dopamine receptor physiology. *Eur. J. Endocrinol.* 156(Suppl. 1), S13–S21. doi: 10.1530/eje.1.02353

Pollock, D. M., Keith, T. L., and Highsmith, R. F. (1995). Endothelin receptors and calcium signaling. *FASEB J.* 9, 1196–1204.

Poole-Wilson, P. A., Swedberg, K., Cleland, J. G., Di Lenarda, A., Hanrath, P., Komajda, M., et al. (2003). Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the carvedilol or metoprolol european trial (COMET): randomised controlled trial. *Lancet* 362, 7–13. doi: 10.1016/S0140-6736(03)13800-7

Rajagopal, S., Rajagopal, K., and Lefkowitz, R. J. (2010). Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat. Rev. Drug Discov.* 9, 373–386. doi: 10.1038/nrd3024

Rashid, A. J., O'dowd, B. F., Verma, V., and George, S. R. (2007). Neuronal Gq/11-coupled dopamine receptors: an uncharted role for dopamine. *Trends Pharmacol. Sci.* 28, 551–555. doi: 10.1016/j.tips.2007.10.001

Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., et al. (2007). Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450, 383–387. doi: 10.1038/nature06325

Renault, N., Laurent, X., Farce, A., El Bakali, J., Mansouri, R., Gervois, P., et al. (2013). Virtual screening of CB(2) receptor agonists from bayesian network and high-throughput docking: structural insights into agonist-modulated GPCR features. *Chem. Biol. Drug Des.* 81, 442–454. doi: 10.1111/cbdd.12095

Rominger, D. H., Cowan, C. L., Gowen-Macdonald, W., and Violin, J. D. (2014). Biased ligands: pathway validation for novel GPCR therapeutics. *Curr. Opin. Pharmacol.* 16, 108–115. doi: 10.1016/j.coph.2014.04.002

Rosenbaum, D. M., Cherezov, V., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., et al. (2007). GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* 318, 1266–1273. doi: 10.1126/science.1150609

Sabio, M., Jones, K., and Topiol, S. (2008). Use of the X-ray structure of the beta2-adrenergic receptor for drug discovery. *Part* 2: identification of active compounds. *Bioorg. Med. Chem. Lett.* 18, 5391–5395. doi: 10.1016/j.bmcl.2008.09.046

Saito, Y., and Berk, B. C. (2001). Transactivation: a novel signaling pathway from angiotensin II to tyrosine kinase receptors. *J. Mol. Cell Cardiol.* 33, 3–7. doi: 10.1006/jmcc.2000

Sbai, O., Monnier, C., Dode, C., Pin, J. P., Hardelin, J. P., and Rondard, P. (2014). Biased signaling through G-protein-coupled PROKR2 receptors harboring missense mutations. *FASEB J.* 28, 3734–3744. doi: 10.1096/fj.13-243402

Scherrer, G., Imamachi, N., Cao, Y. Q., Contet, C., Mennicken, F., O'donnell, D., et al. (2009). Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* 137, 1148–1159. doi: 10.1016/j.cell.2009.04.019

Scheerer, P., Park, J. H., Hildebrand, P. W., Kim, Y. J., Krauss, N., Choe, H. W., et al. (2008). Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 455, 497–502. doi: 10.1038/nature07330

Scholten, D. J., Canals, M., Maussang, D., Roumen, L., Smit, M. J., Wijtmans, M., et al. (2012). Pharmacological modulation of chemokine receptor function. *Br. J. Pharmacol.* 165, 1617–1643. doi: 10.1111/j.1476-5381.2011.01551.x

Serrano-Vega, M. J., Magnani, F., Shibata, Y., and Tate, C. G. (2008). Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. *Proc. Natl. Acad. Sci. U.S.A.* 105, 877–882. doi: 10.1073/pnas.0711253105

Shapiro, D. A., Renock, S., Arrington, E., Chiodo, L. A., Liu, L. X., Sibley, D. R., et al. (2003). Aripiprazole, a novel atypical antipsychotic drug with a unique and robust pharmacology. *Neuropsychopharmacology* 28, 1400–1411. doi: 10.1038/sj.npp.1300203

Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., et al. (2011). Structure of the human histamine H1 receptor complex with doxepin. *Nature* 475, 65–70. doi: 10.1038/nature10236

Sigalov, A. B. (2012). "Monovalen" ligands that trigger TLR-4 and TCR are not necessarily truly monovalent. *Mol. Immunol.* 51, 356–362. doi: 10.1016/j.molimm.2012.03.031

Sinisi, A. A., Asci, R., Bellastella, G., Maione, L., Esposito, D., Elefante, A., et al. (2008). Homozygous mutation in the prokineticin-receptor2 gene (Val274Asp) presenting as reversible Kallmann syndrome and persistent oligozoospermia: case report. *Hum. Reprod.* 23, 2380–2384. doi: 10.1093/humrep/den247

Siu, F. Y., He, M., De Graaf, C., Han, G. W., Yang, D., Zhang, Z., et al. (2013). Structure of the human glucagon class B G-protein-coupled receptor. *Nature* 499, 444–449. doi: 10.1038/nature12393

Soergel, D. G., Subach, R. A., Sadler, B., Connell, J., Marion, A. S., Cowan, C. L., et al. (2014). First clinical experience with TRV130: pharmacokinetics and pharmacodynamics in healthy volunteers. *J. Clin. Pharmacol.* 54, 351–357. doi: 10.1002/jcph.207

Sokkar, P., Mohandass, S., and Ramachandran, M. (2011). Multiple templates-based homology modeling enhances structure quality of AT1 receptor: validation by molecular dynamics and antagonist docking. *J. Mol. Model.* 17, 1565–1577. doi: 10.1007/s00894-010-0860-z

Szatkowski, C., Vallet, J., Dormishian, M., Messaddeq, N., Valet, P., Boulberdaa, M., et al. (2013). Prokineticin receptor 1 as a novel suppressor of preadipocyte proliferation and differentiation to control obesity. *PLoS ONE* 8:e81175. doi: 10.1371/journal.pone.0081175

Tan, Q., Zhu, Y., Li, J., Chen, Z., Han, G. W., Kufareva, I., et al. (2013). Structure of the CCR5 chemokine IV entry inhibitor maraviroc complex. *Science* 341, 1387–1390. doi: 10.1126/science.1241475

Thompson, A. A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., et al. (2012). Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* 485, 395–399. doi: 10.1038/nature11085

Topiol, S., and Sabio, M. (2008). Use of the X-ray structure of the Beta2-adrenergic receptor for drug discovery. *Bioorg. Med. Chem. Lett.* 18, 1598–1602. doi: 10.1016/j.bmcl.2008.01.063

Torres, P. U. (2006). Cinacalcet HCl: a novel treatment for secondary hyperparathyroidism caused by chronic kidney disease. *J. Ren. Nutr.* 16, 253–258. doi: 10.1053/j.jrn.2006.04.010

Urban, J. D., Vargas, G. A., Von Zastrow, M., and Mailman, R. B. (2007). Aripiprazole has functionally selective actions at dopamine D2 receptor-mediated signaling pathways. *Neuropsychopharmacology* 32, 67–77. doi: 10.1038/sj.npp.1301071

Valant, C., May, L. T., Aurelio, L., Chuo, C. H., White, P. J., Baltos, J. A., et al. (2014). Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4614–4619. doi: 10.1073/pnas.1320962111

Vallone, D., Picetti, R., and Borrelli, E. (2000). Structure and function of dopamine receptors. *Neurosci. Biobehav. Rev.* 24, 125–132. doi: 10.1016/S0149-7634(99)00063-9

Vass, M., Schmidt, E., Horti, F., and Keseru, G. M. (2014). Virtual fragment screening on GPCRs: a case study on dopamine D3 and histamine H4 receptors. *Eur. J. Med. Chem.* 77, 38–46. doi: 10.1016/j.ejmech.2014.02.034

Vilar, S., Karpiak, J., and Costanzi, S. (2010). Ligand and structure-based models for the prediction of ligand-receptor affinities and virtual screenings: development and application to the beta(2)-adrenergic receptor. *J. Comput. Chem.* 31, 707–720. doi: 10.1002/jcc.21346

Violin, J. D., Crombie, A. L., Soergel, D. G., and Lark, M. W. (2014). Biased ligands at G-protein-coupled receptors: promise and progress. *Trends Pharmacol. Sci.* 35, 308–316. doi: 10.1016/j.tips.2014.04.007

Violin, J. D., and Lefkowitz, R. J. (2007). Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol. Sci.* 28, 416–422. doi: 10.1016/j.tips.2007.06.006

Wacker, D., Wang, C., Katritch, V., Han, G. W., Huang, X. P., Vardy, E., et al. (2013). Structural features for functional selectivity at serotonin receptors. *Science* 340, 615–619. doi: 10.1126/science.1232808

Waldhoer, M., Fong, J., Jones, R. M., Lunzer, M. M., Sharma, S. K., Kostenis, E., et al. (2005). A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9050–9055. doi: 10.1073/pnas.0501112102

Wang, C., Jiang, Y., Ma, J., Wu, H., Wacker, D., Katritch, V., et al. (2013a). Structural basis for molecular recognition at serotonin receptors. *Science* 340, 610–614. doi: 10.1126/science.1232807

Wang, C., Wu, H., Katritch, V., Han, G. W., Huang, X. P., Liu, W., et al. (2013b). Structure of the human smoothened receptor bound to an antitumour agent. *Nature* 497, 338–343. doi: 10.1038/nature12167

Wang, H., Duffy, R. A., Boykow, G. C., Chackalamannil, S., and Madison, V. S. (2008). Identification of novel cannabinoid CB1 receptor antagonists by using virtual screening with a pharmacophore model. *J. Med. Chem.* 51, 2439–2446. doi: 10.1021/jm701519h

Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., et al. (2008). Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* 454, 486–491. doi: 10.1038/nature07101

Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., et al. (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10782–10787. doi: 10.1073/pnas.1834556100

Weiss, D. R., Ahn, S., Sassano, M. F., Kleist, A., Zhu, X., Strachan, R., et al. (2013). Conformation guides molecular efficacy in docking screens of activated beta-2 adrenergic G protein coupled receptor. *ACS Chem. Biol.* 8, 1018–1026. doi: 10.1021/cb400103f

Whalen, E. J., Rajagopal, S., and Lefkowitz, R. J. (2011). Therapeutic potential of beta-arrestin- and G protein-biased agonists. *Trends Mol. Med.* 17, 126–139. doi: 10.1016/j.molmed.2010.11.004

White, J. F., Noinaj, N., Shibata, Y., Love, J., Kloss, B., Xu, F., et al. (2012). Structure of the agonist-bound neurotensin receptor. *Nature* 490, 508–513. doi: 10.1038/nature11558

Williams, R., Zhou, Y., Niswender, C. M., Luo, Q., Conn, P. J., Lindsley, C. W., et al. (2010). Re-exploration of the PHCCC Scaffold: discovery of improved positive allosteric modulators of mGluR4. *ACS Chem. Neurosci.* 1, 411–419. doi: 10.1021/cn9000318

Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., et al. (2010). Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330, 1066–1071. doi: 10.1126/science.1194396

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., et al. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* 485, 327–332. doi: 10.1038/nature10939

Xing, R. J., Wang, J., Pan, L., and Cheng, M. S. (2009). A selective pharmacophore model for beta(2)-adrenoceptor agonists. *Molecules* 14, 4486–4496. doi: 10.3390/molecules14114486

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

Zhang, C., Srinivasan, Y., Arlow, D. H., Fung, J. J., Palmer, D., Zheng, Y., et al. (2012). High-resolution crystal structure of human protease-activated receptor 1. *Nature* 492, 387–392. doi: 10.1038/nature11701

Zhang, J., Zhang, K., Gao, Z. G., Paoletta, S., Zhang, D., Han, G. W., et al. (2014). Agonist-bound structure of the human P2Y12 receptor. *Nature* 509, 119–122. doi: 10.1038/nature13288

Zhang, Y., Gilliam, A., Maitra, R., Damaj, M. I., Tajuba, J. M., Seltzman, H. H., et al. (2010). Synthesis and biological evaluation of bivalent ligands for the cannabinoid 1 receptor. *J. Med. Chem.* 53, 7048–7060. doi: 10.1021/jm1006676

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Biological redundancy of endogenous GPCR ligands in the gut and the potential for endogenous functional selectivity

Georgina L. Thompson[1], Meritxell Canals[1] and Daniel P. Poole[1,2] *

[1] Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Parkville, VIC, Australia
[2] Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, VIC, Australia

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Jakub Fichna, Medical University of Lodz, Poland
Pamela J. Hornby, Johnson & Johnson, USA

***Correspondence:**
Daniel P. Poole, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, 381 Royal Parade, Parkville, VIC 3052, Australia
e-mail: daniel.poole@monash.edu

This review focuses on the existence and function of multiple endogenous agonists of the somatostatin and opioid receptors with an emphasis on their expression in the gastrointestinal tract. These agonists generally arise from the proteolytic cleavage of prepropeptides during peptide maturation or from degradation of peptides by extracellular or intracellular endopeptidases. In other examples, endogenous peptide agonists for the same G protein-coupled receptors can be products of distinct genes but contain high sequence homology. This apparent biological redundancy has recently been challenged by the realization that different ligands may engender distinct receptor conformations linked to different intracellular signaling profiles and, as such the existence of distinct ligands may underlie mechanisms to finely tune physiological responses. We propose that further characterization of signaling pathways activated by these endogenous ligands will provide invaluable insight into the mechanisms governing biased agonism. Moreover, these ligands may prove useful in the design of novel therapeutic tools to target distinct signaling pathways, thereby favoring desirable effects and limiting detrimental on-target effects. Finally we will discuss the limitations of this area of research and we will highlight the difficulties that need to be addressed when examining endogenous bias in tissues and in animals.

Keywords: biased agonism, enteric nervous system, G protein-coupled receptor, somatostatin, somatostatin receptor, opioid, opioid receptor

ENDOGENOUS BIASED AGONISM

The last decade has witnessed the experimental confirmation of previous theoretical concepts demonstrating that GPCRs exist in many temporally related micro-conformations (Deupi and Kobilka, 2010). Mechanistically, this inherent plasticity is in line with recent biophysical studies indicating that GPCRs can adopt multiple active states that can be differentially stabilized by chemically distinct classes of ligands (Hofmann et al., 2009; Bokoch et al., 2010). Such plasticity allows GPCRs to mediate a spectrum of acute signaling and longer-term regulatory behaviors that can be activated in a ligand-specific manner. Indeed, it is now established that different agonists do not uniformly activate all cellular signaling pathways linked to a given receptor. Rather, different ligands binding to the same receptor stabilize distinct receptor conformations linked to different signaling pathways and physiological outcomes. This paradigm whereby different ligands, binding to the same GPCR in an identical cellular background, promote distinct receptor conformational states linked to a different functional outcome has been termed *biased agonism* or *functional selectivity*. Therapeutically, biased agonism provides new avenues for the development of drugs that are not only receptor-specific but also 'pathway-specific.' As such it has opened the field to the discovery of ligands that selectively activate signaling pathways mediating desired physiological effects whilst minimizing 'on-target' side-effects that are elicited by activation of other signaling pathways *via* the same receptor.

To date, most descriptions of biased agonism have focused on the differential effects of synthetic drugs. However, there are several functionally important GPCR families that bind to multiple endogenous agonists [for example chemokine, somatostatin (SST), and opioid receptors (ORs)]. Although this has been traditionally attributed to the redundancy of some biological systems, biased agonism could represent an added layer of control to engender finely tuned physiological responses. Indeed, recent reports have already highlighted the potential for functional selectivity across the chemokine receptor family (Rajagopal et al., 2013; Zweemer et al., 2014).

In this focused review we provide an overview of the existing literature regarding two of these GPCR families with multiple endogenous peptide ligands, opioids and SST, in the context of the gastrointestinal tract (GIT). The opioid system is a prototypical example of potential biological redundancy, and it also represents one of the first examples where functional selectivity of synthetic drugs has been reported in the context of gut physiology. On the other hand, SST receptors are therapeutic targets in treating GI disease (e.g., diarrhea, bleeding varices, neuroendocrine tumors) and SSTs and related peptides also represent a well-characterized system where multiple endogenous ligands of the same receptor exist within the GIT (Zhao et al., 2013). Importantly, these two receptor systems also reveal different mechanisms that can explain distinct physiological outcomes derived from activation of the same receptor by different ligands.

THE SOMATOSTATIN SYSTEM OF THE GUT

There are five members of the SST receptor family ($SSTR_{1-5}$) and their distribution in the GIT has been recently reviewed (Van Op den Bosch et al., 2009).

Somatostatin, originally known as somatotropin release-inhibiting factor (SRIF), was first identified and characterized as a cyclic tetradecapeptide (Brazeau et al., 1973). It was predicted that SST-14 was a product of a larger peptide precursor and that other forms with potential biological activity were likely to exist. Indeed, it is now known that SST arises from maturation of preprosomatostatin (PPSST), and that all PPSST derivatives originate from the *SST* gene. The removal of a 24 amino acid signal sequence forms prosomatostatin (PSST), which is further C-terminally cleaved to form the biologically active peptides SST-14, SST-25, and SST-28 (Bohlen et al., 1980; Esch et al., 1980; Brazeau et al., 1981). SST-28, the longest peptide, was identified and characterized as an N-terminally extended variant of SST-14 (Pradayrol et al., 1978, 1980; Bohlen et al., 1980) and biological conversion of SST-28 to SST-14 was later confirmed (Zingg and Patel, 1983). Other cleavage products arising from PSST processing include PSST(1–32; Schmidt et al., 1985) and PSST(1–64; Bersani et al., 1989), for which little information regarding function and expression is available.

N-terminal cleavage of PSST also occurs, but the resulting peptides do not contain the SST-14 sequence and are therefore not considered to be SSTs (Benoit et al., 1990). These include SST-28(1–12) and antrin, which contains amino acids 1–10 of PSST [PPSST(25–34)]. Antrin, first identified in the gastric antrum (Benoit et al., 1987), is present in all SST-producing tissues. However, a functional role for this peptide has yet to be ascribed. Most recently, a bioinformatics approach predicted the existence of a novel 13mer PPSST cleavage product [PPSST(31–43)], which was subsequently confirmed by immunoaffinity purification and called neuronostatin (Samson et al., 2008). Neuronostatin is encoded by PSST and is highly conserved across vertebrates. Unlike SST and cortistatin (CST, see below), neuronostatin is not cyclic and is amidated at the C-terminus.

Biological activity of SST variants is conferred through a common Phe-Trp-Lys-Thr (FWKT) motif within the C-terminus (amino acids 7–10; Patel and Srikant, 1997). This sequence is also present in non-SST peptides that share a high-degree of sequence homology with SST. These include CST and thrittene. CST and SST are encoded by distinct genes, and genetic deletion of SST has no effect on the expression of CST. CST is a derivative of the 112 amino acid preproCST (PPCST) precursor (de Lecea et al., 1996), which is converted to proCST by signal peptide cleavage, resulting in the formation of hCST17 and hCST29 (Puebla et al., 1999). CST shares 11 amino acids in common with SST-14 including residues required for interaction with SSTRs and two key cysteines that enable formation of the cyclic peptide structure (Francis et al., 1990). Although they share sequence homology, structure, and affinity for SSTRs, there are clear differences in the ability of CST and SST-14 to stimulate SSTR2 endocytosis and signaling (Liu et al., 2005). Notably, CST is significantly less effective at inhibiting cAMP production and promoting SSTR2 endocytosis. Furthermore, CST does not exclusively interact with SST receptors and can also activate the MrgX2 and GHS-R1a receptors. Whether there are CST variants or a CST-specific GPCR is unknown. Another endogenous peptide that shares extensive sequence homology with SST is thrittene [SST28(1–13)]. As with CST, thrittene is not derived from PSST and is a product of a distinct gene, as supported by the presence of thrittene-like immunoreactivity in PSST deficient mice (Ensinck et al., 2003). Moreover, thrittene and SST are expressed by distinct cell populations and their release is triggered in response to different stimuli (Ensinck et al., 2002). With the exception of these initial studies nothing is known of the functional role of thrittene, nor if thrittene plays an analogous or discrete role to that of SST. A summary of SST and SST-like peptides is presented in **Table 1**.

DISTRIBUTION OF ENDOGENOUS SSTR LIGANDS IN THE GI TRACT

The GIT is the major source of SST and SST is a regulator of many digestive functions. SSTRs are an important therapeutic target in the treatment of digestive disease. In addition to its established role as a neurotransmitter, SST also acts in a hormonal and paracrine manner to regulate gut function (Low, 2004; Van Op den Bosch et al., 2009).

Somatostatin is expressed by D-cells of the stomach and plays a well-defined role in the control of acid secretion. SST negatively regulates gastrin release from antral G cells and histamine release from enterochromaffin-like cells, and acts directly on parietal cells leading to an SSTR2-dependent inhibition of acid release (Walsh, 1988; Lloyd et al., 1997; Low, 2004). SST-14 within the intestinal wall is mainly expressed by enteric neurons and potentially by extrinsic primary spinal afferents (Traub et al., 1999), although this is still debated (Keast and De Groat, 1992). SST-14 is also produced by macrophages during infection or inflammation as part of an immunoregulatory circuit with SSTR2 (Weinstock and Elliott, 2000). SST-28- distribution appears to be more restricted and is primarily expressed by enteroendocrine D-cells (Ravazzola et al., 1983; Baskin and Ensinck, 1984), consistent with the predominant release of SST-28 from the mucosa (Baldissera et al., 1985).

Myenteric SST-immunoreactivity is localized to a subclass of descending inhibitory interneuron, where it is co-expressed with choline acetyltransferase (Portbury et al., 1995; Song et al., 1997). Physiologically, SST is involved in the migrating myoelectric complex in the jejunum (Abdu et al., 2002) and propagating contractions of the colon (Grider, 2003). These actions are mediated through the SSTR2 receptor, which is expressed by NOS-positive inhibitory motor neurons or descending interneurons (Allen et al., 2002). SST is also an inhibitor of gastric emptying and of gall bladder contractility. SST is expressed by submucosal cholinergic secretomotor/ non-vasodilator neurons (Mongardi Fantaguzzi et al., 2009) and hyperpolarizes submucosal neurons (Shen and Surprenant, 1993) probably *via* SSTR1 and SSTR2 (Foong et al., 2010). In the human intestine SST is expressed by putative intrinsic primary afferent neurons of the submucosal plexus (Beyer et al., 2013).

There is limited information regarding the distribution of 'non-SST' peptides in the gut. Relatively high mRNA expression for CST has been detected through the human GIT (Dalm et al., 2004). However, it should be noted that with the exception of pancreatic delta islet cells (Papotti et al., 2003) and potentially

Table 1 | Endogenous somatostatin (SST) peptide sequences (*sequence not confirmed).

Precursor	Peptide	Sequence
Prosomatostatin (PSST)	SST-28	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
	SST-14	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH
	Neuronostatin (PPSST(31-43))	Leu-Arg-Gln-Phe-Leu-Gln-Lys-Ser-Leu-Ala-Ala-Ala-Ala-NH2
	Antrin (SST-25-34)	Ala-Pro-Ser-Asp-Pro-Arg-Leu-Arg-Gln-Phe-OH
	SST-25	Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
	SST-28(1–14)	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys
	SST-28(1–12)	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu
	PPST 1–64	Ala-Pro-Ser-Asp-Pro-Arg-Leu-Arg-Gln-Phe-Leu-Gln-Lys-Ser-Leu-Ala-Ala-Ala-Ala-Gly-Lys-Gln-Glu-Leu-Ala-Lys-Tyr-Phe-Leu-Ala-Glu-Leu-Leu-Ser-Glu-Pro-Asn-Gln-Thr-Glu-Asn-Asp-Ala-Leu-Glu-Pro-Glu-Asp-Leu-Ser-Gln-Ala-Ala-Glu-Gln-Asp-Glu-Met-Arg-Leu-Glu-Leu-Gln-Arg
	PSST 1–32	Ala-Pro-Ser-Asp-Pro-Arg-Leu-Arg-Gln-Phe-Leu-Gln-Lys-Ser-Leu-Ala-Ala-Ala-Ala-Gly-Lys-Gln-Glu-Leu-Ala-Lys-Tyr-Phe-Leu-Ala-Glu-Leu
Preprocortistatin	Cortistatin-14 (rat)	Pro-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys
	Cortistatin-17 (human)	Asp-Arg-Met-Pro-Cys-Arg-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys
	Cortistatin-29	H-Glu-Gly-Ala-Pro-Pro-Gln-Gln-Ser-Ala-Arg-Arg-Asp-Arg-Met-Pro-Cys-Arg-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys-OH
Unknown	Thrittene (SST28(1–13))	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg*

activated inflammatory cells (Gonzalez-Rey et al., 2006), the distribution of CST within the gut remains unknown. Thrittene-like immunoreactivity has been detected in enteroendocrine cells and enteric neurons and this distribution is distinct to that for SST-14 and SST-28 (Ensinck et al., 2002). This is supported by the differential release of thrittene and SST in response to feeding (Ensinck et al., 2003). Antrin expression was originally believed to be restricted to gastric D-cells, where it is localized to SST-28(1–12) containing secretory granules (Ravazzola et al., 1989; Benoit et al., 1990). However, this was contradicted by Rabbani and Patel (1990), who demonstrated comparable expression of antrin in the jejunal mucosa and pancreas by radioimmunoassay and HPLC.

EVIDENCE FOR DIFFERENCES IN FUNCTION

At present there is little evidence for significant differences in the effects of endogenous SSTs on GI function, although this may reflect the limited endpoints that have been assayed. Exposure of enteric neurons to SST results in activation of inwardly rectifying K^+ currents and to hyperpolarization, leading to inhibition of contractile and secretory activity (Van Op den Bosch et al., 2009). Direct electrophysiological recordings demonstrate no apparent difference in the acute effects of SST-14 and SST-28 on submucosal neurons, with exposure to either agonist leading to hyperpolarization and to rapid desensitization of responses (Shen and Surprenant, 1993). Similarly, there was no significant difference in the SST-14, SST-25, and SST-28 mediated inhibition of contractile activity. These agonists cross-desensitized responses to each other, but not to acetylcholine, suggesting actions at the same receptor (McIntosh et al., 1986). However, there is evidence for differences in the *in vivo* effects of SST-14 and SST-28 on both the stomach and intestine. For example, studies examining the direct effects of SSTR activation on gut function showed that SST-14 is significantly more potent at inhibiting gastric acid secretion than SST-28, despite the longer plasma half-life of SST-28 (Hirst et al., 1982; Seal et al., 1982). Zhao et al. (2013) recently demonstrated that although SST-14 and SST-28 both stimulated endocytosis of SSTR2A in myenteric neurons, there were clear differences in receptor recycling. The apparent retention of SSTR2A following treatment of neurons with SST-28 was attributed to the greater resistance of this peptide to degradation by the endosomal endopeptidase endothelin-converting enzyme 1 (ECE-1). This study did not determine the consequences of this retention or prolonged endosomal SSTR2A signaling on gut function. Moreover, the possible biological activity of SST cleavage products resulting from ECE-1 activity was not examined. Intermediate products of both SST-14 (SST-1–10) and SST-28 (SST-1–24) retained the Phe-Trp-Lys-Thr motif at the extreme N-terminus and may represent novel SSTR agonists produced locally within endosomes. However, absence of a key N-terminal Cys residue suggests that these peptides lack the cyclopeptide structure characteristic of SSTs.

The existence of endogenous ligand bias has been examined at the SSTR2A. Comparison of the responses of SST-14, SST-28 and cortistatin has not showed any evidence of functional selectivity at this receptor. However, potential ligand bias has been suggested for the small molecule ligands that bind SSTR2A, albeit the quantification of this bias is lacking (Nunn et al., 2004; Liu et al., 2008;

Cescato et al., 2010). More recently, we have shown that SST-14 and SST-28 show distinct profiles of receptor trafficking upon internalization (Zhao et al., 2013). After incubation with SST-14, SSTR2A recycled to the plasma membrane, which required the activity of the endosomal peptidase ECE-1, and an intact Golgi. In contrast, SSTR2A activated by SST-28, octreotide, lanreotide, or vapreotide was retained within the Golgi and did not recycle. Although ECE-1 rapidly degraded SST-14, SST-28 was resistant to degradation, and ECE-1 did not degrade the synthetic SST analogs. Thus, although no apparent bias was observed at the level of receptor signaling events, SST-14 and SST-28 differ in the trafficking of the receptor upon internalization. The differential regulation of SSTR2A may explain the different physiological effects of endogenous agonists and could account for the long-lasting therapeutic actions and side effects of clinically used agonists.

THE OPIOID SYSTEM IN THE GUT

Opioids and opiates are agonists of the mu, delta and kappa ORs (MOPr, DOPr, and KOPr). The nociceptin receptor (NOPr) was the last ORs to be cloned and is grouped with the ORs based on their high degree of sequence homology and its low level binding of opioids. The pharmacology and function of ORs has been reviewed extensively and will not be covered in detail in this review (Waldhoer et al., 2004). All receptors are expressed by enteric neurons and other cell types in the GIT and are major regulators of gut function (Wood and Galligan, 2004; Galligan and Akbarali, 2014)

The endogenous ligands for ORs are a large family of at least 20 different small peptides. The endogenous peptides have been detected throughout the central and peripheral nervous system as well as in other tissues, with similar distribution to the ORs. They are involved in numerous physiological processes including nociception, reward processing, and GIT motility and secretion. The distribution and physiological effects of endogenous opioids in the GIT have been the most extensively studied. However, identifying regions where endogenous opioids are expressed and released under normal physiological conditions has been challenging due to the high susceptibility of the peptides to degradation. Additionally, most studies have used antibody-based methods that may not reliably distinguish between different opioid peptides due to their high structural similarity, or HPLC-based methods which provide no detail of the specific cell types that express these peptides. Further complications arise due to interspecific differences and region-dependent variations in expression along the GIT. Nonetheless, most of the endogenous opioids are present in the GIT, and in some cases the distribution and release from discrete regions of the GIT has been thoroughly characterized.

There are three major classes of endogenous opioids (enkephalins, dynorphins, and endorphins), which are synthesized by proteolytic cleavage of precursor proteins; pro-enkephalin, pro-dynorphin, and pro-opiomelanocortin (POMC), respectively. The peptides range from 5 to 30 amino acids in length, and share a common N-terminal tetrapeptide sequence Tyr-Gly-Gly-Phe, with either a Leu or Met in the fifth position. These peptides have varying affinities for all three ORs, but none are highly selective for one receptor subtype (Mansour et al., 1995; Janecka et al., 2004). There are also two additional putative endogenous peptides; endomorphin-1, and endomorphin-2, which are structurally unrelated to the typical opioid peptides and are most selective and potent for MOPr (Zadina et al., 1997). The gene or genes encoding the precursor proteins of endomorphins are unknown (Terskiy et al., 2007), although a *de novo* synthesis mechanism has been proposed as an alternative source (Ronai et al., 2009). The presence of endomorphins in the GIT has not been reported and will not be discussed further in this review.

DISTRIBUTION OF ENDOGENOUS OPIOID RECEPTOR LIGANDS IN THE GI TRACT

Screening of the longitudinal muscle-myenteric plexus of the guinea pig ileum by HPLC identified expression of enkephalins (enk: Leu-enk, Met-enk, Met-enk-Arg-Gly-Leu, Met-enk-Arg-Phe, Metorphamide, and BAM-18) and dynorphins [α-neoendorphin, β-neoendorphin, dynorphin A(1–8), and dynorphin B]. No detectable beta endorphin was present in these preparations (Corbett et al., 1988).

Enkephalins

The enkephalins have been the most widely studied opioid peptides in the GIT. Pro-enkephalin contains four copies of Met-enk and one each of Leu-enk, Met-enk-Arg-Phe, and Met-enk-Arg-Gly-Leu, and several additional opioid peptides may be formed by partial processing of the precursor protein (see **Table 2**; Noda et al., 1982). Expression of at least four enkephalin peptides (Leu-enk, Met-enk, Met-enk-Arg-Phe, and Met-enk-Arg-Gly-Leu) in the GIT has been confirmed (Hughes et al., 1977; Linnoila et al., 1978; Tang et al., 1982; Giraud et al., 1984). Immunohistochemical studies demonstrate expression throughout the human GIT, with highest levels detected in the *muscularis externa* (Polak et al., 1977; Ferri et al., 1986, 1988). A similar expression pattern has been observed in rodents (Keast et al., 1985). Enkephalin-derived peptides are mainly found in the cell bodies of myenteric neurons and in nerve fibers within the myenteric plexus and circular muscle (Elde et al., 1976; Jessen et al., 1980; Schultzberg et al., 1980; Furness et al., 1983). There is evidence that immunoreactivities for Leu-enk and Met-enk are expressed by distinct neuronal populations within the enteric nervous system (Linnoila et al., 1978; Larsson et al., 1979; Larsson and Stengaard-Pendersen, 1982). The morphology and distribution of Enk-containing myenteric neurons has been examined in detail. Approximately 23% of myenteric neurons express Enk-immunoreactivity (Furness et al., 1983). These are morphologically Dogiel Type I inhibitory or excitatory motor neurons and are also immunoreactive for ChAT and/or substance P (Furness et al., 1983; Bornstein et al., 1984; Costa et al., 1985; Pfannkuche et al., 1998). Leu-enk-positive myenteric neurons of the human intestine have been described morphologically as 'stubby neurons' and are proposed to represent motor neurons or ascending interneurons (Brehmer et al., 2005). Examples of OR and enkephalin labeling in the intestine are presented in **Figure 1**.

There are a small number of neurons that express enkephalin-immunoreactivity in the submucosal plexus and fibers in the mucosa (Furness et al., 1985; Keast et al., 1985; Pfannkuche

Table 2 | Endogenous opioid peptide sequences.

Precursor	Peptide	Sequence
Pro-Enkephalin	Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
	Met-enkephalin	Tyr-Gly-Gly-Phe-Met
	Met-enkephalin-Arg-Phe	Tyr-Gly-Gly-Phe-Met-Arg-Phe
	Met-enkephalin-Arg-Gly-Leu	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
	Metorphamide	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val
	BAM 12	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu
	BAM 18	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln
	BAM 22	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly
	Peptide E	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu
	Peptide F	Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met
Pro-Dynorphin	Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
	Dynorphin B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	Big Dynorphin (Dyn A/B 1-32)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	Dynorphin A 1–13	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
	Dynorphin A (1–8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile
	Dynorphin A (1–6)	Tyr-Gly-Gly-Phe-Leu-Arg
	Leumorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val
	α-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
	β-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Pro-Opiomelanocortin	α-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Val-Thr-Leu
	β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Val-Thr-Leu-Phe-Lys-Asn-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Unknown	Endomorphin 1	Tyr-Pro-Trp-Phe
	Endomorphin 2	Tyr-Pro-Phe-Phe

et al., 1998), and in enteroendocrine cells (Mimoda et al., 1998). However, it is possible that the enkephalin detected in these regions is due to detection of dynorphins or dynorphin derived Leu-enk which is highly expressed in these regions as discussed later in this review. Expression of other enkephalin derivatives including Met-enk-Arg-Phe (Bu'Lock et al., 1983) and Met-enk-Arg-Gly-Leu (Wang and Lindberg, 1986) by enteric neurons has also been demonstrated. Other sites where preproenkephalin and its derivatives are expressed include enteroendocrine cells (Bu'Lock et al., 1983; Nihei and Iwanaga, 1985; Kokrashvili et al., 2009), extrinsic afferents (Steele and Costa, 1990) and immune cells including CD4+ T cells (Boue et al., 2014).

Dynorphins

There is good evidence that opioid peptides derived from pro-dynorphin (dynorphins), are present in the GIT. Pre-pro-dynorphin mRNA is expressed in the myenteric and mucosal layers to varying levels throughout the GIT (Yuferov et al., 1998). Pro-dynorphin contains three opioid peptides, dynorphin A, dynorphin B, and α-neoendorphin, which can all be further processed to shorter opioid peptides including Leu-Enk (see **Table 2**; Horikawa et al., 1983). Dynorphins have been detected in the GIT of various species, including the full length Dyn A (1–17), Dyn A (1–13), Dyn A (1–8), Dyn B, and α-neoendorphin (Vincent et al., 1984; Wolter, 1986; Steele et al., 1989; Murphy and Turner, 1990; Spampinato et al., 1992). Dynorphins are present in all layers of the gut wall throughout the entire human GIT, although information regarding cellular sites of expression is lacking (Spampinato et al., 1988). Immunohistochemistry studies performed mainly in guinea pigs indicate that dynorphins are widely expressed by submucosal and myenteric neurons (Vincent et al., 1984; Wolter, 1986; Steele and Costa, 1990). Dynorphins are co-expressed with enkephalins in a subpopulation of Dogiel type I myenteric neurons (Costa et al., 1985; Furness et al., 1985; Steele and Costa, 1990). It is possible that this may reflect conversion of dynorphin to Leu-enk in these

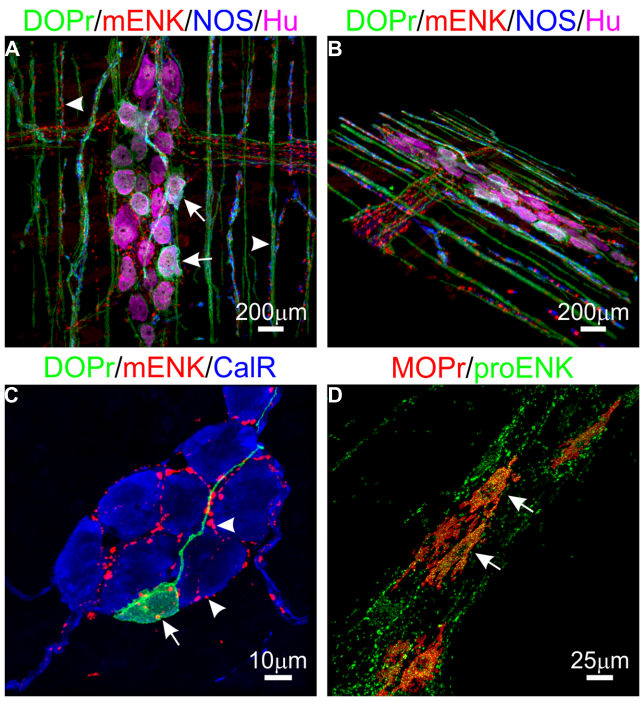

FIGURE 1 | Expression of opioid receptors (ORs) and enkephalin in the enteric nervous system. (A,B) Distribution of the delta opioid receptor (DOPr, green), met-enkephalin (mENK, red), nitric oxide synthase (NOS, blue), and the pan-neuronal marker HuC/D (Hu, magenta) in the myenteric plexus (arrows) and circular muscle nerve fibers (arrowheads) of the mouse distal colon. **(C)** Example of a DOPr-positive submucosal neuron (arrow) and association with mENK-immunoreactive nerve varicosities (arrowheads) in the mouse distal colon. **(D)** Overlap between immunoreactivities for the Mu opioid receptor (MOPr, red) and proenkephalin (proENK, green) in myenteric neurons of the guinea pig ileum. Images have been modified using Imaris 7.4.2 software (Bitplane). Scale bars are as indicated.

neurons rather than co-expression of pro-enkephalin. There are also reports of dynorphin expression by enterochromaffin cells (Cetin, 1988).

Endorphins

The endorphins are formed from the precursor peptide POMC, which also contains several other non-opioid peptide hormones (Eipper and Mains, 1978). POMC contains only one opioid peptide, β-endorphin, which can be cleaved to form α-endorphin. Although β-endorphin has been detected in the GIT (Orwoll and Kendall, 1980; DeBold et al., 1988), the localization of endorphin expression still remains uncertain. There is some evidence of β-endorphin expression, and of other POMC peptides, by myenteric neurons, nerve fibers within the circular muscle and enteroendocrine cells (Schultzberg et al., 1980; Leander et al., 1984; Wolter, 1985b; Kokrashvili et al., 2009; Miller and Hirning, 2010). Another major source of β-endorphin in the gut are immune cells, particularly those associated with inflammatory bowel disease or irritable bowel syndrome (Verma-Gandhu et al., 2006; Hughes et al., 2013). It should be noted that the distribution of β-endorphin in the GIT is controversial, as the specificity of the antisera used in many of these studies has been questioned (Sundler et al., 1981). Hence whilst there is certainly β-endorphin present in the GIT, the question of its origin remains unresolved.

Other OR agonists are also produced endogenously in the GIT. These include morphine and codeine-like compounds (Schulz et al., 1977; Laux-Biehlmann et al., 2013) and the pre-dermorphin derivatives dermorphin and dermenkephalin (Mor et al., 1989, 1990).

Even though the distribution of the different classes of endogenous opioids in the GIT has been fairly well established, there is very little known about individual levels of the different peptides within each class. The expression of proteases that synthesize and degrade endogenous opioids may have varying levels of expression in different cell types, which would result in different production and degradation rates. As such, the mixture of opioid peptides derived from the same precursor will be variable in different cell populations. Differential proteolytic processing of pro-enkephalin and pro-dynorphin peptides occurs in various regions of the brain and other tissues, leading to variations in the relative proportions of peptides derived from the same precursors (Cone et al., 1983; Zamir et al., 1984; Yakovleva et al., 2006). Differential processing of precursors may also occur in the different cell populations within the GIT. In rat duodenum, specific antisera against Dyn A (1–17) and Dyn A (1–8) stain two distinct populations of neurons, one which contains both peptides and one with only Dyn A (1–8), indicating that Dyn A (1–8) may be synthesized *via* distinct proteases or at varying rates in distinct neuronal populations (Wolter, 1985a).

FUNCTION OF ENDOGENOUS OPIOID RECEPTOR LIGANDS IN THE GI TRACT

Endogenous opioids play an important regulatory role in normal gut physiology, primarily through activation of ORs on enteric neurons (Wood and Galligan, 2004). When applied exogenously, the physiological effects of endogenous opioids are the same as the effects of other opioids, they hyperpolarize enteric neurons leading to inhibition of GIT motility and secretion and ultimately cause constipation (Miller and Hirning, 2010). On the other hand, the effects of endogenous peptides when released intrinsically under normal physiological conditions are unclear. Release of enkephalin- and dynorphin-derived peptides has been detected in intestinal tissue preparations during peristalsis or after electrical stimulation. These include Leu-enk, Met-enk, Met-enk-Arg-Phe, Met-enk-Arg-Gly-Leu, metorphamide (Schulz et al., 1977; Corbett et al., 1991), α-neoendorphin (Majeed et al., 1987) and Dyn A (Kromer et al., 1981; Donnerer et al., 1984). In addition, studies using opioid antagonists, mainly naloxone, have shown that inhibition of opioid activity increases non-propagating intestinal motility (Sanger and Tuladhar, 2004). Altogether, this shows that endogenous opioids play a subtle but important role in control of GIT motility by suppressing activity. There is also evidence that the endogenous peptides either contribute to, or protect against, the development of pathophysiological conditions. Levels of endogenous opioids in the GIT have been shown to increase under pathological conditions, including inflammatory bowel disease, and not only inhibit gastrointestinal motility, but also provide visceral antinociception. β-endorphin levels have been shown to increase in a

model of chronic inflammatory bowel disease in mice, suppressing inflammation-associated hyperexcitability of colonic primary spinal afferents (Hughes et al., 2013; Valdez-Morales et al., 2013). In addition, T Lymphocytes can release β-endorphin and induce expression of β-endorphin in the myenteric plexus in mice with immunodeficiency-related visceral hyperalgesia (Verma-Gandhu et al., 2006, 2007). Surgical intervention has also been shown to increase dynorphin expression in the dorsal root ganglia of mice (Romero et al., 2012), and stimulate release of opioid peptides from enteric neurons after abdominal surgery in guinea pigs (Patierno et al., 2005). This may contribute in part to post-operative ileus, although sympathetic pathways are likely to play a more significant role. A greater understanding of the involvement of endogenous opioids in GIT pathophysiology is important as the opioid system is not only a potential target for treatment, but the enhanced production and release of endogenous opioids may also alter the effectiveness of opioid-based therapeutics.

Although the global physiological effects of endogenous opioids in the GIT have been widely studied, the role of individual peptides in the control of normal GIT functions or pathophysiological conditions in discrete regions is still not clear. There are specific distributions of endogenous opioids throughout the GIT. However, since all endogenous opioids can activate all ORs, the specific ORs through which endogenous opioids exert their actions or the specific signaling mechanisms behind these functions is unknown. The physiological significance of such diversity and structural organization of opioid peptides suggests that individual endogenous peptides may serve distinct physiological roles. The diversity in physiological effects can in part be achieved by activation of the different ORs. However, as there are far more endogenous opioids than there are receptors and little receptor selectivity, it is probable that the diversity in endogenous opioids exists to fine tune OR-mediated effects through biased agonism.

BIASED AGONISM AT THE OPIOID RECEPTORS

Opioid receptors are prototypical GPCRs where biased agonism displayed by synthetic and exogenous ligands has been widely explored. Indeed, this reflects the extensive knowledge of opioid physiology and the desire to generate opioid-based analgesics devoid of limiting side effects such as respiratory depression or constipation.

In addition to the ideal separation of therapeutic and clinically limiting side effects, two key observations in the actions of morphine at MOPr have sparked the search for biased agonists at this receptor. First, morphine is relatively poor at inducing MOPr internalization, in spite of its efficacy in mediating G-protein activation, and second, morphine-induced respiratory depression and constipation were attenuated in a β-arrestin knock-out mouse, while analgesia was enhanced. Altogether these reports have sparked the search for potentially different signaling mechanisms that mediate the diverse physiological actions of ORs. Similarly, reports of biased agonism by exogenous ligands have also been described for the other OR subtypes, DOPr (Charfi et al., 2014), and KOPr (Melief et al., 2010). However, the potential for endogenous bias at the OR family has not received much attention. This is despite the fact that, as highlighted above, there is significant biological redundancy in the opioid system. In a systematic approach to evaluate biased agonism at the mu-OR, McPherson et al. (2010; Rivero et al., 2012) examined the signaling bias of a wide range of ligands including endogenous opioid peptides and synthetic opioids. In these and subsequent studies, endomorphin-2 as well as endomorphin-1 showed statistically significant bias toward β-arrestin2 recruitment and away from G protein activation. However, as neither the gene nor the precursor protein of endomorphin1 and two has yet been identified, their classification as endogenous opioids is still a matter of debate.

Opioid receptors have also been reported to form homo- and hetero-dimers. Importantly, it has been suggested that these dimers may indeed form a new signaling entity where the intracellular signaling resulting from the activation of heterodimers may be different from that elicited by the individual protomers or homodimers (Waldhoer et al., 2005; Rozenfeld and Devi, 2007; Gomes et al., 2013). Moreover, some of these dimers have been demonstrated to exist *in vivo* (Massotte, 2014). Although such mechanisms of engendering distinct intracellular signals would not fall into the definition of biased agonism, it is another paradigm to take into account in the context of the differential actions of endogenous opioid peptides.

IDENTIFICATION AND QUANTIFICATION OF BIASED AGONISM: CHALLENGES AND LIMITATIONS

Although biased agonism offers the potential of safer and more effective therapeutics, there are still significant limitations for its detection, quantification and, importantly, its translation into differential physiological responses.

QUANTIFICATION OF BIASED AGONISM

Analytical tools for the detection and quantification of biased agonism are necessary in order to effectively inform future drug development efforts aimed in this direction. The majority of studies to date on biased agonism have used largely qualitative observations, such as reversals in agonist potency orders or maximal agonist effects between different pathways. However, such approaches are not optimal. The potency of a ligand is determined by both its affinity for the receptor state coupled to that particular pathway as well as its intrinsic efficacy for generating a response in that pathway. In contrast, the maximal effect of a ligand at saturating concentrations is only determined by intrinsic efficacy. In addition, contributors to system bias, signal amplification, and receptor expression need to be taken into account as they have markedly different effects on potencies and efficacies of differently efficacious ligands. Therefore, the observed response of an agonist at a given pathway is not only the result of unique ligand-induced receptor conformations, rather it is affected by "system bias," which reflects the differing coupling efficiencies of the receptor to a given signaling pathway, and by "observation bias," which results from differing assay sensitivity and conditions (Kenakin et al., 2012; Kenakin and Christopoulos, 2013a). It is the bias imposed by the ligand on the receptor that is the only source of bias that allows the signaling bias profiles of ligands in different cell types to be compared. It is

therefore important to quantify signaling bias in such a way that it excludes system and observation bias, in order to reveal the unique signaling profile that is induced by the different ligands.

Several analytical approaches have been described to quantify biased agonism (reviewed by Kenakin and Christopoulos, 2013b). The relative transduction ratio (Kenakin et al., 2012) is one of the most robust and widely applicable methods for bias quantification. This method applies the operational model of agonism first derived by Black and Leff (1983) to concentration-response curves and estimates a "transduction coefficient" which is comprised of the functional equilibrium dissociation constant (a measure of the affinity for the receptor coupled to a particular effector protein or signaling pathway) and the intrinsic efficacy of the agonist in activating a particular signaling response and receptor density. This coefficient is thus an overall measure of the relative 'power' of an agonist to induce a response. In order to eliminate the effects of system and observation bias, normalization to a reference agonist is required. Finally, these normalized transduction coefficients can be compared across two signaling pathways for a given agonist to obtain the "relative transduction ratio" as measures of agonist bias. It is, however, important to highlight that key factors need to be considered [reference ligand, cellular content and pluridimensionality of efficacy, (Thompson et al., 2014)] which influence the identification and quantification of biased agonism and that need to be taken into account when interpreting information obtained from studying biased signaling *in vitro*.

EXAMINATION OF ENDOGENOUS BIAS IN A PHYSIOLOGICAL SETTING

Potentially insurmountable difficulties may prevent the examination of endogenous ligand bias in tissues and *in vivo*. First and foremost, multiple agonists for the same receptor exist, and these may be coexpressed (e.g., enkephalins), precluding differential release protocols. Selective stimulation of release may be possible in cases where agonists are expressed by distinct cells or neuronal subtypes (e.g., enteric neurons *vs.* enteroendocrine cells). Peptides may differ with respect to their susceptibility to degradation, complicating interpretation of studies of duration or magnitude of effects. Furthermore, these peptides may vary in their relative affinities to receptors of interest. The endpoints that are measured are often indirect and result from activation of complex reflex pathways involving a number of transmitters. For example, suppression of electrically evoked intestinal contractions, such as occurs in response to OR agonists (Wood and Galligan, 2004) may not reveal subtle agonist-dependent differences. Most of the current descriptions of biased agonism rely on direct measurements from cells (e.g., pERK1/2, cAMP accumulation, β-arrestin-recruitment), which are difficult to assay in enteric neurons. Moreover, the effects of exogenous agonist application may not reflect what occurs physiologically, as location of receptors and ligands may mean that such interactions never occur.

Other factors to consider when translating data derived from heterologous cell lines to enteric neurons, tissues, or *in vivo* studies include not only species, but also regional differences, and the relative expression of key regulatory proteins in the cellular environment examined. These factors are most apparent in the case of the ORs. The distribution of ORs in the gut differs between species. For example, there is limited evidence for functional DOPr expression in the guinea pig ileum (Johnson et al., 1987), whereas there is prominent DOPr expression in the mouse ileum (Poole et al., 2011). There may also be differences in the regional distribution of ORs with respect to both the relative numbers of positive neurons and in the neuronal types that express these receptors, as we have previously demonstrated for the DOPr (Poole et al., 2011). Interestingly, this does not appear to be the case for MOPr expression in the guinea pig ileum and colon where similar neuronal populations express the receptor (Poole et al., unpublished). It is worth noting that these differences in distribution are unlikely to have an effect in the detection of bias, as measurements are likely to be performed in the same tissue preparation. However, species and regional differences in OR expression will affect the potential for heterodimerization of ORs, which may influence the pharmacological profiles of any responses to agonists (Rozenfeld and Devi, 2007). Perhaps of greater importance is the relative expression of key modulatory proteins including β-arrestins and GRKs, which influence OR signaling in enteric neurons. This is highlighted by a number of recent studies using knockout mice. β-arrestin 2 deficient mice exhibit reduced constipatory effects of morphine and loperamide based on assays of fecal output and colonic transit (Raehal et al., 2005). Similarly, GRK6$^{-/-}$ mice also display significantly diminished opiate-induced inhibition of colonic transit relative to wildtype mice (Raehal et al., 2009). Deletion of either β-arrestin 2 or GRK6 did not affect morphine-induced inhibition of small intestinal transit, suggesting region-dependent regulation of neuronal MOR. β-arrestins are also integral to the development of opiate tolerance in the intestine, with deletion of β-arrestin 2 promoting acute morphine tolerance in the colon (Maguma et al., 2012; Akbarali et al., 2014). These studies highlight that OR regulation and physiological function can differ markedly between regions of the GIT and the difficulty in translating data obtained from model cell systems to the physiological setting.

In summary, we have provided an overview of the expression and distribution of endogenous ligands for two major therapeutically relevant classes of GPCRs in the GIT. We have provided evidence for functional selectivity of these ligands and have discussed potential issues related to translation of cell line-derived data to the organ and whole animal levels. Therapeutically, the targeting of selective release of endogenous peptides is probably not a realistic goal. However, understanding the fundamental basis for ligand bias and determining whether differences in the expression and release of endogenous ligands underlie the development and maintenance of disease may be more promising avenues to address and to provide mechanistic insight for the development of safer therapies.

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REFERENCES

Abdu, F., Hicks, G. A., Hennig, G., Allen, J. P., and Grundy, D. (2002). Somatostatin sst(2) receptors inhibit peristalsis in the rat and mouse jejunum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 282, G624–G633. doi: 10.1152/ajpgi.00354.2001

Akbarali, H. I., Inkisar, A., and Dewey, W. L. (2014). Site and mechanism of morphine tolerance in the gastrointestinal tract. *Neurogastroenterol. Motil.* 26, 1361–1367. doi: 10.1111/nmo.12443

Allen, J. P., Canty, A. J., Schulz, S., Humphrey, P. P., Emson, P. C., and Young, H. M. (2002). Identification of cells expressing somatostatin receptor 2 in the gastrointestinal tract of Sstr2 knockout/lacZ knockin mice. *J. Comp. Neurol.* 454, 329–340. doi: 10.1002/cne.10466

Baldissera, F. G., Nielsen, O. V., and Holst, J. J. (1985). The intestinal mucosa preferentially releases somatostatin-28 in pigs. *Regul. Pept.* 11, 251–262. doi: 10.1016/0167-0115(85)90057-6

Baskin, D. G., and Ensinck, J. W. (1984). Somatostatin in epithelial cells of intestinal mucosa is present primarily as somatostatin 28. *Peptides* 5, 615–621. doi: 10.1016/0196-9781(84)90092-5

Benoit, R., Esch, F., Bennett, H. P., Ling, N., Ravazzola, M., Orci, L., et al. (1990). Processing of prosomatostatin. *Metabolism* 39, 22–25. doi: 10.1016/0026-0495(90)90202-N

Benoit, R., Ling, N., and Esch, F. (1987). A new prosomatostatin-derived peptide reveals a pattern for prohormone cleavage at monobasic sites. *Science* 238, 1126–1129. doi: 10.1126/science.2891188

Bersani, M., Thim, L., Baldissera, F. G., and Holst, J. J. (1989). Prosomatostatin 1-64 is a major product of somatostatin gene expression in pancreas and gut. *J. Biol. Chem.* 264, 10633–10636.

Beyer, J., Jabari, S., Rau, T. T., Neuhuber, W., and Brehmer, A. (2013). Substance P- and choline acetyltransferase immunoreactivities in somatostatin-containing, human submucosal neurons. *Histochem. Cell Biol.* 140, 157–167. doi: 10.1007/s00418-013-1078-9

Black, J. W., and Leff, P. (1983). Operational models of pharmacological agonism. *Proc. R. Soc. Lond. B Biol. Sci.* 220, 141–162. doi: 10.1098/rspb.1983.0093

Bohlen, P., Brazeau, P., Benoit, R., Ling, N., Esch, F., and Guillemin, R. (1980). Isolation and amino acid composition of two somatostatin-like peptides from ovine hypothalamus: somatostatin-28 and somatostatin-25. *Biochem. Biophys. Res. Commun.* 96, 725–734. doi: 10.1016/0006-291X(80)91415-1

Bokoch, M. P., Zou, Y., Rasmussen, S. G., Liu, C. W., Nygaard, R., Rosenbaum, D. M., et al. (2010). Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature* 463, 108–112. doi: 10.1038/nature08650

Bornstein, J. C., Costa, M., Furness, J. B., and Lees, G. M. (1984). Electrophysiology and enkephalin immunoreactivity of identified myenteric plexus neurones of guinea-pig small intestine. *J. Physiol.* 351, 313–325.

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

Brazeau, P., Ling, N., Esch, F., Bohlen, P., Benoit, R., and Guillemin, R. (1981). High biological activity of the synthetic replicates of somatostatin-28 and somatostatin-25. *Regul. Pept.* 1, 255–264. doi: 10.1016/0167-0115(81)90048-3

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., et al. (1973). Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* 179, 77–79. doi: 10.1126/science.179.4068.77

Brehmer, A., Lindig, T. M., Schrodl, F., Neuhuber, W., Ditterich, D., Rexer, M., et al. (2005). Morphology of enkephalin-immunoreactive myenteric neurons in the human gut. *Histochem. Cell Biol.* 123, 131–138. doi: 10.1007/s00418-005-0757-6

Bu'Lock, A. J., Vaillant, C., and Dockray, G. J. (1983). Immunohistochemical studies on the gastrointestinal tract using antisera to Met-enkephalin and Met-enkephalin Arg6Phe7. *J. Histochem. Cytochem.* 31, 1356–1362. doi: 10.1177/31.12.6355286

Cescato, R., Loesch, K. A., Waser, B., Macke, H. R., Rivier, J. E., Reubi, J. C., et al. (2010). Agonist-biased signaling at the sst2A receptor: the multi-somatostatin analogs KE108 and SOM230 activate and antagonize distinct signaling pathways. *Mol. Endocrinol.* 24, 240–249. doi: 10.1210/me.2009-0321

Cetin, Y. (1988). Enterochromaffin (EC-) cells of the mammalian gastro-entero-pancreatic (GEP) endocrine system: cellular source of pro-dynorphin-derived peptides. *Cell Tissue Res.* 253, 173–179. doi: 10.1007/BF00221752

Charfi, I., Audet, N., Bagheri Tudashki, H., and Pineyro, G. (2014). Identifying ligand-specific signalling within biased responses: focus on delta opioid receptor ligands. *Br. J. Pharmacol.* doi: 10.1111/bph.12705 [Epub ahead of print].

Cone, R. I., Weber, E., Barchas, J. D., and Goldstein, A. (1983). Regional distribution of dynorphin and neo-endorphin peptides in rat brain, spinal cord, and pituitary. *J. Neurosci.* 3, 2146–2152.

Corbett, A. D., Gillan, M. G., and Kosterlitz, H. W. (1991). Electrically-induced release of opioid peptides from the guinea-pig myenteric plexus preparation. *J. Recept. Res.* 11, 665–673. doi: 10.3109/10799899109066434

Corbett, A. D., Mcknight, A. T., and Kosterlitz, H. W. (1988). Tissue content of opioid peptides in the myenteric plexus-longitudinal muscle of guinea-pig small intestine. *J. Neurochem.* 51, 32–37. doi: 10.1111/j.1471-4159.1988.tb04831.x

Costa, M., Furness, J. B., and Cuello, A. C. (1985). Separate populations of opioid containing neurons in the guinea-pig intestine. *Neuropeptides* 5, 445–448. doi: 10.1016/0143-4179(85)90050-2

Dalm, V. A., Van Hagen, P. M., De Krijger, R. R., Kros, J. M., Van Koetsveld, P. M., Van Der Lely, A. J., et al. (2004). Distribution pattern of somatostatin and cortistatin mRNA in human central and peripheral tissues. *Clin. Endocrinol.* 60, 625–629. doi: 10.1111/j.1365-2265.2004.02024.x

DeBold, C. R., Nicholson, W. E., and Orth, D. N. (1988). Immunoreactive proopiomelanocortin (POMC) peptides and POMC-like messenger ribonucleic acid are present in many rat nonpituitary tissues. *Endocrinology* 122, 2648–2657. doi: 10.1210/endo-122-6–2648

de Lecea, L., Criado, J. R., Prospero-Garcia, O., Gautvik, K. M., Schweitzer, P., Danielson, P. E., et al. (1996). A cortical neuropeptide with neuronal depressant and sleep-modulating properties. *Nature* 381, 242–245. doi: 10.1038/381242a0

Deupi, X., and Kobilka, B. K. (2010). Energy landscapes as a tool to integrate GPCR structure, dynamics, and function. *Physiology (Bethesda)* 25, 293–303. doi: 10.1152/physiol.00002.2010

Donnerer, J., Holzer, P., and Lembeck, F. (1984). Release of dynorphin, somatostatin and substance P from the vascularly perfused small intestine of the guinea-pig during peristalsis. *Br. J. Pharmacol.* 83, 919–925. doi: 10.1111/j.1476-5381.1984.tb16532.x

Eipper, B. A., and Mains, R. E. (1978). Analysis of the common precursor to corticotropin and endorphin. *J. Biol. Chem.* 253, 5732–5744.

Elde, R., Hokfelt, T., Johansson, O., and Terenius, L. (1976). Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. *Neuroscience* 1, 349–351. doi: 10.1016/0306-4522(76)90063-4

Ensinck, J. W., Baskin, D. G., Vahl, T. P., Vogel, R. E., Laschansky, E. C., Francis, B. H., et al. (2002). Thrittene, homologous with somatostatin-28((1-13)), is a novel peptide in mammalian gut and circulation. *Endocrinology* 143, 2599–2609. doi: 10.1210/endo.143.7.8904

Ensinck, J. W., Laschansky, E. C., Vogel, R. E., and D'alessio, D. A. (2003). Effect of ingested nutrients on the release of thrittene into the human circulation. *J. Clin. Endocrinol. Metab.* 88, 4798–4804. doi: 10.1210/jc.2003–030063

Esch, F., Bohlen, P., Ling, N., Benoit, R., Brazeau, P., and Guillemin, R. (1980). Primary structure of ovine hypothalamic somatostatin-28 and somatostatin-25. *Proc. Natl. Acad. Sci. U.S.A.* 77, 6827–6831. doi: 10.1073/pnas.77.11.6827

Ferri, G. L., Morreale, R. A., and Dockray, G. J. (1986). Met5-enkephalin-Arg6-Gly7-Leu8 immunoreactivity in the human gut. *Peptides* 7, 735–739. doi: 10.1016/0196-9781(86)90087-2

Ferri, G. L., Watkinson, A., and Dockray, G. J. (1988). Proenkephalin A-derived peptides in the human gut. *Gastroenterology* 95, 1011–1017.

Foong, J. P., Parry, L. J., Gwynne, R. M., and Bornstein, J. C. (2010). 5-HT(1A), SST(1), and SST(2) receptors mediate inhibitory postsynaptic potentials in the submucous plexus of the guinea pig ileum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 298, G384–G394. doi: 10.1152/ajpgi.00438.2009

Francis, B. H., Baskin, D. G., Saunders, D. R., and Ensinck, J. W. (1990). Distribution of somatostatin-14 and somatostatin-28 gastrointestinal-pancreatic cells of rats and humans. *Gastroenterology* 99, 1283–1291.

Furness, J. B., Costa, M., Gibbins, I. L., Llewellyn-Smith, I. J., and Oliver, J. R. (1985). Neurochemically similar myenteric and submucous neurons directly traced to the mucosa of the small intestine. *Cell Tissue Res.* 241, 155–163. doi: 10.1007/BF00214637

Furness, J. B., Costa, M., and Miller, R. J. (1983). Distribution and projections of nerves with enkephalin-like immunoreactivity in the guinea-pig small intestine. *Neuroscience* 8, 653–664. doi: 10.1016/0306-4522(83)90001-5

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

Giraud, A. S., Williams, R. G., and Dockray, G. J. (1984). Evidence for different patterns of post-translational processing of pro-enkephalin in the bovine adrenal, colon and striatum indicated by radioimmunoassay using region-specific antisera to Met-Enk-Arg6-Phe7 and Met-Enk-Arg6-Gly7-Leu8. *Neurosci. Lett.* 46, 223–228. doi: 10.1016/0304-3940(84)90446-4

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

Gonzalez-Rey, E., Varela, N., Sheibanie, A. F., Chorny, A., Ganea, D., and Delgado, M. (2006). Cortistatin, an antiinflammatory peptide with therapeutic action in inflammatory bowel disease. *Proc. Natl. Acad. Sci. U.S.A.* 103, 4228–4233. doi: 10.1073/pnas.0508997103

Grider, J. R. (2003). Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J. Pharmacol. Exp. Ther.* 307, 460–467. doi: 10.1124/jpet.103.053512

Hirst, B. H., Conlon, J. M., Coy, D. H., Holland, J., and Shaw, B. (1982). Comparison of the gastric exocrine inhibitory activities and plasma kinetics of somatostatin-28 and somatostatin-14 in cats. *Regul. Pept.* 4, 227–237. doi: 10.1016/0167-0115(82)90115-X

Hofmann, K. P., Scheerer, P., Hildebrand, P. W., Choe, H. W., Park, J. H., Heck, M., et al. (2009). A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem. Sci.* 34, 540–552. doi: 10.1016/j.tibs.2009.07.005

Horikawa, S., Takai, T., Toyosato, M., Takahashi, H., Noda, M., Kakidani, H., et al. (1983). Isolation and structural organization of the human preproenkephalin B gene. *Nature* 306, 611–614. doi: 10.1038/306611a0

Hughes, J., Kosterlitz, H. W., and Smith, T. W. (1977). The distribution of methionine-enkephalin and leucine-enkephalin in the brain and peripheral tissues. *Br. J. Pharmacol.* 61, 639–647. doi: 10.1111/j.1476-5381.1977.tb07557.x

Hughes, P. A., Harrington, A. M., Castro, J., Liebregts, T., Adam, B., Grasby, D. J., et al. (2013). Sensory neuro-immune interactions differ between irritable bowel syndrome subtypes. *Gut* 62, 1456–1465. doi: 10.1136/gutjnl-2011–301856

Janecka, A., Fichna, J., and Janecki, T. (2004). Opioid receptors and their ligands. *Curr. Top. Med. Chem.* 4, 1–17. doi: 10.2174/1568026043451618

Jessen, K. R., Saffrey, M. J., Van Noorden, S., Bloom, S. R., Polak, J. M., and Burnstock, G. (1980). Immunohistochemical studies of the enteric nervous system in tissue culture and in situ: localization of vascoactive intestinal polypeptide (VIP), substance-P and enkephalin immunoreactive nerves in the guinea-pig gut. *Neuroscience* 5, 1717–1735. doi: 10.1016/0306-4522(80)90091-3

Johnson, S. M., Costa, M., Humphreys, C. M., and Shearman, R. (1987). Inhibitory effects of opioids in a circular muscle-myenteric plexus preparation of guinea-pig ileum. *Naunyn Schmiedebergs Arch. Pharmacol.* 336, 419–424. doi: 10.1007/BF00164876

Keast, J. R., and De Groat, W. C. (1992). Segmental distribution and peptide content of primary afferent neurons innervating the urogenital organs and colon of male rats. *J. Comp. Neurol.* 319, 615–623. doi: 10.1002/cne.903190411

Keast, J. R., Furness, J. B., and Costa, M. (1985). Distribution of certain peptide-containing nerve fibres and endocrine cells in the gastrointestinal mucosa in five mammalian species. *J. Comp. Neurol.* 236, 403–422. doi: 10.1002/cne.902360308

Kenakin, T., and Christopoulos, A. (2013a). Measurements of ligand bias and functional affinity. *Nat. Rev. Drug Discov.* 12, 483. doi: 10.1038/nrd3954-c2

Kenakin, T., and Christopoulos, A. (2013b). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov.* 12, 205–216. doi: 10.1038/nrd3954

Kenakin, T., Watson, C., Muniz-Medina, V., Christopoulos, A., and Novick, S. (2012). A simple method for quantifying functional selectivity and agonist bias. *ACS Chem. Neurosci.* 3, 193–203. doi: 10.1021/cn200111m

Kokrashvili, Z., Rodriguez, D., Yevshayeva, V., Zhou, H., Margolskee, R. F., and Mosinger, B. (2009). Release of endogenous opioids from duodenal enteroendocrine cells requires Trpm5. *Gastroenterology* 137, 598–606; 606.e1–e2. doi: 10.1053/j.gastro.2009.02.070

Kromer, W., Hollt, V., Schmidt, H., and Herz, A. (1981). Release of immunoreactive-dynorphin from the isolated guinea-pig small intestine is reduced during peristaltic activity. *Neurosci. Lett.* 25, 53–56. doi: 10.1016/0304-3940(81)90100-2

Larsson, L. I., Childers, S., and Snyder, S. H. (1979). Met- and Leu-enkephalin immunoreactivity in separate neurones. *Nature* 282, 407–410. doi: 10.1038/282407a0

Larsson, L. I., and Stengaard-Pendersen, K. (1982). Immunocytochemical and ultrastructural differentiation between Met-enkephalin-, Leu-enkephalin-, and Met/Leu-enkephalin-immunoreactive neurons of feline gut. *J. Neurosci.* 2, 861–878.

Laux-Biehlmann, A., Mouheiche, J., Veriepe, J., and Goumon, Y. (2013). Endogenous morphine and its metabolites in mammals: history, synthesis, localization and perspectives. *Neuroscience* 233, 95–117. doi: 10.1016/j.neuroscience.2012.12.013

Leander, S., Ekman, R., Uddman, R., Sundler, F., and Hakanson, R. (1984). Neuronal cholecystokinin, gastrin-releasing peptide, neurotensin, and beta-endorphin in the intestine of the guinea pig. Distribution and possible motor functions. *Cell Tissue Res.* 235, 521–531. doi: 10.1007/BF00226949

Linnoila, R. I., Diaugustine, R. P., Miller, R. J., Chang, K. J., and Cuatrecasas, P. (1978). An immunohistochemical and radioimmunological study of the distribution of [met5]- and [leu5]-enkephalin in the gastrointestinal tract. *Neuroscience* 3, 1187–1196. doi: 10.1016/0306-4522(78)90138-0

Liu, Q., Cescato, R., Dewi, D. A., Rivier, J., Reubi, J. C., and Schonbrunn, A. (2005). Receptor signaling and endocytosis are differentially regulated by somatostatin analogs. *Mol. Pharmacol.* 68, 90–101. doi: 10.1124/mol.105.011767

Liu, Q., Dewi, D. A., Liu, W., Bee, M. S., and Schonbrunn, A. (2008). Distinct phosphorylation sites in the SST2A somatostatin receptor control internalization, desensitization, and arrestin binding. *Mol. Pharmacol.* 73, 292–304. doi: 10.1124/mol.107.038570

Lloyd, K. C., Amirmoazzami, S., Friedik, E., Chew, P., and Walsh, J. H. (1997). Somatostatin inhibits gastrin release and acid secretion by activating sst2 in dogs. *Am. J. Physiol.* 272, G1481–G1488.

Low, M. J. (2004). The somatostatin neuroendocrine system: physiology and clinical relevance in gastrointestinal and pancreatic disorders. *Best Pract. Res. Clin. Endocrinol. Metab.* 18, 607–622. doi: 10.1016/j.beem.2004.08.005

Maguma, H. T., Dewey, W. L., and Akbarali, H. I. (2012). Differences in the characteristics of tolerance to mu-opioid receptor agonists in the colon from wild type and beta-arrestin2 knockout mice. *Eur. J. Pharmacol.* 685, 133–140. doi: 10.1016/j.ejphar.2012.04.001

Majeed, N. H., Silberring, J., and Przewlocki, R. (1987). The in vitro release of immunoreactive dynorphin and alpha-neoendorphin from the perfused rat duodenum. *Life Sci.* 41, 497–504. doi: 10.1016/0024-3205(87)90227-X

Mansour, A., Hoversten, M. T., Taylor, L. P., Watson, S. J., and Akil, H. (1995). The cloned mu, delta and kappa receptors and their endogenous ligands: evidence for two opioid peptide recognition cores. *Brain Res.* 700, 89–98. doi: 10.1016/0006-8993(95)00928-J

Massotte, D. (2014). In vivo opioid receptor heteromerization: where do we stand? *Br. J. Pharmacol.* doi: 10.1111/bph.12702 [Epub ahead of print].

McIntosh, C. H., Bakich, V., Kwok, Y. N., and Brown, J. C. (1986). A comparison of the inhibitory effects of somatostatin-14, -25, and -28 on motility of the guinea pig ileum. *Can. J. Physiol. Pharmacol.* 64, 303–306. doi: 10.1139/y86-048

McPherson, J., Rivero, G., Baptist, M., Llorente, J., Al-Sabah, S., Krasel, C., et al. (2010). mu-opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol. Pharmacol.* 78, 756–766. doi: 10.1124/mol.110.066613

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

Miller, R. J., and Hirning, L. D. (2010). "Opioid Peptides of The Gut," in *Comprehensive Physiology*. Hoboken, NJ: John Wiley & Sons, Inc.

Mimoda, T., Kitamura, N., Hondo, E., and Yamada, J. (1998). Immunohistochemical colocalization of serotonin, substance P and Met-enkephalin-Arg6-Gly7-Leu8 in the endocrine cells of the ruminant duodenum. *Anat. Histol. Embryol.* 27, 65–69. doi: 10.1111/j.1439-0264.1998.tb00158.x

Mongardi Fantaguzzi, C., Thacker, M., Chiocchetti, R., and Furness, J. B. (2009). Identification of neuron types in the submucosal ganglia of the mouse ileum. *Cell Tissue Res.* 336, 179–189. doi: 10.1007/s00441-009-0773-2

Mor, A., Delfour, A., Amiche, M., Sagan, S., Nicolas, P., Grassi, J., et al. (1989). Dermorphin and related peptides in rat tissues. *Neuropeptides* 13, 51–57. doi: 10.1016/0143-4179(89)90021-8

Mor, A., Pradelles, P., Delfour, A., Montagne, J. J., Quintero, F. L., Conrath, M., et al. (1990). Evidence for Pro-dermorphin processing products in rat tissues. *Biochem. Biophys. Res. Commun.* 170, 30–38. doi: 10.1016/0006-291X(90)91236-L

Murphy, R., and Turner, C. A. (1990). Isolation and microsequence analysis of guinea pig alpha-neo-endorphin. *Peptides* 11, 65–68. doi: 10.1016/0196-9781(90)90111-H

Nihei, K., and Iwanaga, T. (1985). Localization of Met-enkephalin-Arg6-Gly7-Leu8-like immunoreactivity in the gastrointestinal tract of rat and pig. *J. Histochem. Cytochem.* 33, 1001–1006. doi: 10.1177/33.10.3900192

Noda, M., Teranishi, Y., Takahashi, H., Toyosato, M., Notake, M., Nakanishi, S., et al. (1982). Isolation and structural organization of the human preproenkephalin gene. *Nature* 297, 431–434. doi: 10.1038/297431a0

Nunn, C., Cervia, D., Langenegger, D., Tenaillon, L., Bouhelal, R., and Hoyer, D. (2004). Comparison of functional profiles at human recombinant somatostatin sst2 receptor: simultaneous determination of intracellular Ca2+ and luciferase expression in CHO-K1 cells. *Br. J. Pharmacol.* 142, 150–160. doi: 10.1038/sj.bjp.0705735

Orwoll, E. S., and Kendall, J. W. (1980). Beta-endorphin and adrenocorticotropin in extrapituitary sites: gastrointestinal tract. *Endocrinology* 107, 438–442. doi: 10.1210/endo-107-2-438

Papotti, M., Tarabra, E., Allia, E., Bozzalla-Cassione, F., Broglio, F., Deghenghi, R., et al. (2003). Presence of cortistatin in the human pancreas. *J. Endocrinol. Invest.* 26, RC15–RC18. doi: 10.1007/BF03347348

Patel, Y. C., and Srikant, C. B. (1997). Somatostatin receptors. *Trends Endocrinol. Metab.* 8, 398–405. doi: 10.1016/S1043-2760(97)00168-9

Patierno, S., Zellalem, W., Ho, A., Parsons, C. G., Lloyd, K. C., Tonini, M., et al. (2005). N-methyl-D-aspartate receptors mediate endogenous opioid release in enteric neurons after abdominal surgery. *Gastroenterology* 128, 2009–2019. doi: 10.1053/j.gastro.2005.03.042

Pfannkuche, H., Reiche, D., Firzlaff, U., Sann, H., and Schemann, M. (1998). Enkephalin-immunoreactive subpopulations in the myenteric plexus of the guinea-pig fundus project primarily to the muscle and not to the mucosa. *Cell Tissue Res.* 294, 45–55. doi: 10.1007/s004410051155

Polak, J. M., Bloom, S. R., Sullivan, S. N., Facer, P., and Pearse, A. G. (1977). Enkephalin-like immunoreactivity in the human gastrointestinal tract. *Lancet* 1, 972–974. doi: 10.1016/S0140-6736(77)92277-2

Poole, D. P., Pelayo, J. C., Scherrer, G., Evans, C. J., Kieffer, B. L., and Bunnett, N. W. (2011). Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice. *Gastroenterology* 141, 982–991. doi: 10.1053/j.gastro.2011.05.042

Portbury, A. L., Pompolo, S., Furness, J. B., Stebbing, M. J., Kunze, W. A., Bornstein, J. C., et al. (1995). Cholinergic, somatostatin-immunoreactive interneurons in the guinea pig intestine: morphology, ultrastructure, connections and projections. *J. Anat.* 187, 303–321.

Pradayrol, L., Chayvialle, J. A., Carlquist, M., and Mutt, V. (1978). Isolation of a porcine intestinal peptide with C-terminal somatostatin. *Biochem. Biophys. Res. Commun.* 85, 701–708. doi: 10.1016/0006-291X(78)91218-4

Pradayrol, L., Jornvall, H., Mutt, V., and Ribet, A. (1980). N-terminally extended somatostatin: the primary structure of somatostatin-28. *FEBS Lett.* 109, 55–58. doi: 10.1016/0014-5793(80)81310-X

Puebla, L., Mouchantaf, R., Sasi, R., Khare, S., Bennett, H. P., James, S., et al. (1999). Processing of rat preprocortistatin in mouse AtT-20 cells. *J. Neurochem.* 73, 1273–1277. doi: 10.1046/j.1471-4159.1999.0731273.x

Rabbani, S. N., and Patel, Y. C. (1990). Peptides derived by processing of rat prosomatostatin near the amino-terminus: characterization, tissue distribution, and release. *Endocrinology* 126, 2054–2061. doi: 10.1210/endo-126-4-2054

Raehal, K. M., Schmid, C. L., Medvedev, I. O., Gainetdinov, R. R., Premont, R. T., and Bohn, L. M. (2009). Morphine-induced physiological and behavioral responses in mice lacking G protein-coupled receptor kinase 6. *Drug Alcohol Depend.* 104, 187–196. doi: 10.1016/j.drugalcdep.2009.04.011

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

Rajagopal, S., Bassoni, D. L., Campbell, J. J., Gerard, N. P., Gerard, C., and Wehrman, T. S. (2013). Biased agonism as a mechanism for differential signaling by chemokine receptors. *J. Biol. Chem.* 288, 35039–35048. doi: 10.1074/jbc.M113.479113

Ravazzola, M., Benoit, R., Ling, N., Guillemin, R., and Orci, L. (1983). Immunocytochemical localization of prosomatostatin fragments in maturing and mature secretory granules of pancreatic and gastrointestinal D cells. *Proc. Natl. Acad. Sci. U.S.A.* 80, 215–218. doi: 10.1073/pnas.80.1.215

Ravazzola, M., Benoit, R., Ling, N., and Orci, L. (1989). Prosomatostatin-derived antrin is present in gastric D cells and in portal blood. *J. Clin. Invest.* 83, 362–366. doi: 10.1172/JCI113892

Rivero, G., Llorente, J., Mcpherson, J., Cooke, A., Mundell, S. J., McArdle, C. A., et al. (2012). Endomorphin-2: a biased agonist at the mu-opioid receptor. *Mol. Pharmacol.* 82, 178–188. doi: 10.1124/mol.112.078659

Romero, A., Gonzalez-Cuello, A., Laorden, M. L., Campillo, A., Vasconcelos, N., Romero-Alejo, E., et al. (2012). Effects of surgery and/or remifentanil administration on the expression of pERK1/2, c-Fos and dynorphin in the dorsal root ganglia in mice. *Naunyn Schmiedebergs Arch. Pharmacol.* 385, 397–409. doi: 10.1007/s00210-011-0721-z

Ronai, A. Z., Kiraly, K., Szebeni, A., Szemenyei, E., Prohaszka, Z., Darula, Z., et al. (2009). Immunoreactive endomorphin 2 is generated extracellularly in rat isolated L4,5 dorsal root ganglia by DPP-IV. *Regul. Pept.* 157, 1–2. doi: 10.1016/j.regpep.2009.06.006

Rozenfeld, R., and Devi, L. A. (2007). Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. *FASEB J.* 21, 2455–2465. doi: 10.1096/fj.06-7793com

Samson, W. K., Zhang, J. V., Avsian-Kretchmer, O., Cui, K., Yosten, G. L., Klein, C., et al. (2008). Neuronostatin encoded by the somatostatin gene regulates neuronal, cardiovascular, and metabolic functions. *J. Biol. Chem.* 283, 31949–31959. doi: 10.1074/jbc.M804784200

Sanger, G. J., and Tuladhar, B. R. (2004). The role of endogenous opioids in the control of gastrointestinal motility: predictions from in vitro modelling. *Neurogastroenterol. Motil.* 16(Suppl. 2), 38–45. doi: 10.1111/j.1743-3150.2004.00556.x

Schmidt, W. E., Mutt, V., Kratzin, H., Carlquist, M., Conlon, J. M., and Creutzfeldt, W. (1985). Isolation and characterization of proSS1-32, a peptide derived from the N-terminal region of porcine preprosomatostatin. *FEBS Lett.* 192, 141–146. doi: 10.1016/0014-5793(85)80060-0

Schultzberg, M., Hokfelt, T., Nilsson, G., Terenius, L., Rehfeld, J. F., Brown, M., et al. (1980). Distribution of peptide- and catecholamine-containing neurons in the gastro-intestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine beta-hydroxylase. *Neuroscience* 5, 689–744. doi: 10.1016/0306-4522(80)90166-9

Schulz, R., Wuster, M., Simantov, R., Snyder, S., and Herz, A. (1977). Electrically stimulated release of opiate-like material from the myenteric plexus of the guinea pig ileum. *Eur. J. Pharmacol.* 41, 347–348. doi: 10.1016/0014-2999(77)90331-4

Seal, A., Yamada, T., Debas, H., Hollinshead, J., Osadchey, B., Aponte, G., et al. (1982). Somatostatin-14 and -28: clearance and potency on gastric function in dogs. *Am. J. Physiol.* 243, G97–G102.

Shen, K. Z., and Surprenant, A. (1993). Somatostatin-mediated inhibitory postsynaptic potential in sympathetically denervated guinea-pig submucosal neurones. *J. Physiol.* 470, 619–635.

Song, Z. M., Brookes, S. J., Ramsay, G. A., and Costa, M. (1997). Characterization of myenteric interneurons with somatostatin immunoreactivity in the guinea-pig small intestine. *Neuroscience* 80, 907–923. doi: 10.1016/S0306-4522(96)00605-7

Spampinato, S., Ferri, G. L., Candeletti, S., Romualdi, P., Cavicchini, E., Soimero, L., et al. (1988). Regional distribution of immunoreactive dynorphin A in the human gastrointestinal tract. *Neuropeptides* 11, 101–105. doi: 10.1016/0143-4179(88)90077-7

Spampinato, S., Speroni, E., Canossa, M., Sciarretta, G., Malaguti, P., and Ferri, S. (1992). Dynorphin B-like immunoreactivity in gastroduodenal biopsy specimens from gallstone patients. *Regul. Pept.* 41, 131–138. doi: 10.1016/0167-0115(92)90042-S

Steele, P. A., and Costa, M. (1990). Opioid-like immunoreactive neurons in secretomotor pathways of the guinea-pig ileum. *Neuroscience* 38, 771–786. doi: 10.1016/0306-4522(90)90070-K

Steele, P. A., Turner, C. A., and Murphy, R. (1989). Measurement and chromatographic characterization of prodynorphin-derived peptides in the guinea-pig ileum. *Neuropeptides* 13, 207–213. doi: 10.1016/0143-4179(89)90094-2

Sundler, F., Alumets, J., Ekman, R., Hakanson, R., and Van Wimersma Greidanus, T. B. (1981). Immunoreactive adrenocorticotropic hormone (ACTH) in porcine gut and pancreas: fact or artifact? *J. Histochem. Cytochem.* 29, 1328–1335. doi: 10.1177/29.11.6172464

Tang, J., Yang, H. Y., and Costa, E. (1982). Distribution of met5-enkephalin-Arg6-Phe7 (MEAP) in various tissues of rats and guinea pigs. *Life Sci.* 31, 2303–2306. doi: 10.1016/0024-3205(82)90143-6

Terskiy, A., Wannemacher, K. M., Yadav, P. N., Tsai, M., Tian, B., and Howells, R. D. (2007). Search of the human proteome for endomorphin-1 and endomorphin-2 precursor proteins. *Life Sci.* 81, 1593–1601. doi: 10.1016/j.lfs.2007.09.025

Thompson, G. L., Kelly, E., Christopoulos, A., and Canals, M. (2014). Novel GPCR paradigms at the mu-opioid receptor. *Br. J. Pharmacol.* doi: 10.1111/bph.12600 [Epub ahead of print].

Traub, R. J., Hutchcroft, K., and Gebhart, G. F. (1999). The peptide content of colonic afferents decreases following colonic inflammation. *Peptides* 20, 267–273. doi: 10.1016/S0196-9781(98)00157-0

Valdez-Morales, E., Guerrero-Alba, R., Ochoa-Cortes, F., Benson, J., Spreadbury, I., Hurlbut, D., et al. (2013). Release of endogenous opioids during a chronic IBD model suppresses the excitability of colonic DRG neurons. *Neurogastroenterol. Motil.* 25, 39.e4–46.e4. doi: 10.1111/nmo.12008

Van Op den Bosch, J., Adriaensen, D., Van Nassauw, L., and Timmermans, J. P. (2009). The role(s) of somatostatin, structurally related peptides and somatostatin receptors in the gastrointestinal tract: a review. *Regul. Pept.* 156, 1–8. doi: 10.1016/j.regpep.2009.04.003

Verma-Gandhu, M., Bercik, P., Motomura, Y., Verdu, E. F., Khan, W. I., Blennerhassett, P. A., et al. (2006). CD4+ T-cell modulation of visceral nociception in mice. *Gastroenterology* 130, 1721–1728. doi: 10.1053/j.gastro.2006.01.045

Verma-Gandhu, M., Verdu, E. F., Cohen-Lyons, D., and Collins, S. M. (2007). Lymphocyte-mediated regulation of beta-endorphin in the myenteric plexus. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292, G344–G348. doi: 10.1152/ajpgi.00318.2006

Vincent, S. R., Dalsgaard, C. J., Schultzberg, M., Hokfelt, T., Christensson, I., and Terenius, L. (1984). Dynorphin-immunoreactive neurons in the autonomic nervous system. *Neuroscience* 11, 973–987. doi: 10.1016/0306-4522(84)90208-2

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

Waldhoer, M., Fong, J., Jones, R. M., Lunzer, M. M., Sharma, S. K., Kostenis, E., et al. (2005). A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9050–9055. doi: 10.1073/pnas.0501112102

Walsh, J. H. (1988). Peptides as regulators of gastric acid secretion. *Annu. Rev. Physiol.* 50, 41–63. doi: 10.1146/annurev.ph.50.030188.000353

Wang, Y. N., and Lindberg, I. (1986). Distribution and characterization of the opioid octapeptide met5-enkephalin-arg6-gly7-leu8 in the gastrointestinal tract of the rat. *Cell Tissue Res.* 244, 77–85. doi: 10.1007/BF00218384

Weinstock, J. V., and Elliott, D. (2000). The somatostatin immunoregulatory circuit present at sites of chronic inflammation. *Eur. J. Endocrinol.* 143(Suppl. 1), S15–S19. doi: 10.1530/eje.0.143S015

Wolter, H. J. (1985a). Colocalization of dynorphin-A(1-17) and dynorphin-A(1-8) within some perikarya of rat duodenum: immunohistochemical evidence for the presence of two separate dynorphinergic systems. *Biochem. Biophys. Res. Commun.* 130, 774–780. doi: 10.1016/0006-291X(85)90483-8

Wolter, H. J. (1985b). Ultrastructural evidence for beta-endorphin-like immunoreactivity in the nervous system of the rat duodenum. *Brain Res.* 334, 194–199. doi: 10.1016/0006-8993(85)90588-8

Wolter, H. J. (1986). Identification of the tridecapeptide dynorphin B (rimorphin) within perikarya of rat duodenum. *Life Sci.* 39, 727–730. doi: 10.1016/0024-3205(86)90020-2

Wood, J. D., and Galligan, J. J. (2004). Function of opioids in the enteric nervous system. *Neurogastroenterol. Motil.* 16(Suppl. 2), 17–28. doi: 10.1111/j.1743-3150.2004.00554.x

Yakovleva, T., Bazov, I., Cebers, G., Marinova, Z., Hara, Y., Ahmed, A., et al. (2006). Prodynorphin storage and processing in axon terminals and dendrites. *FASEB J.* 20, 2124–2126. doi: 10.1096/fj.06-6174fje

Yuferov, V. P., Culpepper-Morgan, J. A., Laforge, K. S., Ho, A., and Kreek, M. J. (1998). Regional quantitation of preprodynorphin mRNA in guinea pig gastrointestinal tract. *Neurochem. Res.* 23, 505–511. doi: 10.1023/A:1022426400545

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

Zamir, N., Weber, E., Palkovits, M., and Brownstein, M. (1984). Differential processing of prodynorphin and proenkephalin in specific regions of the rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 81, 6886–6889. doi: 10.1073/pnas.81.21.6886

Zhao, P., Canals, M., Murphy, J. E., Klingler, D., Eriksson, E. M., Pelayo, J. C., et al. (2013). Agonist-biased trafficking of somatostatin receptor 2A in enteric neurons. *J. Biol. Chem.* 288, 25689–25700. doi: 10.1074/jbc.M113.496414

Zingg, H. H., and Patel, Y. C. (1983). Processing of somatostatin-28 to somatostatin-14 by rat hypothalamic synaptosomal membranes. *Life Sci.* 33, 1241–1247. doi: 10.1016/0024-3205(83)90004-8

Zweemer, A. J., Toraskar, J., Heitman, L. H., and IJzerman, A. P. (2014). Bias in chemokine receptor signalling. *Trends Immunol.* 35, 243–252. doi: 10.1016/j.it.2014.02.004

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Family a GPCR heteromers in animal models

Javier González-Maeso*

Departments of Psychiatry and Neurology, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA
**Correspondence: javier.maeso@mssm.edu*

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Ralf Jockers, University of Paris, France

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INTRODUCTION

G protein-coupled receptors (GPCRs) were assumed to exist and function in the plasma membrane as monomeric proteins that became activated by binding of one agonist ligand to one receptor molecule (Bourne et al., 1990). However, although previous findings based on rather indirect measures such as radioligand binding had suggested a direct interaction of two receptors with each other (Limbird et al., 1975; Ferre et al., 1991), it was the application of a protein-protein interaction assay by bioluminescence resonance energy transfer (BRET) that revealed the phenomenon of molecular proximity between beta2-adrenergic receptors in living cells (Angers et al., 2000). Since then, this topic has been a major subject of research and numerous in silico and *in vitro* studies have suggested expression of family A GPCRs as homodimers and higher-order homomers in heterologous expression systems. However, the demonstration that reconstitution of a single beta2-adrenergic receptor molecule into lipoprotein particles leads to efficient activation of G proteins raised concerns about the functional significance of family A GPCR homomers (Whorton et al., 2007), and this is currently a controversial topic (for an extensive review on GPCR homodimers/homomers, see Milligan, 2013; see also Bouvier and Hebert, 2014; Lambert and Javitch, 2014).

Another fundamental yet relatively independent question is that related to expression of different GPCR subtypes as heteromers. It is well accepted that the family C GABA$_B$ receptor needs two protomers (GABA$_B$-R1 and GABA$_B$-R2) to reach the plasma membrane as a functional dimeric receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). On the other hand, although multiple lines of evidence indicate that family A GPCR heteromers may exist, particularly in tissue cultures (González-Maeso, 2011; Ferre et al., 2014), only relatively recent studies started to test this formulation in whole animal models.

FAMILY A GPCR HETEROMERS IN WHOLE ANIMAL MODELS

One of the main limitations of the classical techniques used to define GPCR heteromeric formation is the translation of findings obtained in cellulo into physiological or behavioral assays in whole animal models. In this context, co-immunoprecipitation is an approach commonly used to examine protein-protein interaction in native tissue (Milligan and Bouvier, 2005). GPCR antibodies are usually neither specific nor sensitive and therefore validation assays in knockout mice are often required (Fribourg et al., 2011; Moreno et al., 2012). Considering this, it is also clear that demonstration of co-immunoprecipitation in native tissues does not imply the existence of a heteromeric assembly, as they may form part of same protein complex through for example PDZ domain-binding motifs at the end of the C-terminal tails of both receptor types together with adaptor proteins (Magalhaes et al., 2010). Remarkably, there are only a few studies that have investigated GPCR heteromeric formation in living animals, and due to the lack of biophysical methods applicable to study protein-protein interactions in preclinical models, their experimental approaches were mostly focused on signaling and behavioral outcomes rather than on the existence of molecular proximity between different GPCR subtypes.

Although it does not measure molecular proximity, an attractive approach to define whether heteromeric formation is involved in behavioral phenotypes is the use of peptides that disrupt receptor complex formation. These peptides tested *in vivo* are usually selected according to findings previously obtained in heterologous expression systems. As an example, it was demonstrated that the G_s-coupled dopamine D_1 receptor and the $G_{i/o}$-coupled dopamine D_2 form a receptor complex that induces Ca^{2+} release via a $G_{q/11}$-dependent pathway (Lee et al., 2004), and that the region of Met-257—Glu-271 (intracellular loop 3; $D2_{IL3\text{-}29\text{-}2}$) but not Asn-243—Ile-256 (intracellular loop 3; $D2_{IL3\text{-}29\text{-}1}$) of the dopamine D_2 receptor can pull-down the dopamine D_1 receptor. Based on a Tat-tagged peptide approach, it was shown that intracerebroventricular administration of the peptide $D2_{IL3\text{-}29\text{-}2}$, which disrupts heteromeric formation between dopamine D_1 and D_2 receptors *in vitro*, induces antidepressant-like effects in rats (Pei et al., 2010). More recent findings using serial deletions and point mutations further demonstrate that dopamine D_1 receptor carboxyl tail residues Glu-404 and Glu-405 are critical in mediating the interaction with the D_2 receptor, and that administration of a disrupting peptide Tat-D1 modulates depression-like behavior in rats such as forced swim test (Hasbi et al., 2014). A similar approach was used to block the association as a GPCR heteromer between the mu-opioid receptor isoform MOR1D and the gastrin-releasing peptide receptor (GRPR) in the spinal cord (Liu et al., 2011). The authors demonstrated

that the C-terminus of MOR1D is critical for MOR1D-GRPR heteromeric formation. Using a Tat-fusion peptide, they also found that a motif consisting of seven amino acids of the MOR1D C-terminus (RNEEPSS) attenuates morphine-induced scratching, but not morphine-induced analgesia.

The question of whether GPCR heteromers exist *ex vivo* has been addressed using time-resolved Förster resonance energy transfer FRET (TR-FRET) in plasma membrane preparations of mouse brain. It was found that the dopamine D_2 receptor and the ghrelin receptor (GHSR1a) co-localize in mouse striatum, hippocampus and hypothalamus (Kern et al., 2012). When membrane preparations from hypothalamus were incubated with red-ghrelin (acceptor fluorophore) and an anti-D_2 receptor antibody together with a europium cryptate-labeled secondary antibody (donor fluorophore), a significantly TR-FRET signal was observed. Although TR-FRET signal is eliminated in hypothalamic membrane preparations of GHSR1a knockout mice, which supports specificity, these findings were observed *ex vivo* in plasma membrane preparations and further investigation will be necessary to confirm the existence of GHSR1a-D_2 heteromeric formation in hypothalamus *in vivo*.

Another indirect approach to test whether GPCR heteromeric formation affects behavioral phenotypes is the use of chimeric constructs that according to biophysical assays in tissue culture do not form heteromeric complexes. Examples include the 5-HT_{2A}-mGlu2 heteromeric receptor complex (Gonzalez-Maeso et al., 2008) and the MT_1-MT_2 melatonin heteromeric receptor complex (Baba et al., 2013). Serotonin 5-HT_{2A} and metabotropic glutamate 2 (mGlu2) receptors have been shown to form a GPCR heteromeric complex in HEK293 cells. Using chimeric constructs, it was demonstrated that three residues located at the intracellular end of TM4 of mGlu2 are necessary to form a complex with the 5-HT_{2A} receptor (Ala-$677^{4.40}$, Ala-$681^{4.44}$, Ala-$685^{4.48}$) (Fribourg et al., 2011; Moreno et al., 2012). Head-twitch is a rodent behavior model induced by hallucinogenic 5-HT_{2A} agonist such as lysergic acid diethylamide (LSD) and DOI (Hanks and Gonzalez-Maeso, 2013). This behavior requires expression of 5-HT_{2A} receptor in cortical pyramidal neurons (Gonzalez-Maeso et al., 2007) and is absent in mGlu2 knockout mice (Moreno et al., 2011), which supports that mGlu2 is necessary for 5-HT_{2A}-dependent behavioral events. Using a virally-mediated (HSV) over-expression approach, it was demonstrated that the head-twitch response induced by the hallucinogenic 5-HT_{2A} receptor agonist DOI was rescued in mGlu2 knockout mice over-expressing wild-type mGlu2 in frontal cortex, and that this did not occur in mGlu2 knockout mice over-expressing mGlu2deltaTM4N—a mGlu2/mGlu3 chimeric construct that according to previous findings *in vitro* and in cellulo does not form the 5-HT_{2A}-mGlu2 receptor heteromer (Moreno et al., 2012). A similar approach was used to investigate function of the MT_1-MT_2 melatonin heteromeric receptor complex *in vivo* in mouse (Baba et al., 2013). The electroretinogram (ERG), consisting mainly of an a-wave and a b-wave, is commonly used to assess retinal function. Using transgenic mice that express MT_2-P95L (mutant that does not form the MT_1-MT_2 heteromeric receptor complex in HEK293 cells), it was shown that control mice responded to melatonin injection with an increase in the amplitude of the a-wave and b-wave, whereas MT_2-P95L did not.

Although these events have been proposed to represent a demonstration of GPCR heteromeric expression, thereby suggesting a new target for drug design, their conclusions in animal models were based largely on indirect approaches that measured phenotypes affected by manipulations such as chimeric constructs or Tat-tagged peptides that impact heteromeric organization *in vitro*. Consequently, it remains unclear as to whether different GPCR subtypes exist in close molecular proximity *in vivo* in whole animal models, or alternatively if these phenotypes result of signaling mechanisms that are independent of GPCR heteromeric formation. Detailed measurement of such molecular proximity *in vivo*, as well as the processes that control GPCR heteromerization in whole animal models, will require further study.

LIMITATIONS, FUTURE DIRECTIONS, AND CONCLUDING REMARKS

Although a wealth of data from *in vitro* and in cellulo models have established the important role of GPCR heteromers in mediating precise and distinct roles in signaling cascades, their influence in the establishment of complex behavioral phenotypes remains to be fully elucidated. For instance, certain physiological and behavioral outcomes could conceivably be altered in the presence of peptides that according to *in vitro* or in cellulo assays disrupt GPCR heteromeric assembly. Similarly, the use of viral-mediated over-expression or transgenic animals could translate into animal models previous findings with receptor mutants that do not form heterocomplexes *in vitro* or in cellulo. However, a more precise understanding of such structural assembly obtained in rodent models will be necessary to fully define whether GPCR heteromers exist and function *in vivo*. Some of these strategies include the use of FRET (McGinty et al., 2011) of BRET (Dragulescu-Andrasi et al., 2011) imaging of protein-protein interactions in living mice.

An important challenge in the fields of GPCR research and molecular pharmacology is to develop an integrated understanding of how various mechanisms communicate with each other to ultimately orchestrate the formation of heteromeric complexes between some but not all GPCR subtypes. Potential mechanisms that are critical for this interaction specificity include specific pairs of residues that govern heteromeric formation, clustering of GPCRs in membrane microdomains, and crosstalk between receptors and a plethora of multidomain scaffolding proteins. Another important question to be addressed by future research is the molecular basis through which GPCR heteromers affect G protein function. For example, it has been shown that drugs that activate the $G_{q/11}$-coupled 5-HT_{2A} receptor induce both $G_{q/11}$- and $G_{i/o}$-dependent signaling in HEK293 cells co-expressing 5-HT_{2A} and the $G_{i/o}$-coupled mGlu2 receptor as a GPCR heteromer (Gonzalez-Maeso et al., 2008). Although findings in knockout mice suggest that co-expression of 5-HT_{2A} and mGlu2 receptors is necessary to activate $G_{q/11}$ and $G_{i/o}$ by 5-HT_{2A}

agonists in mouse frontal cortex membrane preparations (Fribourg et al., 2011), whether heteromeric formation is needed in living mice for this signaling crosstalk remains unknown. Similarly, more work is required both in cellulo and in animal models to solve whether $G_{q/11}$ and $G_{i/o}$ simultaneously or sequentially couple to the 5-HT_{2A}-mGlu2 heteromeric receptor complex upon agonist binding to one of the two promoters.

Another significant limitation to our current understanding of GPCR heteromeric function is the lack of knowledge about physical stability of family A GPCR heteromers in animal models. Previous findings in HEK293 cells convincingly demonstrate that the alpha1B-adrenergic receptor forms higher-order oligomers, and that receptor oligomerization is required for receptor maturation and plasma membrane delivery (Lopez-Gimenez et al., 2007). On the other hand, results based on an experimental approach that recruits beta2-adrenergic receptors into artificial domains on the surface of living HEK293 cells suggest that the components of family A GPCR homomers interact transiently (Fonseca and Lambert, 2009; Gavalas et al., 2013). A similar conclusion has been reached using total internal reflection fluorescence microscopy (TIRFM) to visualize individual molecules in isolated CHO cells—the authors observed a transient association and dissociation of muscarinic M_1 receptor dimers in real time (Hern et al., 2010). Much further work is needed to characterize where along the pathway from synthesis to maturation and degradation do GPCR heteromers form. It also remains uncertain the stability of family A GPCR heteromers both *in vitro* and in whole animal models. Many studies examining homomeric GPCR interfaces report that residues of both TM1 and TM4 form symmetrical interfaces that lead to higher order species in heterologous expression systems (Guo et al., 2005, 2008), and this has been supported further by a number of recent crystal structures (Wu et al., 2012; Huang et al., 2013). However, it remains to be fully elucidated whether different homomeric and heteromeric organizations (e.g., squares and/or parallelograms) might exist in native tissue. These are all key questions that require further technical advances.

In conclusion, although a range of approaches has been applied and this has led to a general appreciation that GPCR heteromers affect receptor trafficking, pharmacology and function in cellulo, much more work is needed to probe the role of GPCR heteromerization *in vivo*. These advances in GPCR heteromeric research are now occurring at a rapid pace and promise to greatly contribute to the future of molecular medicine.

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REFERENCES

Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M., et al. (2000). Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. U.S.A.* 97, 3684–3689. doi: 10.1073/pnas.97.7.3684

Baba, K., Benleulmi-Chaachoua, A., Journe, A. S., Kamal, M., Guillaume, J. L., Dussaud, S., et al. (2013). Heteromeric MT1/MT2 melatonin receptors modulate photoreceptor function. *Sci. Signal* 6, ra89. doi: 10.1126/scisignal.2004302

Bourne, H. R., Sanders, D. A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 348, 125–132. doi: 10.1038/348125a0

Bouvier, M., and Hebert, T. E. (2014). CrossTalk proposal: weighing the evidence for Class A GPCR dimers, the evidence favours dimers. *J. Physiol.* 592, 2439–2441. doi: 10.1113/jphysiol.2014.272252

Dragulescu-Andrasi, A., Chan, C. T., De, A., Massoud, T. F., and Gambhir, S. S. (2011). Bioluminescence resonance energy transfer (BRET) imaging of protein-protein interactions within deep tissues of living subjects. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12060–12065. doi: 10.1073/pnas.1100923108

Ferre, S., Casado, V., Devi, L. A., Filizola, M., Jockers, R., Lohse, M. J., et al. (2014). G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol. Rev.* 66, 413–434. doi: 10.1124/pr.113.008052

Ferre, S., von Euler, G., Johansson, B., Fredholm, B. B., and Fuxe, K. (1991). Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7238–7241. doi: 10.1073/pnas.88.16.7238

Fonseca, J. M., and Lambert, N. A. (2009). Instability of a class a G protein-coupled receptor oligomer interface. *Mol. Pharmacol.* 75, 1296–1299. doi: 10.1124/mol.108.053876

Fribourg, M., Moreno, J. L., Holloway, T., Provasi, D., Baki, L., Mahajan, R., et al. (2011). Decoding the Signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* 147, 1011–1023. doi: 10.1016/j.cell.2011.09.055

Gavalas, A., Lan, T. H., Liu, Q., Correa, I. R. Jr., Javitch, J. A., and Lambert, N. A. (2013). Segregation of family A G protein-coupled receptor protomers in the plasma membrane. *Mol. Pharmacol.* 84, 346–352. doi: 10.1124/mol.113.086868

González-Maeso, J. (2011). GPCR oligomers in pharmacology and signaling. *Mol. Brain* 4:20. doi: 10.1186/1756-6606-4-20

Gonzalez-Maeso, J., Ang, R. L., Yuen, T., Chan, P., Weisstaub, N. V., Lopez-Gimenez, J. F., et al. (2008). Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452, 93–97. doi: 10.1038/nature06612

Gonzalez-Maeso, J., Weisstaub, N. V., Zhou, M., Chan, P., Ivic, L., Ang, R., et al. (2007). Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron* 53, 439–452. doi: 10.1016/j.neuron.2007.01.008

Guo, W., Shi, L., Filizola, M., Weinstein, H., and Javitch, J. A. (2005). Crosstalk in G protein-coupled receptors: changes at the transmembrane homodimer interface determine activation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17495–17500. doi: 10.1073/pnas.0508950102

Guo, W., Urizar, E., Kralikova, M., Mobarec, J. C., Shi, L., Filizola, M., et al. (2008). Dopamine D2 receptors form higher order oligomers at physiological expression levels. *EMBO J.* 27, 2293–2304. doi: 10.1038/emboj.2008.153

Hanks, J. B., and Gonzalez-Maeso, J. (2013). Animal models of serotonergic psychedelics. *ACS Chem. Neurosci.* 4, 33–42. doi: 10.1021/cn300138m

Hasbi, A., Perreault, M. L., Shen, M. Y., Zhang, L., To, R., Fan, T., et al. (2014). A peptide targeting an interaction interface disrupts the dopamine D1-D2 receptor heteromer to block signaling and function *in vitro* and *in vivo*: effective selective antagonism. *FASEB J.* doi: 10.1096/fj.14-254037. [Epub ahead of print].

Hern, J. A., Baig, A. H., Mashanov, G. I., Birdsall, B., Corrie, J. E., Lazareno, S., et al. (2010). Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2693–2698. doi: 10.1073/pnas.0907915107

Huang, J., Chen, S., Zhang, J. J., and Huang, X. Y. (2013). Crystal structure of oligomeric beta1-adrenergic G protein-coupled receptors in ligand-free basal state. *Nat. Struct. Mol. Biol.* 20, 419–425. doi: 10.1038/nsmb.2504

Jones, K. A., Borowsky, B., Tamm, J. A., Craig, D. A., Durkin, M. M., Dai, M., et al. (1998). GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature* 396, 674–679. doi: 10.1038/25348

Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., et al. (1998). GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature* 396, 683–687. doi: 10.1038/25360

Kern, A., Albarran-Zeckler, R., Walsh, H. E., and Smith, R. G. (2012). Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. *Neuron* 73, 317–332. doi: 10.1016/j.neuron.2011.10.038

Lambert, N. A., and Javitch, J. A. (2014). CrossTalk opposing view: weighing the evidence for class A GPCR dimers, the jury is still out. *J. Physiol.* 592, 2443–2445. doi: 10.1113/jphysiol.2014.272997

Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lanca, A. J., et al. (2004). Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. *J. Biol. Chem.* 279, 35671–35678. doi: 10.1074/jbc.M401923200

Limbird, L. E., Meyts, P. D., and Lefkowitz, R. J. (1975). Beta-adrenergic receptors: evidence for negative cooperativity. *Biochem. Biophys. Res. Commun.* 64, 1160–1168. doi: 10.1016/0006-291X(75)90815-3

Liu, X.-Y., Liu, Z.-C., Sun, Y.-G., Ross, M., Kim, S., Tsai, F.-F., et al. (2011). Unidirectional cross-activation of GRPR by MOR1D uncouples itch and analgesia induced by opioids. *Cell* 147, 447–458. doi: 10.1016/j.cell.2011.08.043

Lopez-Gimenez, J. F., Canals, M., Pediani, J. D., and Milligan, G. (2007). The alpha1b-adrenoceptor exists as a higher-order oligomer: effective oligomerization is required for receptor maturation, surface delivery, and function. *Mol. Pharmacol.* 71, 1015–1029. doi: 10.1124/mol.106.033035

Magalhaes, A. C., Holmes, K. D., Dale, L. B., Comps-Agrar, L., Lee, D., Yadav, P. N., et al. (2010). CRF receptor 1 regulates anxiety behavior via sensitization of 5-HT2 receptor signaling. *Nat. Neurosci.* 13, 622–629. doi: 10.1038/nn.2529

McGinty, J., Stuckey, D. W., Soloviev, V. Y., Laine, R., Wylezinska-Arridge, M., Wells, D. J., et al. (2011). *In vivo* fluorescence lifetime tomography of a FRET probe expressed in mouse. *Biomed. Opt. Express* 2, 1907–1917. doi: 10.1364/BOE.2.001907

Milligan, G. (2013). The prevalence, maintenance and relevance of GPCR oligomerization. *Mol. Pharmacol.* 84, 158–169. doi: 10.1124/mol.113.084780

Milligan, G., and Bouvier, M. (2005). Methods to monitor the quaternary structure of G protein-coupled receptors. *FEBS J.* 272, 2914–2925. doi: 10.1111/j.1742-4658.2005.04731.x

Moreno, J. L., Holloway, T., Albizu, L., Sealfon, S. C., and Gonzalez-Maeso, J. (2011). Metabotropic glutamate mGlu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT2A receptor agonists. *Neurosci. Lett.* 493, 76–79. doi: 10.1016/j.neulet.2011.01.046

Moreno, J. L., Muguruza, C., Umali, A., Mortillo, S., Holloway, T., Pilar-Cuellar, F., et al. (2012). Identification of three residues essential for 5-HT2A-mGlu2 receptor heteromerization and its psychoactive behavioral function. *J. Biol. Chem.* 287, 44301–44319. doi: 10.1074/jbc.M112.413161

Pei, L., Li, S., Wang, M., Diwan, M., Anisman, H., Fletcher, P. J., et al. (2010). Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. *Nat. Med.* 16, 1393–1395. doi: 10.1038/nm.2263

White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., et al. (1998). Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* 396, 679–682. doi: 10.1038/25354

Whorton, M. R., Bokoch, M. P., Rasmussen, S. G., Huang, B., Zare, R. N., Kobilka, B., et al. (2007). A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7682–7687. doi: 10.1073/pnas.0611448104

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., et al. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* 485, doi: 10.1038/nature10939

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Antibodies to probe endogenous G protein-coupled receptor heteromer expression, regulation, and function

Ivone Gomes[1], Achla Gupta[1], Ittai Bushlin[1] and Lakshmi A. Devi[1,2]*

[1] *Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, NY, USA*
[2] *The Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute, Mexico
Sergi Ferre, National Institutes of Health, USA

***Correspondence:**
Lakshmi A. Devi, Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, Annenberg Building 19-84, One Gustave L. Levy Place, New York, NY 10029, USA
e-mail: lakshmi.devi@mssm.edu

Over the last decade an increasing number of studies have focused on the ability of G protein-coupled receptors to form heteromers and explored how receptor heteromerization modulates the binding, signaling and trafficking properties of individual receptors. Most of these studies were carried out in heterologous cells expressing epitope tagged receptors. Very little information is available about the *in vivo* physiological role of G protein-coupled receptor heteromers due to a lack of tools to detect their presence in endogenous tissue. Recent advances such as the generation of mouse models expressing fluorescently labeled receptors, of TAT based peptides that can disrupt a given heteromer pair, or of heteromer-selective antibodies that recognize the heteromer in endogenous tissue have begun to elucidate the physiological and pathological roles of receptor heteromers. In this review we have focused on heteromer-selective antibodies and describe how a subtractive immunization strategy can be successfully used to generate antibodies that selectively recognize a desired heteromer pair. We also describe the uses of these antibodies to detect the presence of heteromers, to study their properties in endogenous tissues, and to monitor changes in heteromer levels under pathological conditions. Together, these findings suggest that G protein-coupled receptor heteromers represent unique targets for the development of drugs with reduced side-effects.

Keywords: G protein-coupled receptor, dimerization, heteromerization, opioid, cannabinoid, angiotensin

INTRODUCTION

Since the first report showing that metabotropic $GABA_B$ receptors, members of the family C of G protein-coupled receptors (GPCRs), form constitutive heteromers (White et al., 1998; Kuner et al., 1999; Pin et al., 2009) an increasing number of studies have provided evidence suggesting that other GPCRs, particularly those belonging to family A, also heteromerize (Albizu et al., 2010; Gomes et al., 2013a; Hiller et al., 2013; Szafran et al., 2013). However, most of the studies reporting GPCR heteromerization were carried out in heterologous cells co-expressing differentially epitope tagged recombinant receptors. Concerns that heteromerization in heterologous cells could be due to over-expression of individual receptors and that the unique signaling reported for a given heteromer is due to receptor cross-talk via downstream signaling rather than direct receptor-receptor interactions led investigators in the field to propose a set of criteria to be fulfilled in order to consider that a GPCR pair forms an heteromer in endogenous tissue (Ferre et al., 2009): (i) both receptors can be detected in the same subcellular compartment in a cell; (ii) close proximity between the two receptors for direct interactions can be demonstrated through the use of either proximity ligation assays, ligand-based FRET, or heteromer-selective probes such as antibodies only in wild-type tissue; (iii) the receptors can be co-immunoprecipitated from wild-type but not from tissue lacking one of the receptors; (iv) the heteromer pair exhibits a "biochemical fingerprint" in wild-type tissue that matches that seen in heterologous cells co-expressing both receptors but not cells expressing only one of the receptors; and (v) heteromer formation can be disrupted by agents such as TAT peptides and this leads to alterations in the "biochemical fingerprint" to one that resembles that of individual receptor protomers (Ferre et al., 2009).

In order to detect and map the presence of a GPCR heteromer in endogenous tissue, sensitive and selective tools are needed. Such tools could help not only to monitor heteromer levels under physiological and pathological conditions but also to tease apart the contribution of receptor homomers and heteromers to a given physiological response. In order to address this need our laboratory undertook the challenge to generate monoclonal antibodies that selectively recognize a given heteromer pair. Since monoclonal antibodies recognize a single epitope and are highly specific, they would not only facilitate detection of the targeted heteromer in endogenous tissue but would also permit studies to elucidate the contribution of the heteromer to signaling in tissues/membranes expressing both receptors. In general it is easy to generate antibodies to immunodominant and abundant epitopes; however this task is more challenging when using epitopes that are likely to be rare or less immunodominant. This would be the case with "heteromer-selective" epitopes where very little is known about the "heteromer" interface. We therefore decided to use a subtractive immunization strategy to improve our changes of raising such antibodies. This strategy has been successfully used in the cancer field to generate monoclonal antibodies that can

specifically block metastasis but not proliferation of cancer cells (Brooks et al., 1993), antibodies that can discriminate proteins that have a similar sequence (Sleister and Rao, 2002), or antibodies that could be used as diagnostic tools in certain types of cancer (Trefzer et al., 2000; Yasumoto et al., 2012). In this review we describe the strategy used to generate and characterize antibodies selective to either μOR-δOR, δOR-κOR, δOR-CB1R, and AT1R-CB1R heteromers (**Table 1**).

GENERATION OF HETEROMER-SELECTIVE ANTIBODIES USING A SUBTRACTIVE IMMUNIZATION STRATEGY

An important requirement to generate antibodies that can selectively recognize a given GPCR heteromer is the immunogen. An ideal immunogen would be a synthetic peptide that mimics the heteromeric region between two GPCRs since the latter would be distinct and unique compared to the homomeric regions. However, not much is known about the heteromer interface or a unique region shared by heteromers. Hence we used membranes from cells expressing the heteromer pair of interest as the immunogen. Given the likelihood that the heteromeric epitopes would be of very low abundance and of low immunogenicity, thereby preventing their detection by antibody producing cells, direct immunization with such membranes would have a low probability of successfully generating heteromer-selective antibodies. Therefore in order to improve our chances of generating heteromer-selective antibodies we used a subtractive immunization strategy (Salata et al., 1992; Sleister and Rao, 2001, 2002) that involves two major steps: (i) tolerization of mice to unwanted epitopes, and (ii) immunization with membranes expressing the heteromer pair of interest. Tolerization to unwanted epitopes can be achieved by immunizing mice with an emulsion of membranes from cells used to express the heteromer pair in combination with complete Freund's adjuvant (Gomes et al., 2013b); these cells can be CHO or HEK-293 cells that are usually used in GPCR co-expression studies as well as cells that endogenously express one of the receptor protomers (Gomes et al., 2013b). The mice are then treated for the next 3 days with cyclophosphamide to kill activated antibody producing cells (Gomes et al., 2013b). Every 15 days mice are administered with booster injections comprised of membrane emulsions in Freund's incomplete adjuvant followed by the 3 day treatment with cyclophosphamide (Gomes et al., 2013b). Booster injections are repeated until a consistently low titer is observed by ELISA with the membranes used for the tolerization step (Gomes et al., 2013b). Once animals are tolerized they are immunized with membranes expressing the heteromer pair of interest (Gomes et al., 2013b). Booster injections are repeated until a high titer is obtained by ELISA (using membranes that co-express both receptors). Animals are killed and individual spleens used to generate monoclonal antibodies using standard protocols (Gomes et al., 2013b). Once monoclonal antibodies are obtained, individual clones are tested for heteromer selectivity by ELISA, immunofluorescence or Western blot analysis using (i) cells that express individual receptors; (ii) cells that co-express both of the protomers of interest; (iii) cells that co-express one of the receptor protomers with a different partner GPCR; and (iv) tissues from wild-type and from animals lacking each of the receptor protomers (Gomes et al., 2013b). An antibody is considered to be heteromer-selective only if it gives a signal with cells or tissues co-expressing both of the protomers of interest. It is to be noted that heteromer selectivity may be observed with one screening procedure such as ELISA but not with another such as Western blotting or immunofluorescence since either heat denaturation of membrane proteins (as in the case of Western blot analysis) and/or tissue fixation (in the case of immunofluorescence studies) could mask the epitope identified by the antibody. Thus one needs to be careful about selecting the screening technique to allow for detection of the antigen under the assay of choice. Using this subtractive immunization strategy we successfully generated antibodies selective for either μOR-δOR, δOR-κOR, δOR-CB1R, or AT1R-CB1R heteromers (Gupta et al., 2010; Rozenfeld et al., 2011; Berg et al., 2012; Bushlin et al., 2012). In the following sections we describe these heteromer pairs and the studies carried out using heteromer selective antibodies.

μOR-δOR HETEROMERIZATION

A number of early studies proposed heteromerization between μOR and δOR based on interactions between these receptors. For example, pharmacological studies showed that morphine (a μOR agonist) shifted competitive radiolabeled leucine-enkephalin displacement curves by unlabeled leucine-enkephalin (a δOR agonist) into non-competitive curves (Rothman and Westfall, 1982). In addition behavioral studies showed that δOR agonists (endogenous peptides or synthetic agonists) could potentiate μOR-mediated antinociception while potent δOR antagonists attenuated not only morphine-mediated antinociception but also the development of tolerance to this drug (reviewed in Fujita et al., 2014a). Furthermore studies showed that chronic treatment with morphine increases surface expression of δOR in either cultured cortical or dorsal root ganglion neurons and in the dorsal horn of the spinal cord of wild-type but not in mice lacking μOR (Cahill et al., 2001; Morinville et al., 2003; Gendron et al., 2006). Studies with animals lacking either μOR or δOR further support interactions between these receptors. These studies indicate that δOR-mediated antinociception requires the presence of functional μOR (Matthes et al., 1996, 1998) and that δOR contributes to the development of tolerance to morphine (Zhu et al., 1999). The latter observation is also supported by studies using antisense oligonucleotides to decrease δOR expression in the brain (Sanchez-Blazquez et al., 1997). In addition, it has been reported that treatment with a selective δOR antagonist, naltriben, reduces the rewarding effects of morphine as measured using the morphine conditioned place preference test and this is accompanied by increases in δOR levels at the post-synaptic density fraction (Billa et al., 2010). Taken together these studies suggested receptor-receptor interactions between μOR and δOR.

A major requirement for two receptors to directly interact with each other is that they be localized not only to the same cell but also to the same subcellular compartment. Early evidence for the presence of μOR and δOR in the same cell came from electrophysiological and radiolabeled binding studies using either neurons or neuroblastoma cell lines (Egan and North, 1981; Zieglgansberger et al., 1982; Yu et al., 1986; Kazmi and Mishra, 1987; Baumhaker et al., 1993; Palazzi et al., 1996). In addition, a number of immunohistochemical studies showed that both

Table 1 | Genertion and characterization of heteromer-selective antibodies and their potential therapeutic applications.

Heteromer pair detected	Antibodies generation scheme	Heteromer selectivity tested using	Properties	Potential therapeutic applications	Ref.
μOR-δOR	Tolerization: HEK293 membranes Immunization: HEK293 membranes expressing μOR-δOR	(i) Membranes from HEK293 cells alone, cells expressing either μOR, δOR or μOR-δOR; (ii) Membranes from cells expressing either μOR or δOR in combination with other GPCRs; (iii) Membranes from cells expressing different ratios of μOR and δOR; (iv) Membranes from cells expressing chimeric μOR-δOR constructs; (v) Membranes from wild-type, μOR k/o or δOR k/o tissue	(a) Detects changes in heteromer levels in endogenous tissue; (b) Blocks heteromer-mediated binding & signaling	Tolerance	Gupta et al., 2010
κOR-δOR	Tolerization: HEK293 membranes Immunization: HEK293 membranes expressing κOR-δOR	(i) Membranes from HEK293 cells alone, cells expressing either κOR, δOR, or κOR-δOR; (ii) HEK293 cells expressing either κOR, δOR, CB1R, μOR-δOR or κOR-δOR; (iii) Neuro2A cells expressing either CB1R-AT1R, CB1R-CBR2, CB1R-μOR, CB1R-δOR; and CB1R-κOR	Potentiates DPDPE- mediated antinocipeption during thermal allodynia	Antinociception	Berg et al., 2012
δOR-CB1R	Tolerization: Neuro2A* membranes Immunization: Neuro2A* membranes expressing δOR	(i) Membranes from Neuro2A* cells alone or in combination with either δOR, μOR, κOR, CB2R or AT1R; (ii) Membranes from HEK293 cells expressing either δOR alone or in combination with either μOR or κOR; (iii) Cortical membranes from either wild-type, CB1R k/o or δOR mice	(a) Detects changes in heteromer levels in endogenous tissue; (b) Blocks CB1R agonist-mediated increases in δOR activity	Neuropathic pain	Bushlin et al., 2012
CB1R-AT1R	Tolerization: Neuro2A* membranes Immunization: Neuro2A* membranes expressing AT1R	(i) Membranes from HEK293 expressing either CB1R, AT1R or CB1R-AT1R; (ii) HEK293 cells expressing different ratios of CB1R and AT1R; (iii) HEK293 cells expressing either CB1R, AT1R, or CB1R-CB2R, CB1R-δOR, CB1R-μOR, CB1R-κOR, μOR-δOR or κOR- δOR (iv) Membranes from Neuro2A* cells alone or in combination with AT1R (CB1R-AT1R cells) and CB1R-AT1R cells where CB1R levels were reduced by expression of RNAi	(a) Detects changes in heteromer levels in activated HSCs; (b) Blocks angiotensin II-mediated signaling only in cells co-expressing CB1R-AT1R; (c) Decreases secretion of fibrogenic proteins from activated HSCs	Treatment of liver fibrosis	Rozenfeld et al., 2011

**Neuro2A cells endogenously express CB1R.*

AT1R, angiotensin type 1 receptor; CB1R, cannabinoid type 1 receptor; CB2R, cannabinoid type 2 receptor; ELISA, enzyme-linked immunosorbent assay; HSCs, hepatic stellate cells; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; k/o, knockout; n.d., not determined.

receptors were present in the same subcellular compartment in the brain and spinal cord by using receptor-selective antibodies (Arvidsson et al., 1995; Cheng et al., 1997; Wang and Pickel, 2001). Controversy arose about the co-localization of μOR and δOR in the dorsal root ganglions of the spinal cord primarily due to data with mice with a knockin of eGFP-tagged δOR that showed that both receptors were segregated from each other (<5% neurons showed receptor colocalization) with μOR being expressed in small peptidergic neurons where it was involved in inhibition of pain induced by noxious heat while δOR was expressed in medium-sized non-peptidergic and large myelinated neurons where it was involved in inhibition of pain induced by mechanical stimuli (Scherrer et al., 2009). However, previous and recent observations questioned the lack of μOR and δOR colocalization. For example, support for colocalization came from (i) studies using either immunogold electron microscopy (Cheng et al., 1997), single-cell PCR, *in situ* hybridization or immunostaining to demonstrate the presence of μOR and δOR in small peptidergic DRG neurons (Wang et al., 2010); (ii) studies showing that *myc*-tagged δOR is present in CGRP-containing large dense core vesicles while eGFP-tagged δOR is present at the cell surface when expressed in small DRGs (Zhang and Bao, 2012) suggesting that the C-terminal GFP might affect receptor trafficking; (iii) studies treating peptidergic nociceptors expressing μOR and δOR with selective agonists that prevent substance P release induced by formalin or capsaicin treatment and this could be blocked by receptor selective antagonists (Beaudry et al., 2011); (iv) studies using electrophysiological recordings from a wide range of neurons in the spinal trigeminal nucleus of anesthetized animals showing that activation of either μOR or δOR relieves both thermal- or mechanical induced pain with same potency (Normandin et al., 2013); and (v) studies showing colocalization of μOR and δOR in the plasma membrane of a small population of CGRP-containing neurons in eGFP-tagged δOR knockin mice (Bardoni et al., 2014). Additional support for colocalization comes from mice expressing eGFP-tagged δOR and mCherry-tagged μOR. These mice show that ∼40% of eGFP-tagged δOR positive and ∼30% of mCherry-tagged μOR positive DRGs co-express the two receptors (Erbs et al., 2014). In addition, these mice show colocalization of μOR and δOR in neurocircuits involved in survival, pain regulation, as well as food intake, water consumption and sexual behavior (Erbs et al., 2014). In the hippocampus co-expression of eGFP-tagged δOR and mCherry-tagged μOR is detected in GABAergic interneurons and formation of μOR-δOR interacting complexes was demonstrated by co-immunoprecipitation studies (Erbs et al., 2014). Taken together these results demonstrate substantial co-localization of μOR or δOR in the brain and spinal cord.

In order to detect the presence of μOR-δOR heteromers in endogenous tissue our laboratory generated heteromer-selective antibodies (**Table 1**) using a subtractive immunization strategy (Gupta et al., 2010). ELISA with these antibodies show that they detect an epitope present only in cells co-expressing μOR and δOR and not in cells expressing individual receptors or co-expressing either μOR or δOR in combination with other GPCRs (Gupta et al., 2010). Moreover, the signal obtained in ELISA is reduced when the antibodies are pre-incubated with membranes co-expressing μOR and δOR but not with membranes expressing individual receptors (Gupta et al., 2010). In addition, these antibodies recognize an epitope present only in membranes from wild-type mice but not from mice lacking either μOR or δOR (Gupta et al., 2010). Furthermore, the heteromer-selective antibodies showed better recognition of co-expressed wild-type receptors compared to co-expressed chimeric receptors where regions of μOR were substituted with δOR and *vice-versa* (Gupta et al., 2010). Taken together these results indicate that the antibodies selectively recognize the μOR-δOR heteromer.

The μOR-δOR heteromer-selective antibodies can be used for immunohistochemical studies to detect the presence of these heteromers in endogenous tissue or primary DRG cultures (Gupta et al., 2010). An interesting finding with these antibodies is that chronic treatment with escalating doses of morphine under conditions that lead to the development of antinociceptive tolerance leads to an increase in μOR-δOR heteromers in select brain regions from wild-type but not from mice lacking either μOR or δOR (Gupta et al., 2010). These regions include the medial nucleus of the trapezoid body (MNTB), an auditory relay nucleus and the rostral ventral medulla (RVM), a key relay nucleus involved in pain perception (Gupta et al., 2010). Similar increases in μOR-δOR heteromers were also observed in the cell bodies and dendrites of primary DRG neurons following 48 h treatment with morphine (**Figure 1**). More recently μOR-δOR heteromer-selective antibodies were used to detect the presence of these heteromers in ileal tissue (Fujita et al., 2014b).

Another criteria that a μOR and δOR heteromer has to fulfill is that both receptor protomers have to be in close enough proximity to directly interact. Co-immunoprecipitation studies using either antibodies to epitope tags or to endogenous receptors show that μOR and δOR form interacting complexes only in spinal cord membranes from wild-type (but not from mice lacking one of the receptors) as well as in cells co-expressing both receptors (George et al., 2000; Gomes et al., 2000, 2004). In addition we find that the μOR-δOR heteromer-selective antibodies can immunoprecipitate the heteromer from primary dorsal root ganglion (DRG) neurons as well as from cells co-expressing both receptors (Gupta et al., 2010). That μOR and δOR are in close proximity to directly interact was further supported by proximity based assays showing that the two receptors are <100Å in live cells co-expressing both receptors (Gomes et al., 2004; Hasbi et al., 2007).

A third criteria that the μOR-δOR heteromer has to fulfill is that it exhibits a unique "biochemical fingerprint" that is seen only in cells/tissues expressing both receptors. The "biochemical fingerprint" for μOR-δOR heteromers consists of changes in ligand binding and signaling properties. These include (i) the binding affinity of selective synthetic agonists is decreased while that of endogenous peptidic agonists is increased (George et al., 2000); (ii) occupancy of a receptor protomer allosterically modulates the binding and signaling profile of the partner protomer (Gomes et al., 2000, 2004, 2011); (iii) the μOR-δOR heteromer signals via either pertussis toxin insensitive Gαz (George et al., 2000; Fan et al., 2005; Hasbi et al., 2007), pertussis toxin sensitive Ca^{+2} signaling (Charles et al., 2003), or β-arrestin2 (Rozenfeld and Devi, 2007) compared to individual receptor homomers that signal via pertussis sensitive Gαi. A related point supporting

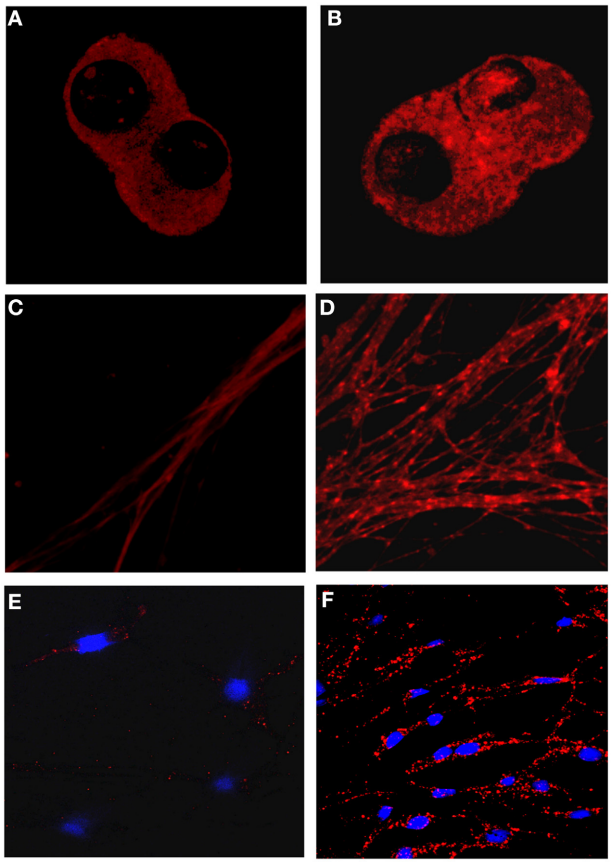

FIGURE 1 | Detection of μOR-δOR heteromers in primary dorsal root ganglion neurons using heteromer-selective antibodies. (A–D) Primary dorsal root ganglion neurons (DRGs) from embryonic rats were treated without **(A,C)** or with 10 μM morphine **(B,D)** for 48 h. μOR-δOR heteromers were visualized in the cell bodies **(A,B)** or in dendrites **(B,D)** using heteromer-selective antibodies (red). **(E,F)** Primary DRGs from adult rats were treated without **(E)** or with 10 μM morphine **(F)** for 48 h and μOR-δOR heteromers visualized using heteromer-selective antibodies (red). Morphine treatment increases μOR-δOR heteromer levels. Blue color represents nuclear DAPI staining.

receptor-receptor interactions is changes in maturation, endocytosis and degradation. For example, a study showed that co-expression of μOR and δOR leads to retention of the heteromer in the Golgi and that increased cell surface expression of μOR-δOR heteromers requires the expression of a chaperone protein, receptor transport protein-4 (Decaillot et al., 2008). Moreover, the presence of receptor transport protein-4 protects the μOR-δOR heteromer from ubiquitination and degradation (Decaillot et al., 2008). Another study showed that morphine and the opioid antagonists naltrexone and naltriben could serve as chemical chaperones that increase the cell surface expression of μOR-δOR heteromers (Gupta et al., 2010). With regards to heteromer internalization one study used cells that expressed μOR and where δOR expression was induced by treatment with ponasterone A treatment to show that the receptor protomers internalized independently from each other (Law et al., 2005). However, other studies showed that treatment with some μOR or δOR agonists (DAMGO, methadone, Deltorphin II, SNC80) but not others (morphine, DPDPE, DSLET) could induce μOR-δOR heteromer internalization (Hasbi et al., 2007; He et al., 2011; Milan-Lobo and Whistler, 2011). Interestingly, internalized heteromers are degraded (He et al., 2011; Milan-Lobo and Whistler, 2011) while internalized receptor homomers are recycled to the cell surface (Milan-Lobo and Whistler, 2011). Taken together these studies indicate that μOR-δOR heteromers exhibit unique properties compared to individual receptor homomers.

A final and important criteria for a μOR-δOR heteromer is the development of unique reagents that selectively target or disrupt the biochemical fingerprint of the heteromer. Several such reagents have been generated including (i) TAT fused peptides that disrupt μOR-δOR heteromerization *in vitro* as well as *in vivo* (He et al., 2011; Kabli et al., 2014); (ii) bivalent ligands that are more potent than morphine and without significant development of tolerance and dependence (Daniels et al., 2005); (iii) heteromer-selective antibodies that block μOR-δOR heteromer-mediated signaling (Gupta et al., 2010); and (iv) a small molecule μOR-δOR biased agonist, CYM51050, that is as potent as morphine but with lower development of tolerance (Gomes et al., 2013c). In the case of TAT fused peptides, a peptide fused to the transmembrane domain 1 of μOR disrupted the formation of μOR-δOR heteromers both in heterologous cells and in the spinal cord (He et al., 2011). Disruption of μOR-δOR heteromers in the spinal cord, in turn, led to an increase in morphine-mediated analgesia (He et al., 2011). Another peptide that could disrupt the formation of μOR-δOR heteromers in heterologous cells comprised of a TAT peptide fused to a sequence corresponding to the distal carboxyl terminal tail of δOR (Kabli et al., 2014). Interestingly, intra-accumbens administration of this TAT peptide attenuated the antidepressant and antianxiolytic effects of the δOR agonist UFP-512 (Kabli et al., 2014). In the case of bivalent ligands a compound comprising a δOR antagonist that is separated from a μOR agonist by a 21-atom spacer arm has been synthesized and named MDAN21 (Daniels et al., 2005). Studies show that MDAN21-mediated antinociception is 100 times more potent than that of morphine and that chronic administration of this compound does not lead to the development of tolerance and dependence (Daniels et al., 2005). In addition, MDAN21 prevents the internalization of μOR-δOR heteromers probably by occupying both protomers and immobilizing the heteromer at the cell surface (Yekkirala et al., 2013). Other bivalent ligands consisting of high-affinity μOR ligands (oxymorphone or naltrexone) linked by a spacer arm to low-affinity δOR ligands (ENTI or DM-SNC80 respectively) have also been synthesized (Harvey et al., 2012); however not much is known about the analgesic effects of these ligands and whether their administration leads to side-effects. In the case of monoclonal μOR-δOR heteromer-selective antibodies studies show that they can block the ability of low concentrations of a δOR selective antagonist, TIPPψ, to potentiate the binding and signaling by DAMGO, a selective μOR agonist (Gupta et al., 2010). More recently, a small molecule μOR-δOR biased agonist, CYM51050, was identified by high-throughput screening of a small molecule library using a β-arrestin recruitment assay (Gomes et al., 2013c). Studies with CYM51010 show that it is more efficacious at activating G-proteins and recruiting

β-arrestin in cells expressing the μOR-δOR heteromer compared to cells expressing either μOR or δOR homomers (Gomes et al., 2013c). In addition, while the antinociceptive activity of CYM51010 is similar to that of morphine, chronic administration of this biased agonist results in lower antinociceptive tolerance compared to morphine (Gomes et al., 2013c). That the signaling and antinociceptive effects of CYM51010 are mostly mediated via μOR-δOR heteromers is supported by the observation that they can be partly but significantly blocked by μOR-δOR heteromer-selective antibodies (Gomes et al., 2013c). Taken together, these unique heteromer targeting reagents show that μOR-δOR heteromers occur *in vivo* and that the heteromer-selective antibodies are useful not only in detecting the presence of an heteromer in endogenous tissue under normal and pathological conditions but also to study the properties of the heteromers and to identify heteromer selective ligands.

κOR-δOR HETEROMERIZATION

Localization studies examining heteromerization between δOR and κ opioid receptors (κOR) found them to be expressed in the same neuroblastoma cell line (Baumhaker et al., 1993) and co-expressed in axons of the dorsal horn of the spinal cord (Wessendorf and Dooyema, 2001). Co-immunoprecipitation studies using lysates from cells expressing differentially epitope tagged receptors (Jordan and Devi, 1999) or from peripheral sensory neurons (Berg et al., 2012) show that δOR and κOR form interacting complexes. That these two receptors are in close proximity for direct receptor-receptor interactions was demonstrated through the use of bioluminescence resonance energy transfer assays (BRET) (Ramsay et al., 2002). Signaling studies in cells co-expressing δOR and κOR show a unique "biochemical fingerprint" *in vitro* compared to cells expressing individual receptors (Jordan and Devi, 1999) since they report (i) a decrease in the binding affinity of δOR or κOR agonists; (ii) an increase in the binding affinity of a combination of δOR and κOR agonists or antagonists; (iii) an increase in signaling with a combination of δOR and κOR agonists; and (iv) that etorphine is not able to internalize δOR; etorphine internalizes δOR in cells expressing only this receptor (Jordan and Devi, 1999). However, it is not known whether this "biochemical fingerprint" observed for δOR-κOR heteromers in heterologous cells co-expressing epitope-tagged receptors is also observed in endogenous tissue. Studies with unique reagents targeting δOR-κOR heteromers show that a bivalent ligand, KDN-21, made up of a κOR antagonist, 5′-GNTI, that is joined by a spacer arm to a δOR antagonist, naltrindole, exhibits antagonistic activity but has no antinociceptive activity (Bhushan et al., 2004). Another reagent, 6′-guanidinonaltrindole (6′-GNTI) was initially identified as a δOR-κOR selective agonist that exhibits antinociceptive activity when administered intrathecally (i.t.) but not intracerebroventricularly (i.c.v) (Waldhoer et al., 2005). However, recent studies have reported that 6′-GNTI exhibits biased agonistic activity for κOR both in heterologous cells and striatal neurons (Rives et al., 2012; Schmid et al., 2013). This brings into question the selectivity of this compound for δOR-κOR heteromers. Finally, a δOR-κOR heteromer selective antibody has been generated and characterized (**Table 1**). Although not much is known about the ability of this antibody to block heteromer-mediated binding and signaling, it has been useful in demonstrating a role for δOR-κOR heteromer function in *vivo* (Berg et al., 2012). Administration of the δOR-κOR heteromer selective antibody into the hind paw of rats potentiated the antinociceptive response of a subthreshold dose of DPDPE such that the latter now gave nearly the maximal possible antinociceptive response required to inhibit the thermal allodynia produced by PGE2 (Berg et al., 2012). Since, treatment with the κOR antagonist, nor-BNI, also increases the antinociceptive response of a subthreshold dose of DPDPE although not to the same extent as the δOR-κOR heteromer selective antibody (Berg et al., 2012), these results suggest that either drugs targeting the δOR-κOR heteromer or a combination of the heteromer-selective antibody with DPDPE would be more effective in the treatment of thermal allodynia.

δOR-CB1R HETEROMERIZATION

A number of early studies suggested interactions between δOR and CB1 cannabinoid receptors (CB1R). These included (i) additive effects on signaling in N18TG neuroblastoma cells by a combination of opioid and cannabinoid ligands (Shapira et al., 1998); (ii) release of leucine-enkephalin during Δ^9-THC-mediated antinociception (Welch and Eads, 1999); (iii) signal cross-desensitization between CB1R and δOR (Shapira et al., 1998); (iv) the anxiolytic effects of the CB1R agonist, Δ^9-THC, could be blocked by the δOR antagonist, naltrindole (Berrendero and Maldonado, 2002); (v) increases in the levels and activity of CB1R in some brain regions of δOR knockout mice (Berrendero et al., 2003); and (vi) increases in δOR activity in CB1R knockout mice (Uriguen et al., 2005). Co-localization studies demonstrated the presence of CB1R and δOR in the same neuroblastoma cell line (Shapira et al., 1998) and within the cell bodies and processes of primary cortical neurons (Rozenfeld et al., 2012). Moreover, co-immunoprecipitation studies detect the formation of interacting CB1R-δOR complexes only in cells that co-express both receptors (Rozenfeld et al., 2012) and proximity based assays show that both receptors are in close proximity for direct receptor-receptor interactions in live cells (Rios et al., 2006). Examination of unique signaling showed that the δOR-CB1R heteromer exhibits a distinct biochemical fingerprint in heterologous cells in that (i) the presence of δOR or low concentrations of δOR ligands decreases the signaling potency of a CB1R agonist in heterologous cells and this is not seen in cells with a knockdown of δOR levels (Rozenfeld et al., 2012); (ii) the activity of CB1R is increased in cortical membranes from δOR knockout mice (Rozenfeld et al., 2012); (iii) in the presence of δOR a CB1R agonist activates a pathway involving phospholipase C (PLC) and β-arrestin2 while in the absence of δOR it activates Gαi/o-mediated signaling (Rozenfeld et al., 2012); (iv) in cells co-expressing CB1R and δOR activation of CB1R leads to accumulation of phosphorylated ERk1/2 in centrosomes (Rozenfeld et al., 2012); (v) activation of CB1R promotes increased cell survival only in cells co-expressing CB1R and δOR (Rozenfeld et al., 2012); and (vi) treatment with a CB1R antagonist decreases the survival of primary cortical neurons from wild-type but not from δOR knockout mice (Rozenfeld et al., 2012). Additional studies supporting heteromerization between δOR and CB1R include those examining the maturation and

trafficking of the two receptors. These studies show that co-expression of δOR changes the localization of CB1R from an intracellular compartment to the cell surface and this involves increased association with the adaptor protein AP-2 (Rozenfeld et al., 2012) and, reducing δOR levels in F11 cells that co-express CB1R and δOR leads to a decrease in the surface expression of CB1R (Rozenfeld et al., 2012).

To date reagents that selectively disrupt the δOR-CB1R heteromer or ligands targeting this heteromer have not been developed. However, antibodies that selectively recognize δOR-CB1R heteromers have been generated and characterized (**Table 1**). The δOR-CB1R heteromer-selective antibody was used to examine the regulation of the heteromer during neuropathic pain. We detected changes in heteromer levels 14 days after induction of neuropathic pain. Specifically, the antibody detected significant increases in δOR-CB1R heteromer levels in cortex, hypothalamus and midbrain of animals exhibiting neuropathic pain (Bushlin et al., 2012). This antibody was also useful in determining the heteromer-selective fingerprint in that it could block CB1R agonist-mediated increases in δOR activity and this was seen only in membranes from animals with neuropathic pain (Bushlin et al., 2012). Taken together, these studies indicate that the δOR-CB1R heteromer could be a novel therapeutic target in the treatment of neuropathic pain. Moreover, the δOR-CB1R heteromer-selective antibody could also be a potential therapeutic for the treatment of neuropathic pain given that it could block heteromer- mediated signaling.

CB1R-AT1R HETEROMERIZATION

Studies showing an increase in CB1R levels in liver cells that also express AT1 angiotensin receptors (AT1R) suggested possible interactions between these receptors (Teixeira-Clerc et al., 2006; Mallat and Lotersztajn, 2008; Siegmund and Schwabe, 2008; Lanthier et al., 2009). Co-localization of CB1R with AT1R has been demonstrated in hepatic stellate cells activated in response to chronic ethanol administration (Rozenfeld et al., 2011). Furthermore, co-immunoprecipitation studies show that CB1R and AT1R form interacting complexes in these cells (Rozenfeld et al., 2011). Examination of the biochemical profile of the CB1R-AT1R heteromer shows that the AT1R agonist induces a rapid and robust increase in ERK1/2 phosphorylation via $G_{\alpha i}$ instead of $G_{\alpha q}$ in cells co-expressing both receptors, and this is reduced either by decreasing the levels of CB1R using siRNA, or by inhibiting the activity of diacylglycerol lipase, the enzyme involved in the synthesis of the endocannabinoid 2-arachidonoylglycerol (Rozenfeld et al., 2011). In addition, CB1R ligands modulate AT1R-mediated increases in ERK1/2 phosphorylation with agonists potentiating and antagonists blocking signaling (Rozenfeld et al., 2011). Moreover, in cells co-expressing CB1R and AT1R phosphorylation of ERK1/2 by a CB1R agonist is only detected in the presence of a very low non-signaling dose of an AT1R agonist (Rozenfeld et al., 2011). Another interesting feature of cells co-expressing CB1R and AT1R is that although CB1R activation does not lead to increases in intracellular Ca^{+2} levels, activation of AT1R induces increases via $G_{\alpha q}$ but this requires the presence of CB1R since it is attenuated following siRNA-mediated knockdown of CB1R (Rozenfeld et al., 2011). Additional support for direct interactions between CB1R and AT1R comes from studies examining the maturation of these receptors. These studies show that the expression of AT1R changes the localization of CB1R from an intracellular compartment to the plasma membrane in Neuro 2A cells (Rozenfeld et al., 2011).

Although reagents that selectively disrupt CB1R-AT1R heteromers and ligands that selectively target this heteromer pair have not as yet been generated, antibodies that selectively recognize this heteromer have (**Table 1**). The CB1R-AT1R heteromer-selective antibody was used to examine the heteromer signaling fingerprint. The antibody can block angiotensin II-mediated ERK1/2 phosphorylation only in cells expressing the heteromer but not when CB1R levels are reduced in these cells by siRNA treatment; this indicates that ERK1/2 phosphorylation by angiotensin II is mediated via the CB1R-AT1R heteromer (Rozenfeld et al., 2011). In addition, the antibody can block the secretion of fibrogenic proteins including α-SMA from activated hepatic stellate cells obtained from rats chronically treated with ethanol (Rozenfeld et al., 2011). This together with observations indicating that the profibrogenic activity of AT1R in ethanol induced liver fibrosis requires the presence of CB1R (Rozenfeld et al., 2011) suggest that the CB1R-AT1R heteromer represents a novel therapeutic target for the treatment of liver fibrosis and that the CB1R-AT1R heteromer by its ability to block the secretion of fibrogenic proteins could potentially be used as a therapeutic to treat liver fibrosis.

CONCLUSIONS

In this review we describe how a subtractive immunization strategy can be successfully used to generate monoclonal antibodies that are selective for a given heteromer pair and that can be useful for examination of endogenous heteromers. Even though the procedure is time consuming, and requires a number of controls during screening procedures for determining the heteromer-selectivity of the antibodies, there are many advantages to developing the heteromer selective antibodies. These include the fact that they recognize a unique epitope that is present only in cells/tissues expressing the heteromer of interest, and thus they could be used to map the targeted heteromer in endogenous tissue and to monitor changes in heteromer levels during pathological conditions. In addition, heteromer-selective antibodies are also useful to discriminate the contribution of the heteromer from individual receptor homomers for a given signaling response. Finally, in select cases heteromer-selective antibodies have been useful to block a biological response. In this case the antibodies could be used as therapeutic targets in pathological conditions where heteromer levels are upregulated in addition to being useful in the identification of heteromer-selective/biased ligands. Thus, heteromer-selective antibodies represent unique and invaluable tools that would help in our understanding of the physiological roles of GPCR heteromers in endogenous tissues.

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REFERENCES

Albizu, L., Moreno, J. L., Gonzalez-Maeso, J., and Sealfon, S. C. (2010). Heteromerization of G protein-coupled receptors: relevance to neurological disorders and neurotherapeutics. *CNS Neurol. Disord. Drug Targets* 9, 636–650. doi: 10.2174/187152710793361586

Arvidsson, U., Riedl, M., Chakrabarti, S., Lee, J. H., Nakano, A. H., Dado, R. J., et al. (1995). Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. *J. Neurosci.* 15(Pt 1), 3328–3341.

Bardoni, R., Tawfik, V. L., Wang, D., Francois, A., Solorzano, C., Shuster, S. A., et al. (2014). Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. *Neuron* 81, 1312–1327. doi: 10.1016/j.neuron.2014.01.044

Baumhaker, Y., Wollman, Y., Goldstein, M. N., and Sarne, Y. (1993). Evidence for mu-, delta-, and kappa-opioid receptors in a human neuroblastoma cell line. *Life Sci.* 52, PL205–PL210. doi: 10.1016/0024-3205(93)90061-7

Beaudry, H., Dubois, D., and Gendron, L. (2011). Activation of spinal mu- and delta-opioid receptors potently inhibits substance P release induced by peripheral noxious stimuli. *J. Neurosci.* 31, 13068–13077. doi: 10.1523/JNEUROSCI.1817-11.2011

Berg, K. A., Rowan, M. P., Gupta, A., Sanchez, T. A., Silva, M., Gomes, I., et al. (2012). Allosteric interactions between delta and kappa opioid receptors in peripheral sensory neurons. *Mol. Pharmacol.* 81, 264–272. doi: 10.1124/mol.111.072702

Berrendero, F., and Maldonado, R. (2002). Involvement of the opioid system in the anxiolytic-like effects induced by Delta(9)-tetrahydrocannabinol. *Psychopharmacology (Berl.)* 163, 111–117. doi: 10.1007/s00213-002-1144-9

Berrendero, F., Mendizabal, V., Murtra, P., Kieffer, B. L., and Maldonado, R. (2003). Cannabinoid receptor and WIN 55 212-2-stimulated [35S]-GTPgammaS binding in the brain of mu-, delta- and kappa-opioid receptor knockout mice. *Eur. J. Neurosci.* 18, 2197–2202. doi: 10.1046/j.1460-9568.2003.02951.x

Bhushan, R. G., Sharma, S. K., Xie, Z., Daniels, D. J., and Portoghese, P. S. (2004). A bivalent ligand (KDN-21) reveals spinal delta and kappa opioid receptors are organized as heterodimers that give rise to delta(1) and kappa(2) phenotypes. Selective targeting of delta-kappa heterodimers. *J. Med. Chem.* 47, 2969–2972. doi: 10.1021/jm0342358

Billa, S. K., Xia, Y., and Moron, J. A. (2010). Disruption of morphine-conditioned place preference by a delta2-opioid receptor antagonist: study of mu-opioid and delta-opioid receptor expression at the synapse. *Eur. J. Neurosci.* 32, 625–631. doi: 10.1111/j.1460-9568.2010.07314.x

Brooks, P. C., Lin, J. M., French, D. L., and Quigley, J. P. (1993). Subtractive immunization yields monoclonal antibodies that specifically inhibit metastasis. *J. Cell Biol.* 122, 1351–1359. doi: 10.1083/jcb.122.6.1351

Bushlin, I., Gupta, A., Stockton, S. D. Jr., Miller, L. K., and Devi, L. A. (2012). Dimerization with cannabinoid receptors allosterically modulates delta opioid receptor activity during neuropathic pain. *PLoS ONE* 7:e49789. doi: 10.1371/journal.pone.0049789

Cahill, C. M., Morinville, A., Lee, M. C., Vincent, J. P., Collier, B., and Beaudet, A. (2001). Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta-mediated antinociception. *J. Neurosci.* 21, 7598–7607. Available online at: http://www.jneurosci.org/content/21/19/7598.long

Charles, A. C., Mostovskaya, N., Asas, K., Evans, C. J., Dankovich, M. L., and Hales, T. G. (2003). Coexpression of delta-opioid receptors with micro receptors in GH3 cells changes the functional response to micro agonists from inhibitory to excitatory. *Mol. Pharmacol.* 63, 89–95. doi: 10.1124/mol.63.1.89

Cheng, P. Y., Liu-Chen, L. Y., and Pickel, V. M. (1997). Dual ultrastructural immunocytochemical labeling of mu and delta opioid receptors in the superficial layers of the rat cervical spinal cord. *Brain Res.* 778, 367–380. doi: 10.1016/S0006-8993(97)00891-3

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

Decaillot, F. M., Rozenfeld, R., Gupta, A., and Devi, L. A. (2008). Cell surface targeting of mu-delta opioid receptor heterodimers by RTP4. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16045–16050. doi: 10.1073/pnas.0804106105

Egan, T. M., and North, R. A. (1981). Both mu and delta opiate receptors exist on the same neuron. *Science* 214, 923–924. doi: 10.1126/science.6272393

Erbs, E., Faget, L., Scherrer, G., Matifas, A., Filliol, D., Vonesch, J. L., et al. (2014). A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* doi: 10.1007/s00429-014-0717-9. [Epub ahead of print].

Fan, T., Varghese, G., Nguyen, T., Tse, R., O'Dowd, B. F., and George, S. R. (2005). A role for the distal carboxyl tails in generating the novel pharmacology and G protein activation profile of mu and delta opioid receptor hetero-oligomers. *J. Biol. Chem.* 280, 38478–38488. doi: 10.1074/jbc.M505644200

Ferre, S., Baler, R., Bouvier, M., Caron, M. G., Devi, L. A., Durroux, T., et al. (2009). Building a new conceptual framework for receptor heteromers. *Nat. Chem. Biol.* 5, 131–134. doi: 10.1038/nchembio0309-131

Fujita, W., Gomes, I., and Devi, L. A. (2014a). Heteromers of mu-delta opioid receptors: new pharmacology and novel therapeutic possibilities. *Br. J. Pharmacol.* doi: 10.1111/bph.12663. [Epub ahead of print].

Fujita, W., Gomes, I., Dove, L. S., Prohaska, D., McIntyre, G., and Devi, L. A. (2014b). Molecular characterization of eluxadoline as a potential ligand targeting mu-delta opioid receptor heteromers. *Biochem. Pharmacol.* doi: 10.1016/j.bcp.2014.09.015. [Epub ahead of print].

Gendron, L., Lucido, A. L., Mennicken, F., O'Donnell, D., Vincent, J. P., Stroh, T., et al. (2006). Morphine and pain-related stimuli enhance cell surface availability of somatic delta-opioid receptors in rat dorsal root ganglia. *J. Neurosci.* 26, 953–962. doi: 10.1523/JNEUROSCI.3598-05.2006

George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., et al. (2000). Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J. Biol. Chem.* 275, 26128–26135. doi: 10.1074/jbc.M000345200

Gomes, I., Fujita, W., Chandrakala, M. V., and Devi, L. A. (2013a). Disease-specific heteromerization of G-protein-coupled receptors that target drugs of abuse. *Prog. Mol. Biol. Transl. Sci.* 117, 207–265. doi: 10.1016/B978-0-12-386931-9.00009-X

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

Gomes, I., Gupta, A., and Devi, L. A. (2013b). G-protein-coupled heteromers: regulation in disease. *Methods Enzymol.* 521, 219–238. doi: 10.1016/B978-0-12-391862-8.00012-0

Gomes, I., Gupta, A., Filipovska, J., Szeto, H. H., Pintar, J. E., and Devi, L. A. (2004). A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 101, 5135–5139. doi: 10.1073/pnas.0307601101

Gomes, I., Ijzerman, A. P., Ye, K., Maillet, E. L., and Devi, L. A. (2011). G protein-coupled receptor heteromerization: a role in allosteric modulation of ligand binding. *Mol. Pharmacol.* 79, 1044–1052. doi: 10.1124/mol.110.070847

Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, N., Nagy, V., and Devi, L. A. (2000). Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. *J. Neurosci.* 20:RC110. Available online at: http://www.jneurosci.org/content/20/22/RC110.long

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

Harvey, J. H., Long, D. H., England, P. M., and Whistler, J. L. (2012). Tuned-affinity bivalent ligands for the characterization of opioid receptor heteromers. *ACS Med. Chem. Lett.* 3, 640–644. doi: 10.1021/ml300083p

Hasbi, A., Nguyen, T., Fan, T., Cheng, R., Rashid, A., Alijaniaram, M., et al. (2007). Trafficking of preassembled opioid mu-delta heterooligomer-Gz signaling complexes to the plasma membrane: coregulation by agonists. *Biochemistry* 46, 12997–13009. doi: 10.1021/bi701436w

He, S. Q., Zhang, Z. N., Guan, J. S., Liu, H. R., Zhao, B., Wang, H. B., et al. (2011). Facilitation of mu-opioid receptor activity by preventing delta-opioid receptor-mediated codegradation. *Neuron* 69, 120–131. doi: 10.1016/j.neuron.2010.12.001

Hiller, C., Kuhhorn, J., and Gmeiner, P. (2013). Class A G-protein-coupled receptor (GPCR) dimers and bivalent ligands. *J. Med. Chem.* 56, 6542–6559. doi: 10.1021/jm4004335

Jordan, B. A., and Devi, L. A. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399, 697–700. doi: 10.1038/21441

Kabli, N., Nguyen, T., Balboni, G., O'Dowd, B. F., and George, S. R. (2014). Antidepressant-like and anxiolytic-like effects following activation of the

mu-delta opioid receptor heteromer in the nucleus accumbens. *Mol. Psychiatry* 19, 986–994. doi: 10.1038/mp.2013.115

Kazmi, S. M., and Mishra, R. K. (1987). Comparative pharmacological properties and functional coupling of mu and delta opioid receptor sites in human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol.* 32, 109–118.

Kuner, R., Kohr, G., Grunewald, S., Eisenhardt, G., Bach, A., and Kornau, H. C. (1999). Role of heteromer formation in GABAB receptor function. *Science* 283, 74–77. doi: 10.1126/science.283.5398.74

Lanthier, N., Horsmans, Y., and Leclercq, I. A. (2009). The metabolic syndrome: how it may influence hepatic stellate cell activation and hepatic fibrosis. *Curr. Opin. Clin. Nutr. Metab. Care* 12, 404–411. doi: 10.1097/MCO.0b013e32832c7819

Law, P. Y., Erickson-Herbrandson, L. J., Zha, Q. Q., Solberg, J., Chu, J., Sarre, A., et al. (2005). Heterodimerization of mu- and delta-opioid receptors occurs at the cell surface only and requires receptor-G protein interactions. *J. Biol. Chem.* 280, 11152–11164. doi: 10.1074/jbc.M500171200

Mallat, A., and Lotersztajn, S. (2008). Endocannabinoids and liver disease. I. Endocannabinoids and their receptors in the liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G9–G12. doi: 10.1152/ajpgi.00467.2007

Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383, 819–823. doi: 10.1038/383819a0

Matthes, H. W., Smadja, C., Valverde, O., Vonesch, J. L., Foutz, A. S., Boudinot, E., et al. (1998). Activity of the delta-opioid receptor is partially reduced, whereas activity of the kappa-receptor is maintained in mice lacking the mu-receptor. *J. Neurosci.* 18, 7285–7295.

Milan-Lobo, L., and Whistler, J. L. (2011). Heteromerization of the mu- and delta-opioid receptors produces ligand-biased antagonism and alters mu-receptor trafficking. *J. Pharmacol. Exp. Ther.* 337, 868–875. doi: 10.1124/jpet.111.179093

Morinville, A., Cahill, C. M., Esdaile, M. J., Aibak, H., Collier, B., Kieffer, B. L., et al. (2003). Regulation of delta-opioid receptor trafficking via mu-opioid receptor stimulation: evidence from mu-opioid receptor knock-out mice. *J. Neurosci.* 23, 4888–4898. Available online at: http://www.jneurosci.org/content/23/12/4888.long

Normandin, A., Luccarini, P., Molat, J. L., Gendron, L., and Dallel, R. (2013). Spinal mu and delta opioids inhibit both thermal and mechanical pain in rats. *J. Neurosci.* 33, 11703–11714. doi: 10.1523/JNEUROSCI.1631-13.2013

Palazzi, E., Ceppi, E., Guglielmetti, F., Catozzi, L., Amoroso, D., and Groppetti, A. (1996). Biochemical evidence of functional interaction between mu- and delta-opioid receptors in SK-N-BE neuroblastoma cell line. *J. Neurochem.* 67, 138–144. doi: 10.1046/j.1471-4159.1996.67010138.x

Pin, J. P., Comps-Agrar, L., Maurel, D., Monnier, C., Rives, M. L., Trinquet, E., et al. (2009). G-protein-coupled receptor oligomers: two or more for what? Lessons from mGlu, and GABAB receptors. *J. Physiol.* 587(Pt 22), 5337–5344. doi: 10.1113/jphysiol.2009.179978

Ramsay, D., Kellett, E., McVey, M., Rees, S., and Milligan, G. (2002). Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. *Biochem. J.* 365(Pt 2), 429–440. doi: 10.1042/BJ20020251

Rios, C., Gomes, I., and Devi, L. A. (2006). mu opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. *Br. J. Pharmacol.* 148, 387–395. doi: 10.1038/sj.bjp.0706757

Rives, M. L., Rossillo, M., Liu-Chen, L. Y., and Javitch, J. A. (2012). 6′-Guanidinonaltrindole (6′-GNTI) is a G protein-biased kappa-opioid receptor agonist that inhibits arrestin recruitment. *J. Biol. Chem.* 287, 27050–27054. doi: 10.1074/jbc.C112.387332

Rothman, R. B., and Westfall, T. C. (1982). Morphine allosterically modulates the binding of [3H]leucine enkephalin to a particulate fraction of rat brain. *Mol. Pharmacol.* 21, 538–547.

Rozenfeld, R., Bushlin, I., Gomes, I., Tzavaras, N., Gupta, A., Neves, S., et al. (2012). Receptor heteromerization expands the repertoire of cannabinoid signaling in rodent neurons. *PLoS ONE* 7:e29239. doi: 10.1371/journal.pone.0029239

Rozenfeld, R., and Devi, L. A. (2007). Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. *FASEB J.* 21, 2455–2465. doi: 10.1096/fj.06-7793com

Rozenfeld, R., Gupta, A., Gagnidze, K., Lim, M. P., Gomes, I., Lee-Ramos, D., et al. (2011). AT1R-CBR heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II. *EMBO J.* 30, 2350–2363. doi: 10.1038/emboj.2011.139

Salata, R. A., Malhotra, I. J., Hampson, R. K., Ayers, D. F., Tomich, C. S., and Rottman, F. M. (1992). Application of an immune-tolerizing procedure to generate monoclonal antibodies specific to an alternate protein isoform of bovine growth hormone. *Anal. Biochem.* 207, 142–149. doi: 10.1016/0003-2697(92)90515-9

Sanchez-Blazquez, P., Garcia-Espana, A., and Garzon, J. (1997). Antisense oligodeoxynucleotides to opioid mu and delta receptors reduced morphine dependence in mice: role of delta-2 opioid receptors. *J. Pharmacol. Exp. Ther.* 280, 1423–1431.

Scherrer, G., Imamachi, N., Cao, Y. Q., Contet, C., Mennicken, F., O'Donnell, D., et al. (2009). Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* 137, 1148–1159. doi: 10.1016/j.cell.2009.04.019

Schmid, C. L., Streicher, J. M., Groer, C. E., Munro, T. A., Zhou, L., and Bohn, L. M. (2013). Functional selectivity of 6′-guanidinonaltrindole (6′-GNTI) at kappa-opioid receptors in striatal neurons. *J. Biol. Chem.* 288, 22387–22398. doi: 10.1074/jbc.M113.476234

Shapira, M., Gafni, M., and Sarne, Y. (1998). Independence of, and interactions between, cannabinoid and opioid signal transduction pathways in N18TG2 cells. *Brain Res.* 806, 26–35. doi: 10.1016/S0006-8993(98)00697-0

Siegmund, S. V., and Schwabe, R. F. (2008). Endocannabinoids and liver disease. II. Endocannabinoids in the pathogenesis and treatment of liver fibrosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G357–G362. doi: 10.1152/ajpgi.00456.2007

Sleister, H. M., and Rao, A. G. (2001). Strategies to generate antibodies capable of distinguishing between proteins with >90% amino acid identity. *J. Immunol. Methods* 252, 121–129. doi: 10.1016/S0022-1759(01)00346-5

Sleister, H. M., and Rao, A. G. (2002). Subtractive immunization: a tool for the generation of discriminatory antibodies to proteins of similar sequence. *J. Immunol. Methods* 261, 213–220. doi: 10.1016/S0022-1759(01)00567-1

Szafran, K., Faron-Gorecka, A., Kolasa, M., Kusmider, M., Solich, J., Zurawek, D., et al. (2013). Potential role of G protein-coupled receptor (GPCR) heterodimerization in neuropsychiatric disorders: a focus on depression. *Pharmacol. Rep.* 65, 1498–1505. doi: 10.1016/S1734-1140(13)71510-X

Teixeira-Clerc, F., Julien, B., Grenard, P., Tran Van Nhieu, J., Deveaux, V., Li, L., et al. (2006). CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat. Med.* 12, 671–676. doi: 10.1038/nm1421

Trefzer, U., Rietz, N., Chen, Y., Audring, H., Herberth, G., Siegel, P., et al. (2000). SM5-1: a new monoclonal antibody which is highly sensitive and specific for melanocytic lesions. *Arch. Dermatol. Res.* 292, 583–589. doi: 10.1007/s004030000186

Uriguen, L., Berrendero, F., Ledent, C., Maldonado, R., and Manzanares, J. (2005). Kappa- and delta-opioid receptor functional activities are increased in the caudate putamen of cannabinoid CB1 receptor knockout mice. *Eur. J. Neurosci.* 22, 2106–2110. doi: 10.1111/j.1460-9568.2005.04372.x

Waldhoer, M., Fong, J., Jones, R. M., Lunzer, M. M., Sharma, S. K., Kostenis, E., et al. (2005). A heterodimer-selective agonist shows *in vivo* relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9050–9055. doi: 10.1073/pnas.0501112102

Wang, H. B., Zhao, B., Zhong, Y. Q., Li, K. C., Li, Z. Y., Wang, Q., et al. (2010). Coexpression of delta- and mu-opioid receptors in nociceptive sensory neurons. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13117–13122. doi: 10.1073/pnas.1008382107

Wang, H., and Pickel, V. M. (2001). Preferential cytoplasmic localization of delta-opioid receptors in rat striatal patches: comparison with plasmalemmal mu-opioid receptors. *J. Neurosci.* 21, 3242–3250. Available online at: http://www.jneurosci.org/content/21/9/3242.long

Welch, S. P., and Eads, M. (1999). Synergistic interactions of endogenous opioids and cannabinoid systems. *Brain Res.* 848, 183–190. doi: 10.1016/S0006-8993(99)01908-3

Wessendorf, M. W., and Dooyema, J. (2001). Coexistence of kappa- and delta-opioid receptors in rat spinal cord axons. *Neurosci. Lett.* 298, 151–154. doi: 10.1016/S0304-3940(00)01700-6

White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., et al. (1998). Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* 396, 679–682. doi: 10.1038/25354

Yasumoto, M., Hamabashiri, M., Akiba, J., Ogasawara, S., Naito, Y., Taira, T., et al. (2012). The utility of a novel antibody in the pathological diagnosis of pancreatic acinar cell carcinoma. *J. Clin. Pathol.* 65, 327–332. doi: 10.1136/jclinpath-2011-200442

Yekkirala, A. S., Kalyuzhny, A. E., and Portoghese, P. S. (2013). An immunocytochemical-derived correlate for evaluating the bridging of heteromeric mu-delta opioid protomers by bivalent ligands. *ACS Chem. Biol.* 8, 1412–1416. doi: 10.1021/cb400113d

Yu, V. C., Richards, M. L., and Sadee, W. (1986). A human neuroblastoma cell line expresses mu and delta opioid receptor sites. *J. Biol. Chem.* 261, 1065–1070.

Zhang, X., and Bao, L. (2012). Interaction and regulatory functions of mu- and delta-opioid receptors in nociceptive afferent neurons. *Neurosci. Bull.* 28, 121–130. doi: 10.1007/s12264-012-1206-x

Zhu, Y., King, M. A., Schuller, A. G., Nitsche, J. F., Reidl, M., Elde, R. P., et al. (1999). Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron* 24, 243–252. doi: 10.1016/S0896-6273(00)80836-3

Zieglgansberger, W., French, E. D., Mercuri, N., Pelayo, F., and Williams, J. T. (1982). Multiple opiate receptors on neurons of the mammalian central nervous system. *In vivo* and *in vitro* studies. *Life Sci.* 31, 2343–2346. doi: 10.1016/0024-3205(82)90152-7

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Fluorescent knock-in mice to decipher the physiopathological role of G protein-coupled receptors

Rhian A. Ceredig and Dominique Massotte*

CNRS, Institut des Neurosciences Cellulaires et Intégratives, UPR 3212, Strasbourg, France

Edited by:
Marianthi Papakosta, Pfizer, USA

Reviewed by:
Enrico Sanna, University of Cagliari, Italy
Ioannis N. Charalampopoulos, University of Crete, Greece

****Correspondence:***
Dominique Massotte, CNRS, Institut des Neurosciences Cellulaires et Intégratives, UPR 3212, 5 rue Blaise Pascal, 67084 Strasbourg Cedex 03, France
e-mail: d.massotte@unistra.fr

G protein-coupled receptors (GPCRs) modulate most physiological functions but are also critically involved in numerous pathological states. Approximately a third of marketed drugs target GPCRs, which places this family of receptors in the main arena of pharmacological pre-clinical and clinical research. The complexity of GPCR function demands comprehensive appraisal in native environment to collect in-depth knowledge of receptor physiopathological roles and assess the potential of therapeutic molecules. Identifying neurons expressing endogenous GPCRs is therefore essential to locate them within functional circuits whereas GPCR visualization with subcellular resolution is required to get insight into agonist-induced trafficking. Both remain frequently poorly investigated because direct visualization of endogenous receptors is often hampered by the lack of appropriate tools. Also, monitoring intracellular trafficking requires real-time visualization to gather in-depth knowledge. In this context, knock-in mice expressing a fluorescent protein or a fluorescent version of a GPCR under the control of the endogenous promoter not only help to decipher neuroanatomical circuits but also enable real-time monitoring with subcellular resolution thus providing invaluable information on their trafficking in response to a physiological or a pharmacological challenge. This review will present the animal models and discuss their contribution to the understanding of the physiopathological role of GPCRs. We will also address the drawbacks associated with this methodological approach and browse future directions.

Keywords: G protein-coupled receptors, fluorescent protein, knock-in, mouse model, drug design, biased agonism, receptor trafficking

INTRODUCTION

G protein-coupled-receptors (GPCRs) are proteins composed of seven transmembrane alpha helices with an extracellular *N*-terminus and an intracellular *C*-terminus (Rosenbaum et al., 2009). They represent one of the largest gene families in mammals and humans (Lagerström and Schiöth, 2008, and references therein). GPCRs can respond to various stimuli such as photons, ions, lipids, peptides, odorants, nucleotides, hormones, or neurotransmitters (Congreve et al., 2014). There are five human GPCR families: Rhodopsin, Secretin, Adhesion, Glutamate, and Frizzled/Taste2 with the rhodopsin receptor family being the largest. More than half of the 800 human GPCRs are classified as chemosensory taste or olfactory receptors (Lagerström and Schiöth, 2008; Heng et al., 2013). The remaining human GPCRs -roughly 370- may be involved in pathophysiological processes and are therefore potentially drugable targets. Indeed, metabolic, inflammatory, infectious or neurodegenerative diseases as well as cancer all involve a plethora of GPCRs (Heng et al., 2013). As many GPCRs belong to neuromodulatory systems (van den Pol, 2012), a large number of them are targeted by drugs in the context of nervous system disorders such as pain, drug addiction, anxiety, depression, sleep disorders, and neuroendocrine deregulation (Heng et al., 2013). Altogether, GPCRs represent the targets of about one third of marketed drugs (Overington et al., 2006).

Understanding the roles of GPCRs requires both in depth small scale investigation and overview. Indeed, GPCR expression, function, modulation, and trafficking properties remain difficult to fathom and reflect the complex, highly regulated pathways in which they are involved. The study of GPCRs in physiology and disease therefore requires integrative and functional systems. This is especially true when considering the central nervous system (CNS) where neuronal networks are complex and intermingled. It is therefore of utmost importance to identify and delineate cells that express the GPCR of interest. In the majority of studies, mapping GPCR expression was overcast by poor antibody specificity. The measure of this limitation was only fully appreciated when genetically modified mice which were deficient for the GPCR of interest became available, emphasizing the insufficient specificity of the commonly used antibodies, thereby prompting the search for new technologies to monitor receptor trafficking, decipher activated intracellular signaling cascades or investigate functional outcomes of GPCR activation in integrated systems, and particularly in neuronal networks (Marder, 2012). Among the options which were being explored, fluorescent proteins (FPs) isolated from natural organisms attracted special interest as they appeared to be very promising tools to achieve these goals. There are many advantages to using fluorescent molecular tags; the inherent fluorescence is directly visible, chemically resistant to fixation and can be used in time-course studies in living cells for tracking receptor trafficking events (Kallal and Benovic, 2000).

The Green FP (GFP) was the first FP used in biology. This protein is composed of 238 amino acids (roughly 27 kDa) and was

isolated from the jellyfish *Aequorea victoria* (Shimomura et al., 1962, for review see Tsien, 1998). A mutant form of GFP called enhanced GFP (eGFP) was later generated, with improved quantum yield efficiency and higher solubility, making eGFP a popular reporter molecule (Cormack et al., 1997). The additional mutants that were created offer a large palette of fluorescence, ranging from violet to far red, thus opening new perspectives, including the possibility of co-expressing two or more FP in the same cell, whereby protein interactions could be investigated (Heim and Tsien, 1996). Likewise, this can be achieved by simultaneously expressing eGFP and mcherry, a stable monomeric mutant derived from the red fluorescent protein (RFP) DsRed, the latter was isolated from the coral *Discosoma sp.* (Campbell et al., 2002; Shaner et al., 2004). Additional variants derived from the GFP or DsRed were also generated and possess fast maturation, improved pH stability and photostability (reviewed in Shaner et al., 2007; Subach et al., 2009). The development of these FPs has been paralleled by technological advances in the field of live cell imaging that have brought high quality approaches for analysis of biological processes in a time- and space-dependent manner (Nienhaus and Nienhaus, 2014).

Validation of drug targets and pharmacological mechanisms cannot be achieved without *in vivo* preclinical studies for which mouse models provide a mammalian background and genetic tools of great value (Doyle et al., 2012; Bradley et al., 2014). In order to address GPCR function *in vivo,* tracking endogenous receptors with FPs therefore represents indisputable added value. In the following sections, we will review and comment on the use of FPs that has helped to shed light on endogenous GPCR function *in vivo.*

IN VIVO EXPRESSION OF FP UNDER GPCR PROMOTER

FROM TRANSGENIC TO KNOCK-IN MOUSE LINES

Transgenic mouse lines expressing FPs under the control of promoters for a GPCR or an endogenous peptide were created. A number of reporter mice generated using bacterial artificial chromosomes (BACs) were part of a project called gene expression nervous system atlas (GENSAT) http://www.gensat.org/index.html (Gong et al., 2003) that produced an important set of data relative to gene expression which could be used for deciphering the developmental implications and network dynamics of selected genes of interest. On the account that specific CNS genes are most often expressed in a particular cell population or anatomically defined structure, tandem dimer Tomato (td-Tomato), a RFP, or eGFP-labeling of these cells renders analysis of the anatomical, physiological and biomolecular properties of a chosen subtype of neurons accessible. Overall, transgenic reporter mouse lines have proven to be extremely useful for the precise mapping of GPCR and endogenous ligands expression in the nervous system, and are suitable for analysis of cell populations (Heintz, 2001).

The shortcomings of the transgenic mouse models are, however, manifold (Haruyama et al., 2009). (1) Transgenic expression results in overexpression compared to wild type animals. (2) Low efficiency of transmission to offspring may be caused by mosaic expression of the transgene in founder animals. Indeed, high copy number insertion of transgenes is more vulnerable to epigenetic silencing, which reduces the transgene expression level in successive generations. (3) Expression in unexpected tissues or timeframes may result from transgene insertion in genomic regions containing an endogenous promoter or enhancer. (4) Silencing or ectopic expression can be caused by positional effects. Transgene insertion can take place into transcriptionally inactive regions of the genome, or can be affected by neighboring repressor sites. Transgene insertion being, in essence, random, the possibility of disrupting the normal genome is very high. As a consequence, the erratic nature of the transgene insertion may result in unpredicted and/or detrimental phenotypes and off-target effects. As an example, many groups used BAC transgenic mice expressing eGFP driven by the promoter for either D_1 or D_2 receptors, the dopamine receptor 1 or 2, respectively (Lee et al., 2006; Bertran-Gonzalez et al., 2008; Valjent et al., 2009; Tian et al., 2010; Kramer et al., 2011; Chan et al., 2012). Mainly, work published using these two BAC transgenic mice successfully identified neurons expressing dopamine receptors and delineated dopaminergic connectivity in the CNS. However, Kramer et al. (2011) brought evidence of molecular and behavioral alterations in Drd2-eGFP BAC transgenic mice comprising novel environment hyperactivity, reduced locomotor response to cocaine, and D_2 receptor agonist hypersensitivity. These effects were presumably due to unfortunate insertion of the BAC, which caused receptor overexpression (Kramer et al., 2011).

KNOCK-IN MICE: TOWARD MORE SPECIFIC MODELS

To overcome the limitations associated with the use of transgenic mice, efforts were made to generate knock-in animals in which a FP is introduced at the locus of interest by homologous

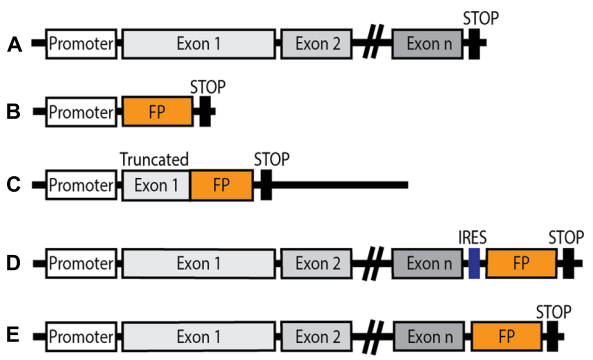

FIGURE 1 | Schematic diagram of genetic constructions of knock-in mice expressing a fluorescent protein (FP) under the control of an endogenous GPCR promoter. (A) Endogenous GPCR gene layout. **(B)** Knock-in FP expressed under the control of the endogenous GPCR promoter: the endogenous GPCR gene is replaced by the FP coding sequence. **(C)** The FP coding sequence is knocked into the truncated gene coding for the native GPCR, resulting in genetic invalidation of the receptor. **(D)** Insertion of an internal ribosomal entry site (IRES) downstream of the endogenous GPCR gene, ahead of the FP coding sequence. Native GPCR expression is maintained, and the FP is also expressed under the control of the endogenous GPCR promoter. **(E)** The FP sequence is inserted in frame in place of the stop codon in the endogenous GPCR gene giving rise to a fluorescent fusion protein in which the FP is fused to the *C*-terminus of the functional GPCR in conditions of native expression.

recombination. Several strategies are used (see **Figure 1**). Models in which an FP is expressed either under the control of an endogenous GPCR promoter are valuable and reliable tools for localization and characterization of cell population which express the GPCR of interest. However, such strategies present a significant drawback since the GPCR is non-functional following partial or total replacement of its coding sequence by the FP coding one. The FP is thus expressed in appropriate cells, but the precise subcellular localization and function of the receptor cannot be examined and the final outcome, in the case of homozygous animals, is the absence of the functional GPCR, equivalent to a knock-out phenotype. This limitation can be circumvented by the introduction of an internal ribosomal entry site (IRES) sequence, whereby expression of the endogenous GPCR is maintained and the chosen FP is expressed under control of the endogenous promoter.

Chemokine receptors

Jung et al. (2000) published the first knock-in mouse in which an FP was expressed under a GPCR promoter. The aim was to track cells which expressed the Fractalkin (CX_3C) chemokine receptor CX_3CR1, using a GFP knock-in strategy by replacing the first 390 bp of exon 2 of the *CX3CR1* gene that encodes the receptor *N*-terminus by a eGFP-coding sequence, enabling direct identification of peripheral blood cells and brain microglia expressing CX_3CR1 (see **Table 1**). In heterozygous animals, CX_3CR1 expression remained detectable because these $CX_3CR1^{+/GFP}$ heterozygous animals possess one allele for fluorescence visualization of cells expressing the GPCR of interest and one allele for expression of the functional receptor. Since CX_3CR1 and its ligand Fractalkin play a role in immunological and inflammatory processes, this model was used to investigate microglia proliferation during early embryonic spinal cord invasion (Rigato et al., 2012) neuron-glia interactions in the context of nerve injury or neuroinflammation (Garcia et al., 2013) and in neurodegenerative diseases such as Alzheimer's disease (Fuhrmann et al., 2010), or Parkinson's disease (Virgone-Carlotta et al., 2013).

A follow-up to this knock-in mouse was published in 2010. In their paper, Saederup et al. (2010) designed a mouse with another single FP, RFP (a DsRed variant) replacing the first 279 base pairs of the open reading frame coding for the chemokine receptor type 2 (CCR2), and crossed the heterozygous $CCR2^{+/RFP}$ and homozygous $CCR2^{RFP/RFP}$ knock-in animals with the previously published $CX_3CR1^{GFP/GFP}$ homozygous animals, in order to obtain heterozygous double knock-in animals $CX_3CR1^{+/GFP}CCR2^{+/RFP}$. The two chemokine receptors are expressed by distinct monocyte populations, therefore the red and green FPs constitute an elegant "two-colored" mouse model which was ideally suited for immunological studies (see **Table 1**). Indeed, because the immune system is constituted of cells that circulate in blood and lymph vessels, mature cells do not constitute a solid organ and are not restricted by connective tissue, therefore immune cell tracking is essential. Both the double heterozygous knock-in animals and the first mouse line ($CX_3CR1^{+/GFP}$ knock-in), were used to study and adequately quantify macrophage and monocyte population dynamics in a model of autoimmune tissue inflammation (experimental autoimmune encephalomyelitis), which recapitulates an animal model of multiple sclerosis (MS). In a subsequent study, the same group unveiled myeloid lineage and microglial chemokine receptor changes at embryonic stages 8.5–13.5, monitored CNS colonization by cells of interest, during development and in an MS model using adult mice (Mizutani et al., 2012). The knock-in models thus yielded exciting and fundamental results relative to the identification of cells expressing the designated GPCRs, and a fine description of cellular population changes in various disease paradigms.

Oxytocin receptors

Yoshida et al. (2009) engineered a mouse line in which a 5′ fragment of exon 3 of the oxytocin receptor (*OTR*) gene was replaced by a sequence coding for Venus FP, a yellow FP variant (Nagai et al., 2002). The recombined allele did not encode functional OTR but heterozygous animals retained radiolabelled oxytocin binding patterns through the intact allele, while enabling direct visualization of Venus in oxytocin expressing cells (Yoshida et al., 2009). Immunohistochemical analysis of brain sections from these animals revealed that there was a high expression of Venus (hence OTR) in monoaminergic areas of the brain in agreement with *in situ* hybridization (ISH) studies (Vaccari et al., 1998). However, the approach provided more sensitive detection of OTR expression by identifying additional areas and cells expressing Venus fluorescence among which serotoninergic ones. This study was the first to show evidence for interaction between oxytonergic and serotonergic systems in a pathway, which modulates anxiety. In a following study, these knock-in mice were used to map OTR expression in the spinal cord; shedding light on the modulatory role of oxytocinergic networks involved in spinal cord functions, such as nociception (Wrobel et al., 2011).

Taste receptors

Sensing of the chemical categories which are responsible for sweet, sour or umami taste is specifically encoded by GPCRs expressed on primary taste neurons (Liman et al., 2014). The taste receptor family 1 (Tas1r) belongs to class C GPCRs and function as obligatory heteromers, meaning that two GPCRs of different subtypes are associated and interact to form a functional entity. The taste receptor family 2 (Tas2r), on the other hand, are currently classified among class A GPCRs (Alexander, 2013).

In order to study the distribution of taste receptors in the mouse gustatory tissue, Voigt and collaborators engineered two knock-in mouse lines which they subsequently crossed in order to obtain double knock-in animals in which the open reading frame encoding the receptor was replaced by the sequence coding for the mcherry or humanized Renilla (hr)GFP under the control of Tas1r1 (umami taste receptor) or Tas2r131 (bitter taste receptor) promoters, respectively (Voigt et al., 2012). This approach permitted identification of cells expressing mcherry under the control of the Tas1r1 promoter in the lingual papillae, soft palate, fungiform and foliate papillae, confirming previous findings (Hoon et al., 1999; Stone et al., 2007) but also in extra-gustatory tissues (lung epithelium, testis, thymus) which had not been investigated

Table 1 | Knock-in mice expressing fluorescent proteins under the control of G protein-coupled receptor (GPCR) endogenous promoters.

Targeted GPCR	Fluorescent protein	Identified cell type	Model	Therapeutic potential	Reference
Insertion of FP sequence at the GPCR gene locus					
Chemokine CX_3CR1	eGFP	Immune cells	Peritonitis Nerve injury	Neuroinflammation Neurodegenerative diseases	Jung et al. (2000)
		Microglia		Population dynamics in embryonic development	Rigato et al. (2012)
		Microglia	Neurodegeneration	Alzheimer	Fuhrmann et al. (2010)
		Microglia	Neuroinflammation	Parkinson	Virgone-Carlotta et al. (2013)
Chemokine CCR2	RFP	Immune cells	Experimental autoimmune encephalomyelitis	Neuroinflammation Neurodegenerative diseases	Saederup et al. (2010)
Chemokine CX_3CR1 x Chemokine CCR2	eGFP RFP	Immune cells	Experimental autoimmune encephalomyelitis	Neuroinflammation Neurodegenerative diseases	Saederup et al. (2010)
		Myeloid cells Microglia	Experimental autoimmune encephalomyelitis	Population dynamics in embryonic development	Mizutani et al. (2012)
Oxytocin	Venus	Brain distribution	Anxiety related	Psychiatric disorders	Yoshimura et al. (2001)
		Spinal cord distribution		Nociception/pain	Wrobel et al. (2011)
Mrgprd	eGFPf	Sensory projections to epidermis		Nociception/pain	Zylka et al. (2005)
		Sensory projections to tooth pulp		Nociception/dental pain	Chung et al. (2012)
Taste TasR1	mcherry	Taste cells in taste buds and peripheral tissue		–	Voigt et al. (2012)
Taste Tas2R131	hrGFP	Taste cells in taste buds and peripheral tissue		–	Voigt et al. (2012)
Taste TasR1 x Taste Tas2R131	mcherry hrGFP	Taste cells in taste buds and peripheral tissue		–	Voigt et al. (2012)
GPCR-IRES-FP expression					
Mas-related Mrgprd	eGFPf	Sensory projections to epidermis		Nociception/pain	Zylka et al. (2005)
Cannabinoid CB1	Td-Tomato	Neurons	Chronic cocaine injection	Drug addiction	Winters et al. (2012)

before (Voigt et al., 2012). Expression of hrGFP under the control of Tas2r131 promoter was in accordance with previously findings describing taste receptor distributions (Behrens et al., 2007), showing abundant hrGFP expression in taste buds of the posterior tongue, vallate palate and foliate palate. In addition, it uncovered, for the first time, expression restricted to only half of the bitter sensor cells (Voigt et al., 2012). Double knock-in animals lacked both taste receptors, but expressed FPs in the targeted cells [verified by reverse transcription polymerase chain reaction (RT-PCR), ISH and immunohistochemistry]. This genetic labeling technique served for population distribution studies, which was until then unachievable, given the fact that Tasr expression is sparse in cells, and that the available antibodies lack specificity. The double knock-in animals yielded a valuable and detailed cartography of taste receptors in the mouse, and revealed that distinct chemosensory cell populations mediate specific and non-overlapping taste qualities.

Mas-related-G-protein coupled receptors

Mas-related-G-protein coupled receptor member D (Mrgprd) belongs to a GPCR family of approximately 50 members, related to *Mas1* (oncogene-like MAS), called Mrgs. Mrgs are suspected to

be involved in development, regulation and function of nociceptive neurons or nociceptors (Dong et al., 2001) and are expressed in a subset of nociceptors, which are small diameter primary sensory neurons in dorsal root ganglia (DRG) directly involved in processing nociceptive stimuli, especially itch (Liu et al., 2012).

Zylka et al. (2005) observed similar expression patterns of the eGFPf (a farnesylated form that anchors the FP to the cytoplasmic leaflet of the lipid bilayer) in nociceptors, and projections of the sensory neurons to the epidermis using knock-in mice in which the open reading frame coding for Mrgprd is replaced by the sequence encoding the eGFPf or knock-in animals in which the eGFPf sequence is inserted behind an IRES element downstream of the mouse Mrgprd gene (Zylka et al., 2005). This demonstrates that both strategies can be equally used for cellular mapping. In addition, similar projection profiles in the epidermis validated the eGFPf knock-in mouse for axonal tracing by comparison with the widely used human placental alkaline phosphatase tethered to the extracellular surface of the plasma membrane by a glycophosphatidylinositol linkage.

In a later study, the knock-in mouse model expressing eGFPf at the Mrgprd locus was used to identify non-peptidic nociceptive neurons of trigeminal ganglia innervating tooth pulp (Chung et al., 2012). This opens future application of this model to study the role and function of the targeted GPCR in dental pain.

Cannabinoid receptors

The endocannabinoid system plays roles in memory, appetite, stress and immune processes, as well as motivation and emotional responses and modulates the effects of some drugs of abuse (Pertwee, 2006; Tan et al., 2014). In the nucleus accumbens (NAc), a brain structure which has a crucial role in reward processing and a decisive influence on emotional and motivational responses, cannabinoid receptor 1 (CB1) expression is limited but nevertheless essential for cocaine-induced reward in mice (Marsicano and Lutz, 1999). In order to further identify and delineate the cellular and electrophysiological properties of CB1 receptor expressing cells in the NAc, Winters et al. (2012) designed a knock-in mouse line in which an IRES element ensures expression of both CB1 receptors and td-Tomato under the control of the CB1 promoter. Importantly, this mouse line still expressed functional CB1 receptors. Neurons expressing CB1 receptors were readily visualized in the NAc and their distribution was in accordance with previous data on CB1 receptor localization using ISH or immunohistochemistry (Mailleux and Vanderhaeghen, 1992; Tsou et al., 1997). This mouse line enabled to identify of cells and to explicitly demonstrate biochemical and signaling properties of a particular neuronal population of fast-spiking interneurons in the NAc which impacts on the NAc projections and connectivity. Results also revealed functional impact of cocaine on these neurons (Winters et al., 2012).

GPCR-FP FUSION FOR *IN VIVO* FUNCTIONAL AND MAPPING STUDIES

INITIAL VALIDATION OF GPCR-FP FUSIONS IN HETEROLOGOUS SYSTEMS

Fusions between a GPCR and an FP as tools to monitor the GPCR subcellular localization and trafficking were first studied in heterologous systems. Two fusion options were considered: either the FP at the *N*-terminus or at the *C*-terminus. A vast majority of GPCRs do not have cleavable *N*-terminus signal sequences that target them to the plasma membrane. Introduction of a foreign sequence ahead of their *N*-terminus has been shown to disrupt surface addressing, and correct membrane targeting and insertion therefore requires introduction of an additional foreign signal sequence in front of the fusion construct (McDonald et al., 2007). If proper cell surface expression is indeed restored, introduction of such a signal sequence nonetheless strongly impacts on the relative ratio between surface expression and intracellular distribution by substantially increasing the amount of protein at the cell surface (Dunham and Hall, 2009, and references therein). Hence, such fusion proteins are not well suited to mimic the responses of endogenous GPCRs to agonist stimulation and were not used for *in vivo* studies.

Concerns have also been raised regarding in frame insertion of the FP at the *C*-terminus of the GPCR by substitution of the stop codon. The presence of a 27 kDa beta barrel at the intracellular extremity of the GPCR could indeed interfere with intracellular scaffold partners and modify signaling or internalization processes thus defeating the object when studying GPCR signaling properties. However, many studies performed in mammalian cells on a large number of GPCRs strongly suggest that addition of GFP at the *C*-terminus does not significantly affect subcellular distribution in the basal/unstimulated state, ligand binding or agonist-induced receptor phosphorylation and internalization, (for review Kallal and Benovic, 2000). McLean and Milligan (2000) expressed β_1- and β_2-adrenergic receptors fused to a *C*-terminal eGFP mutant in human embryonic kidney (HEK 293) cells. These authors concluded that the presence of the eGFP did not influence ligand binding but decreased the agonist-induced internalization kinetics without affecting the intracellular fate of the receptor. Trafficking of the fusion protein was qualitatively maintained, but was quantitatively slightly modified compared to native proteins. This study therefore supports the use of such fusions to monitor endogenous receptor subcellular localization. Similarly, the genetic construction encoding the delta opioid (DOP) receptor fused with eGFP protein at the *C*-terminus was expressed in transfected HEK 293 cells, and the fusion did not alter opioid ligand binding affinity or signaling (Scherrer et al., 2006). This construct was later successfully used to express a functional DOP-eGFP fusion in mice by knocking the modified sequence into the endogenous DOP receptor locus (Scherrer et al., 2006, see below).

In some cases, however, FP fusion at the GPCR C-terminus had deleterious effects. Defective targeting to the cell surface was reported for the melanocortin 2 receptor fused to the GFP in HEK 293 cells (Roy et al., 2007) and no recycling was observed for the muscarinic M4 receptor fused to a *C*-terminal red variant of GFP in neuroblastoma/glioma hybrid cells (NG108-15 cells; Madziva and Edwardson, 2001). In both cases, impairment was more likely to be due to gross overexpression rather than fusion of the FP to the *C*-terminus. High levels of expression of a GPCR in a non-native environment can indeed artificially elicit properties and interactions that would not occur *in vivo*. Moreover, cell lines

used for heterologous expression may provide different intracellular machinery for complex protein folding or post-translational modifications compared to naturally producing cells. This represents an additional limitation to the study of GPCR functions and prompted to develop *in vivo* approaches.

FROM TRANSGENIC TO KNOCK-IN MOUSE LINES

Papay et al. (2004) reported a transgenic mouse model of a fluorescent tagged GPCR. The construct they described was composed of a 3.4 kb fragment of the mouse endogenous α1B adrenoceptor promoter, the human α1B adrenoceptor coding sequence with *C*-terminal fusion eGFP sequence. The resulting founder lines were characterized, and high expression levels were observed in all tissues that naturally express α1B adrenoceptors by fluorescence microscopy. Binding affinities and internalization profiles were similar to those of endogenous receptors. With this study, Papay et al. (2004) reported the first mouse model expressing a GPCR tagged with eGFP as a transgenic approach for *in vivo* GPCR localization studies. The generation of knock-in animals represented a further improvement by enabling for the first time to track down endogenous receptors, which has opened a new era for pharmacological research.

KNOCK-IN HUMANIZED RHODOPSIN FUSED WITH A FLUORESCENT PROTEIN (hRho-eGFP)

Chan et al. (2004) mouse lines expressing human rhodopsin-eGFP were engineered using different knock-in strategies. All mouse lines showed decreased expression levels of the fusion protein relative to the endogenous mouse rhodopsin. Comparing the different homozygote mouse lines enabled to correlate the decrease in human rhodopsin–eGFP expression to the increased rate of retinal degeneration, providing a model of human diseases. More recently, using a human mutant rhodopsin allele [proline-to-histidine change at codon 23 (P23H) rhodopsin] which induces mislocalization and degradation of the human protein, the research group generated a knock-in mouse line which modeled a common cause of autosomal dominant retinitis pigmentosa (Price et al., 2011). In humans, mutation Q344X is responsible for a severe early onset form of retinitis pigmentosa. The Q344X mutation introduces a premature stop codon that prevents GFP expression in the human rhodopsin-eGFP construct. Knock-in animals expressing this mutant construct were used to monitor eGFP fluorescence recovery as an index of the frequency and timing of somatic mutations in the rhodopsin gene (Sandoval et al., 2014). These mouse lines provided substantial and valuable data concerning rhodopsin distribution in the retina (for references, also see **Table 2**), and were advantageously implemented for non-invasive measurement by illuminating the mouse retina in live animals with blue light (Wensel et al., 2005). They will provide a means to assess the impact of future gene-targeting treatment strategies for retinal degeneration (Gross et al., 2006; Sandoval et al., 2014).

OPIOID RECEPTORS

The opioid system modulates a wide range of physiological states, of which nociception, reward, mood, stress, neuroendocrine physiology, immunity, autonomic functions such as gastro-intestinal transit (Kieffer and Evans, 2009; Walwyn et al., 2010; Chu Sin Chung and Kieffer, 2013; Lutz and Kieffer, 2013). Opioid receptors are members of the class A GPCR family, mu (MOP), delta (DOP) and kappa (KOP) opioid receptors couple to inhibitory heterotrimeric inhibitory G protein, and have high sequence homology (Akil et al., 1998).

Mapping of receptor expression with neuronal resolution

Scherrer et al. (2006) generated a DOP-eGFP knock-in mouse line by homologous recombination in which the coding sequence for the DOP receptor fused to its *C*-terminus to the eGFP was inserted at the *Oprd1* locus.

Delta opioid-eGFP knock-in mice proved very helpful to map DOP receptors in the nervous system and remedy the lack of highly specific antibodies (see **Table 2**). In the peripheral nervous system, DOP-eGFP receptors were detected in cell bodies of specific peripheral sensory neuronal populations which process sensory stimuli, namely mostly in large diameter myelinated (Neurofilament 200 positive), and in small diameter unmyelinated non-peptidergic (Isolectin B4 positive) neurons (Scherrer et al., 2009; Bardoni et al., 2014). The expression pattern of DOP-eGFP receptors was also reported in mechanosensory organs in the skin (Bardoni et al., 2014). Another study focused on the distribution of DOP-eGFP in enteric neurons with DOP-eGFP expression mainly in secretomotor neurons of the submucosal plexus of the digestive tract (Poole et al., 2011). The observed distribution reflects functional roles of DOP receptors in inhibition of intestinal motility and absorption.

In the CNS, DOP-eGFP mapping was performed in the brain and spinal cord (Erbs et al., 2014). Detailed DOP-eGFP expression was also reported in the hippocampus, where functional DOP-eGFP was found to be mainly expressed in GABAergic interneurons, mostly parvalbumin-positive ones (Erbs et al., 2012; Rezai et al., 2013). The DOP-eGFP knock-in mice also enabled to resolve the debate concerning the presence of DOP receptors in principal cells. The absence of colocalization with calbindin (Erbs et al., 2012) and presynaptic expression restricted to afferents to glutamatergic principal cells established that no functional DOP receptors are expressed under basal conditions in those cells (Rezai et al., 2012). These results are consistent with a modulation of principal cell activity in the hippocampus by DOP receptors, and therefore an impact of the receptors in learning and memory.

More recently, a knock-in mouse line expressing a MOP receptor fused with a RFP at the C-terminus, MOP-mcherry, was generated by Erbs et al. (2014). At the *Oprm1* locus, mcherry cDNA was introduced into exon 4 of the MOP gene in frame and 5′ from the stop codon. This FP is monomeric and highly photostable, and the strong red signal of MOP-mcherry fusion protein enabled direct identification of neurons expressing MOP in the nervous system (Erbs et al., 2014). The authors compiled the DOP-eGFP and MOP-mcherry distributions in a neuroanatomical atlas available at http://mordor.ics-mci.fr

Several studies in heterologous systems or cell culture had suggested that MOP and DOP receptors may interact to form heteromers (van Rijn et al., 2010; Rozenfeld et al., 2012; Stockton and Devi, 2012) but their existence *in vivo* remains debated. Co-immunoprecipitation studies performed on tissue from spinal

Table 2 | Knock-in mice expressing GPCR-fluorescent protein fusions.

Fusion construct	Biological readout	Reference
hRhodopsin-eGFP	Retinal degeneration kinetics (model of recessive retinitis pigmentosa)	Chan et al. (2004)
	Distribution, membrane structure, and trafficking of rhodopsin (model of retinitis pigmentosa)	Gross et al. (2006)
P23H-hRhodopsin-eGFP	Degeneration and degradation kinetics of rhodopsin (model of common cause of autosomal dominant retinitis pigmentosa)	Price et al. (2011)
Q344X-hRhodopsin-eGFP	DNA repair in photoreceptors cells during retinogenesis (degeneration and degradation kinetics in a model of severe early-onset of retinitis pigmentosa)	Sandoval et al. (2014)
DOP-eGFP	Receptor distribution:	
	– central nervous system	Scherrer et al. (2006, 2009), Erbs et al. (2014)
	– hippocampus	Erbs et al. (2012), Rezai et al. (2012, 2013)
	– dorsal root ganglia	Scherrer et al. (2009), Bardoni et al. (2014)
	– mechanosensors in the skin	Bardoni et al. (2014)
	– myenteric plexus	Poole et al. (2011)
	Correlation between behavioral desensitization and receptor internalization	Scherrer et al. (2006), Pradhan et al. (2009, 2010)
	Biased agonism at the receptor	
	– pharmacological drugs	Pradhan et al. (2009, 2010)
	– endogenous opioid peptides	Faget et al. (2012)
	Behaviorally controlled receptor subcellular distribution	Faget et al. (2012), Bertran-Gonzalez et al. (2013), Laurent et al. (2014)
MOP-mcherry	Receptor distribution in the central and peripheral nervous systems	Erbs et al. (2014)
MOP-mcherry x DOP-eGFP	MOP-DOP neuronal co-expression in the brain	Erbs et al. (2014)

cord or DRGs also hinted at close physical proximity between the two receptors in these areas (Gomes et al., 2004; Xie et al., 2009). In addition, MOP-DOP heteromers had been detected in some brain areas using specific antibodies (Gupta et al., 2010). Recently, extensive mapping of MOP-DOP neuronal colocalization using double knock-in mice co-expressing DOP-eGFP and MOP-mcherry provided sound data to investigate MOP-DOP physical proximity and functional interactions. In the hippocampus, a brain area where the two receptors are highly co-expressed, co-immunoprecipitation experiments using antibodies raised against the FPs indeed confirmed physical proximity (Erbs et al., 2014). These animals will now be useful to address MOP-DOP specificities in ligand binding, signaling and trafficking as well as functional output and to investigate the potential of MOP-DOP heteromers as a novel therapeutic target.

In vivo trafficking, desensitization and behavioral output

The DOP-eGFP mouse line is the first example of the use of a knock-in line to study GPCR functions *in vivo* (Scherrer et al., 2006). DOP agonist-induced internalization was observed *in vivo* upon activation by the alkaloid [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5- dimethyl-1-piperazinyl)-3-meth oxybenzyl]-N,*N*-diethylbenzamide] (SNC-80) and the endogenous peptide Met-enkephalin (Scherrer et al., 2006). The two agonists induce receptor internalization in heterologous systems with receptor phosphorylation as the first step of a cascade of events leading to termination of G protein dependent signaling, receptor removal from the cell membrane and trafficking to intracellular compartments (Ferguson et al., 1996; von Zastrow and Williams, 2012; Walther and Ferguson, 2013). DOP-eGFP mice revealed that these agonists also induce receptor phosphorylation, internalization via clathrin coated pits *in vivo* and degradation in the lysosomal compartment in the brain (Scherrer et al., 2006; Pradhan et al., 2009; Faget et al., 2012) and peripheral nervous system in the myenteric plexus (Poole et al., 2011) and DRGs (Scherrer et al., 2009). Moreover, these animals prove to be instrumental to decipher molecular mechanisms underlying receptor desensitization leading to a loss of responsiveness of the receptor upon stimulation by an agonist. Scherrer et al. (2006) were indeed able, for the first time, to establish the correlation between receptor

trafficking *in vivo* and the behavioral response: namely that the receptor internalization induced by acute administration of the agonist SNC-80 was responsible for the observed locomotor desensitization. This paper was followed by additional studies exploring the consequences of receptor pharmacological stimulation in more detail, in particular the concept of biased agonism.

G protein-coupled receptors have a flexible and highly dynamic nature (Moreira, 2014) which enables a given ligand to show functional selectivity, that is, preferential activation of signal transduction pathways, otherwise termed biased agonism (Ostrom and Insel, 2004; Giguere et al., 2014; Kenakin, 2014). DOP-eGFP mice offer the possibility of addressing this concept *in vivo* and to link it to a functional response. DOP-eGFP mice were used to analyze the properties of two DOP receptor agonists possessing similar signaling potencies and efficacies but with different internalization profiles (Pradhan et al., 2009). SNC-80 and N,*N*-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide (AR-M100390), with high and low internalization properties respectively, were systemically administered to mice, and receptor trafficking was correlated to induced anti-allodynic effect in the context of inflammatory pain (Pradhan et al., 2009). As expected, acute SNC-80 administration resulted in receptor phosphorylation, decreased G protein coupling and receptor degradation in the lysosomal compartment, leading to desensitization with loss of anti-allodynic properties. On the other hand, acute injection of AR-M100390 did not result in receptor phosphorylation, did not reduce G protein coupling, did not induce receptor internalization or desensitization but retained analgesic properties. This study demonstrated that DOP receptor localization determines its function *in vivo* and highlights the importance of receptor tracking in order to extricate behavioral and cellular correlates of specific agonist properties (Pradhan et al., 2009).

In a following study, DOP-eGFP mice were used to assess the physiological impact of distinct signaling pathway recruitment and/or adaptive responses upon chronic administration of two DOP receptor agonists (Pradhan et al., 2010). Chronic administration of SNC-80, which has high internalization properties, led to marked receptor downregulation and degradation in SNC-80-tolerant animals. Receptor internalization prevented any additional activation through physical disappearance from the cell surface leading to general desensitization, as assessed by thermal and mechanical analgesia, locomotor activity and anxiety-related behavior. On the other hand, chronic administration of AR-M100390, with weak internalization properties, did not cause changes in DOP-eGFP localization and induced tolerance restricted to analgesia, with no effect on locomotor activity or anxiolytic responses. These data show that a selective internalization-independent tolerance was elicited and suggest the occurrence of adaptative mechanisms that are network dependent. These findings reinforce the importance of understanding agonist specific signaling underlying biased agonism and tolerance. Considering that drug design has focused on offering orthosteric or allosteric modulators of GPCRs (Bradley et al., 2014), research groups need to explore the downstream signaling cascades of these drugs in more detail in order to understand and target the molecular events which underlie their efficacy. This is an essential progress for the understanding of drug action and opens new possibilities for drug design.

Direct visualization of the receptor also permitted to decipher the functional role of delta receptors in neuronal networks and to understand the complex relation between behavior and receptor subcellular distribution. Of particular interest is the observation that DOP subcellular distribution is modified in two brain areas involved in the processing of information associated with emotional value or predicted outcome. The CA1 area of the hippocampus is known to operate as a coincidence detector that reflects association of the context with strong emotional stimuli of positive or aversive value (Duncan et al., 2012). Accordingly, increased c-Fos immunoreactivity revealed activation of this region in a drug-context association paradigm, and DOP-eGFP internalization in this area therefore suggested a modulatory role of the receptor in behavioral responses linked to context-induced withdrawal (Faget et al., 2012). Along the same line, persistent increase of DOP-eGFP expression at the cell surface of cholinergic interneurons was induced by conditioned training in the NAc shell, which is involved in decision making and predictive reward evaluation upon pavlovian conditioning (Bertran-Gonzalez et al., 2013; Laurent et al., 2014).

Finally, the knock-in strategy revealed that the DOP-eGFP internalization profile in response to endogenous opioid release is distinct from what is observed upon pharmacological stimulation (Faget et al., 2012). Indeed, only part of the receptor population present at the cell surface underwent internalization under physiological conditions. This observation further highlights the need to take into account the extent of changes that drug administration induces in receptor cellular distribution.

Methodological improvements

Interestingly, DOP-eGFP knock in mice also bring useful technical insight. During the process of acute brain slice preparation for electrophysiological recordings, DOP-eGFP revealed spontaneous receptor internalization (Rezai et al., 2013). This event was likely due to high glutamatergic activity in the hippocampus upon slicing that leads to exitoxicity. Direct visualization of the receptor therefore revealed a bias associated with previously unrecognized receptor trafficking that can now be addressed by initiating optimization of slice preparation conditions for electrophysiological recording (Rezai et al., 2013). This observation may be of particular relevance when addressing cellular responses elicited by drug application.

CONCERNS ABOUT THE USE *IN VIVO* OF GPCR-FP FUSIONS FOR FUNCTIONAL STUDIES

Despite the undeniably wide advances which have been and will be brought by genetically engineered mice encoding fluorescent endogenous GPCRs, concerns were raised regarding the inherent consequences of genetic manipulation. The possibility that the observed localization does not entirely reflect the wild type receptor distribution appears irrelevant since both MOP-mcherry and DOP-eGFP receptor distributions in the brain are in full agreement with reports in mice and rats based on ligand binding (Kitchen et al., 1997; Slowe et al., 1999; Goody et al., 2002; Lesscher et al., 2003), GTPγS incorporation (Tempel and Zukin, 1987; Pradhan

and Clarke, 2005) or mRNA detection [George et al., 1994; Mansour et al., 1995; Cahill et al., 2001; for a review see (LeMerrer et al., 2009)]. Also, in a more detailed study, DOP-eGFP expression in the hippocampus, mainly in parvalbumin-positive GABAergic interneurons (Erbs et al., 2012), was corroborated by ISH studies on DOP receptors (Stumm et al., 2004).

In the peripheral nervous system, despite previous reports suggesting SP-dependent trafficking of DOP receptors to the cell membrane (Guan et al., 2005), Scherrer et al. (2009) reported that DOP-eGFP almost never co-localized with substance P (SP) in peripheral sensory neurons (Scherrer et al., 2009), a finding that was debated by others (Wang et al., 2010). A more recent study addressed this discrepancy by comparing DOP-eGFP cellular distribution to that of the native DOP receptor using an ultrasensitive and specific ISH technique, which can detect single mRNA molecules (Bardoni et al., 2014). Patterns of DOP-eGFP distribution and *Oprd1* mRNA expression were found to be very similar and detectable in the same neuronal populations, namely mostly in large diameter myelinated cells (Neurofilament 200 positive), and in small diameter unmyelinated non-peptidergic neurons (isolectin B4 positive; Bardoni et al., 2014). These data unambiguously confirm that the expression profile of the fluorescent constructs mimics the endogenous one and that fluorescent knock-in mice can be reliably used for mapping receptors in the central and peripheral nervous system.

Regarding functional aspects, there has been no evidence so far of any overt phenotypical or behavioral differences between the DOP receptor knock-in strain and wild type animals (Scherrer et al., 2006; Pradhan et al., 2009, 2010; Rezai et al., 2013), despite a twofold increase in mRNA and protein levels as well as increased G protein activation compared to wild type animals (Scherrer et al., 2006). However, the possibility that the subcellular distribution of the fluorescent fusion does not recapitulate that of the native untagged receptor is still debated. Indeed, high surface expression of DOP-eGFP is observed under basal conditions in several brain regions, particularly in the hippocampus (Scherrer et al., 2009; Erbs et al., 2012, 2014; Faget et al., 2012). This does not correlate with previous studies on wild type receptors using electron microscopy or fluorescent ligands that indicated a predominant intracellular localization under basal conditions and surface recruitment upon chronic morphine or chronic pain condition (Cahill et al., 2001; Morinville et al., 2004; Gendron et al., 2006; for review see Cahill et al., 2007; Gendron et al., 2014). Surface expression of DOP-eGFP, however, varies across CNS regions and neuronal type whereas high fluorescence is always visible within the cytoplasm (Erbs et al., 2014). Accordingly, high surface expression appears to be restricted to some neuronal types such as GABAergic interneurons in the hippocampus or large proprioceptors in DRGs (Scherrer et al., 2006; Erbs et al., 2014). In many areas where DOP receptors are highly expressed such as the striatum, the basal ganglia, the amygdala or the spinal cord, DOP-eGFP is not readily detected at the cell surface (Erbs et al., 2014) suggesting that DOP-eGFP intracellular localization is predominant in those neurons. Importantly, surface expression of DOP-eGFP can be augmented under physiological stimulation (Bertran-Gonzalez et al., 2013; Laurent et al., 2014; see above) or increased upon chronic morphine treatment as previously reported for wild type receptors (Erbs et al., unpublished data), strongly supporting that the fused FP does not impact on the native subcellular distribution of the receptor and that the latter can be modulated according to the physiological state or modified upon pharmacological treatment.

In the case of MOP-mcherry knock-in mice, the red fluorescent signal is stronger inside the cell than at the plasma membrane (Erbs et al., 2014). This distribution reflects actual receptor intracellular distribution, as evidenced by comparison with MOP-specific immunohistochemistry in heterozygous mice, which confirms that the fusion protein does not cause defective receptor localization or surface trafficking (Erbs et al., 2014). Importantly, MOP-mcherry retained unchanged receptor density as well as [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) binding and efficacy and agonist-induced internalization compared to MOP. Moreover, behavioral effects of morphine in knock-in mice were similar to wild type animals: acute and chronic thermal analgesia, physical dependence, sensitization and rewarding properties revealed no significant differences with wild type animals (Erbs et al., 2014). These data suggest that predominant intracellular localization of MOP-mcherry receptors with low expression at the cell surface indeed reflect endogenous wild type receptor subcellular distribution under basal conditions, as observed in enteric neurons (Poole et al., 2011). In addition, internalization kinetics of MOP-mcherry upon activation by the agonist DAMGO in hippocampal primary neuronal cultures (Erbs et al., 2014) were similar to those reported for DAMGO promoted internalization of endogenous wild type receptors in the rat spinal cord (Trafton et al., 2000) and in organotypic cultures of guinea pig ileum (Minnis et al., 2003) or to Fluoro-dermorphin-induced sequestration in rat cortical primary neurons (Lee et al., 2002). This supports once again the use of fluorescent knock-in mice to study endogenous receptor trafficking. Of note, DAMGO promotes Flag-MOP receptor internalization with similar kinetics in transfected striatal primary neurons (Haberstock-Debic et al., 2005), in adenovirus infected primary cultures from DRG (Walwyn et al., 2006) or in neurons of the locus coeruleus in brain slices from transgenic FLAG-MOP receptor mice (Arttamangkul and Quillinan, 2008).

CONCLUSIONS AND IMPACT FOR DRUG DESIGN

Fluorescent knock-in mice represent a substantial technical improvement in basic science. Precise identification and localization of the neurons expressing the GPCR of interest and reliable monitoring of receptor subcellular localization are both essential in understanding the physiopathological roles of endogenous GPCRs. This was greatly anticipated, given the difficulties encountered by many on the grounds of poor specificity of the available antibodies for GPCR targeting. The main surprising finding is maybe that the presence of the FP at the *C*-terminus of the GPCR does not significantly alter the behavioral output: this observation fully validates the technology. However, fluorescent knock-in animals available to date target a handful of class A GPCRs only. The potency of the model being now clearly established, one would expect rapid expansion to

other receptors, in particular those with critical roles in human pathologies. Forefront candidates include class C $GABA_B$ and metabotropic glutamate receptors, both of which are involved in a wide range of neurological disorders such as schizophrenia, neuropathic pain, cerebral ischemia, mood disorders and substance abuse (Benes and Berretta, 2001; Delille et al., 2013; Kumar et al., 2013). Fluorescent knock-in animals would enable to revisit heterodimerization mechanisms, membrane targeting and cellular distribution patterns of these obligatory heterodimers *in vivo*. Furthermore, the relation between multimer scaffold composition, in particular $GABA_B$ auxiliary subunits, and neuronal or synaptic functions could also be readily examined to refine our current understanding of the variations in pharmacological and functional responses mediated by native receptors (Gassmann and Bettler, 2012).

The knock-in mice bearing GPCR-FP fusions already contributed to understanding the fundamental concepts of distinct signaling or regulatory responses recruited by different agonists of the same GPCR. These essential aspects of biased agonism are a growing central concern in drug discovery in the hope of developing strategies that ally high efficacy with low or no side effects. In addition, GPCR-FP fusions could bring considerable knowledge regarding functional aspects of receptor activity and internalization to evaluate the therapeutic potency of allosteric modulators. This very active field of research is mainly targeting class C GPCRs with well identified allosteric and orthosteric binding sites such as metabotropic glutamate or $GABA_B$ receptors but relevance for class A GPCRs is attracting increasing attention (Nickols and Conn, 2014). Direct visualization of the neurons of interest, either by FP under the control of a GPCR promoter or by expression of the GPCR fluorescent construct, also represents a significant breakthrough by making subsequent targeted investigations available. This includes electrophysiological recordings on previously identified cell, cell isolation by fluorescence-activated cell sorting for further biochemical (Western Blotting) and molecular (RT-PCR) downstream analysis or highly specific and efficient immunoprecipitation of the interacting partners. The presence of the FP also gives access to imaging techniques with which receptor population tracking within membranes can be achieved, by fluorescence recovery after photobleaching or fluorescence resonance energy transfer. The latter also opens ways to identify heteromer formation between GPCRs or between a GPCR and a ligand-gated channel and to investigate *in vivo* their intracellular fate and impact on signaling cascades. All these technological developments will undeniably contribute to deepening our current knowledge of GPCR controlled molecular and cellular processes and ultimately will benefit to drug design and screening.

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REFERENCES

Akil, H., Owens, C., Gutstein, H., Taylor, L., Curran, E., and Watson, S. (1998). Endogenous opioids: overview and current issues. *Drug Alcohol Depend.* 51, 127–140. doi: 10.1016/S0376-8716(98)00071-4

Alexander, S. P. (2013). The concise guide to pharmacology 2013/14: G protein coupled receptors. *Br. J. Pharmacol.* 170, 1459–1581. doi: 10.1111/bph.12445

Arttamangkul, S., and Quillinan, N. (2008). Differential activation and trafficking of μ-opioid receptors in brain slices. *Mol. Pharmacol.* 74, 972–979. doi: 10.1124/mol.108.048512

Bardoni, R., Tawfik, V. L., Wang, D., François, A., Solorzano, C., Shuster, S. A., et al. (2014). Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. *Neuron* 81, 1312–1327. doi: 10.1016/j.neuron.2014.01.044

Behrens, M., Foerster, S., Staehler, F., Raguse, J.-D., and Meyerhof, W. (2007). Gustatory expression pattern of the human TAS2R bitter receptor gene family reveals a heterogenous population of bitter responsive taste receptor cells. *J. Neurosci.* 27, 12630–12640. doi: 10.1523/JNEUROSCI.1168-07.2007

Benes, F., and Berretta, S. (2001). GABAergic interneurons: implications for understanding schizophrenia and bipolar disorder. *Neuropsychopharmacology* 25, 1–27. doi: 10.1016/S0893-133X(01)00225-1

Bertran-Gonzalez, J., Bosch, C., Maroteaux, M., Matamales, M., Hervé, D., Valjent, E., et al. (2008). Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol. *J. Neurosci.* 28, 5671–5685. doi: 10.1523/JNEUROSCI.1039-08.2008

Bertran-Gonzalez, J., Laurent, V., Chieng, B. C., Christie, M. J., and Balleine, B. W. (2013). Learning-related translocation of δ-opioid receptors on ventral striatal cholinergic interneurons mediates choice between goal-directed actions. *J. Neurosci.* 33, 16060–16071. doi: 10.1523/JNEUROSCI.1927-13.2013

Bradley, S. J., Riaz, S. A., and Tobin, A. B. (2014). Employing novel animal models in the design of clinically efficacious GPCR ligands. *Curr. Opin. Cell Biol.* 27, 117–125. doi: 10.1016/j.ceb.2013.12.002

Cahill, C. M., Holdridge, S. V., and Morinville, A. (2007). Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. *Trends Pharmacol. Sci.* 28, 23–31. doi: 10.1016/j.tips.2006.11.003

Cahill, C. M., Morinville, A., Lee, M., Vincent, J., Collier, B., and Beaudet, A. (2001). Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta -mediated antinociception. *J. Neurosci.* 21, 7598–7607.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., et al. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7877–7882. doi: 10.1073/pnas.082243699

Chan, C. S., Peterson, J. D., Gertler, T. S., Glajch, K. E., Quintana, R. E., Cui, Q., et al. (2012). Strain-specific regulation of striatal phenotype in Drd2-eGFP BAC transgenic mice. *J. Neurosci.* 32, 9124–9132. doi: 10.1523/JNEUROSCI.0229-12.2012

Chan, F., Bradley, A., Wensel, T. G., and Wilson, J. H. (2004). Knock-in human rhodopsin-GFP fusions as mouse models for human disease and targets for gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9109–9114. doi: 10.1073/pnas.0403149101

Chu Sin Chung, P., and Kieffer, B. L. (2013). Delta opioid receptors in brain function and diseases. *Pharmacol. Ther.* 140, 112–120. doi: 10.1016/j.pharmthera.2013.06.003

Chung, M.-K., Jue, S. S., and Dong, X. (2012). Projection of non-peptidergic afferents to mouse tooth pulp. *J. Dent. Res.* 91, 777–782. doi: 10.1177/0022034512450298

Congreve, M., Dias, J. M., and Marshall, F. H. (2014). Structure-based drug design for G protein-coupled receptors. *Prog. Med. Chem.* 53, 1–63 doi: 10.1016/B978-0-444-63380-4.00001-9

Cormack, B. P., Bertram, G., Egerton, M., Gow, N. A. R., Falkow, S., and Brown, A. J. P. (1997). Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology* 143, 303–311. doi: 10.1099/00221287-143-2-303

Delille, H. K., Mezler, M., and Marek, G. J. (2013). The two faces of the pharmacological interaction of mGlu2 and 5-HT2A – relevance of receptor heterocomplexes and interaction through functional brain pathways. *Neuropharmacology* 70, 296–305. doi: 10.1016/j.neuropharm.2013.02.005

Dong, X., Han, S., Zylka, M. J., Simon, M. I., and Anderson, D. J. (2001). A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* 106, 619–632. doi: 10.1016/S0092-8674(01)00483-4

Doyle, A., McGarry, M. P., Lee, N. A., and Lee, J. J. (2012). The construction of transgenic and gene knockout/knockin mouse models of human disease. *Transgenic Res.* 21, 327–349. doi: 10.1007/s11248-011-9537-3

Duncan, K., Ketz, N., and Davachi, L. (2012). Evidence for area CA1as a match/mismatch detector: a high-resolution fMRI study of the human hippocampus. *Hippocampus* 22, 389–398. doi: 10.1002/hipo.20933

Dunham, J. H., and Hall, R. A. (2009). Enhancement of the surface expression of G protein-coupled receptors. *Trends Biotechnol.* 27, 541–545. doi: 10.1016/j.tibtech.2009.06.005

Erbs, E., Faget, L., Scherrer, G., and Kessler, P. (2012). Distribution of delta opioid receptor-expressing neurons in the mouse hippocampus. *Neuroscience* 221, 203–213. doi: 10.1016/j.neuroscience.2012.06.023

Erbs, E., Faget, L., Scherrer, G., Matifas, A., Filliol, D., Vonesch, J.-L., et al. (2014). A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* doi: 10.1007/s00429-014-0717-9 [Epub ahead of print].

Faget, L., Erbs, E., LeMerrer, J., Scherrer, G., Matifas, A., Benturquia, N., et al. (2012). In vivo visualization of delta opioid receptors upon physiological activation uncovers a distinct internalization profile. *J. Neurosci.* 32, 7301–7310. doi: 10.1523/JNEUROSCI.0185-12.2012

Ferguson, S. S. G., Ill, W. E. D., Colapietro, A., Barak, L. S., Menard, L., and Caron, M. G. (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* 271, 363–366. doi: 10.1126/science.271.5247.363

Fuhrmann, M., Bittner, T., Jung, C. K. E., Burgold, S., Page, R. M., Mitteregger, G., et al. (2010). Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease. *Nat. Neurosci.* 13, 411–413. doi: 10.1038/nn.2511

Garcia, J., Cardona, S., and Cardona, A. (2013). Analyses of microglia effector function using CX3CR1-GFP knock-in mice. *Microglia* 1041, 307–317. doi: 10.1007/978-1-62703-520-0_27

Gassmann, M., and Bettler, B. (2012). Regulation of neuronal GABA(B) receptor functions by subunit composition. *Nat. Rev. Neurosci.* 13, 380–394. doi: 10.1038/nrn3249

Gendron, L., Lucido, A. L., Mennicken, F., O'Donnell, D., Vincent, J.-P., Stroh, T., et al. (2006). Morphine and pain-related stimuli enhance cell surface availability of somatic delta-opioid receptors in rat dorsal root ganglia. *J. Neurosci.* 26, 953–962. doi: 10.1523/JNEUROSCI.3598-05.2006

Gendron, L., Mittal, N., Beaudry, H., and Walwyn, W. (2014). Recent advances on the δ opioid receptor: from trafficking to function. *Br. J. Pharmacol.* doi: 10.1111/bph.12706 [Epub ahead of print].

George, S., Zastawny, R., Brionesurbina, R., Cheng, R., Nguyen, T., Heiber, M., et al. (1994). Distinct distributions of mu, delta and kappa opioid receptor mRNA in rat brain. *Biochem. Biophys. Res. Commun.* 205, 1438–1444. doi: 10.1006/bbrc.1994.2826

Giguere, P. M., Kroeze, W. K., and Roth, B. L. (2014). Tuning up the right signal: chemical and genetic approaches to study GPCR functions. *Curr. Opin. Cell Biol.* 27, 51–55. doi: 10.1016/j.ceb.2013.11.006

Gomes, I., Gupta, A., Filipovska, J., Szeto, H. H., Pintar, J. E., and Devi, L. A. (2004). A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 101, 5135–5139. doi: 10.1073/pnas.0307601101

Gong, S., Zheng, C., Doughty, M. L., Losos, K., Didkovsky, N., Schambra, U. B., et al. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425, 917–925. doi: 10.1038/nature02033

Goody, R. J., Oakley, S. M., Filliol, D., Kieffer, B. L., and Kitchen, I. (2002). Q uantitative autoradiographic mapping of opioid receptors in the brain of d -opioid receptor gene knockout mice. *Brain Res.* 945, 9–19. doi: 10.1016/S0006-8993(02)02452-6

Gross, A. K., Decker, G., Chan, F., Sandoval, I. M., Wilson, J. H., and Wensel, T. G. (2006). Defective development of photoreceptor membranes in a mouse model of recessive retinal degeneration. *Vision Res.* 46, 4510–4518. doi: 10.1016/j.visres.2006.07.012

Guan, J.-S., Xu, Z.-Z., Gao, H., He, S.-Q., Ma, G.-Q., Sun, T., et al. (2005). Interaction with vesicle luminal protachykinin regulates surface expression of delta-opioid receptors and opioid analgesia. *Cell* 122, 619–631. doi: 10.1016/j.cell.2005.06.010

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

Haberstock-Debic, H., Kim, K.-A., Yu, Y. J., and von Zastrow, M. (2005). Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J. Neurosci.* 25, 7847–7857. doi: 10.1523/JNEUROSCI.5045-04.2005

Haruyama, N., Cho, A., and Kulkarni, A. (2009). Overview: engineering transgenic constructs and mice. *Curr. Protoc. Cell Biol.* Unit 19.10(Suppl. 42), 1–12. doi: 10.1002/0471143030.cb1910s42

Heim, R., and Tsien, R. Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* 6, 178–182. doi: 10.1016/S0960-9822(02)00450-5

Heintz, N. (2001). BAC to the future: the use of BAC transgenic mice. *Nat. Rev. Neurosci:* 2, 1–10. doi: 10.1038/35104049

Heng, B. C., Aubel, D., and Fussenegger, M. (2013). An overview of the diverse roles of G-protein coupled receptors (GPCRs) in the pathophysiology of various human diseases. *Biotechnol. Adv.* 31, 1676–1694. doi: 10.1016/j.biotechadv.2013.08.017

Hoon, M., Adler, E., Lindemeier, J., and Battey, J. (1999). Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell* 96, 541–551. doi: 10.1016/S0092-8674(00)80658-3

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., et al. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol.* 20, 4106–4114. doi: 10.1128/MCB.20.11.4106-4114.2000

Kallal, L., and Benovic, J. L. (2000). Using green fluorescent proteins to study G-protein-coupled receptor localization and trafficking. *Trends Pharmacol. Sci.* 21, 175–180. doi: 10.1016/S0165-6147(00)01477-2

Kenakin, T. (2014). What is pharmacological "affinity?" Relevance to biased agonism and antagonism. *Trends Pharmacol. Sci.* 35, 434–441. doi: 10.1016/j.tips.2014.06.003

Kieffer, B. L., and Evans, C. J. (2009). Opioid receptors: from binding sites to visible molecules in vivo. *Neuropharmacology* 56(Suppl. 1), 205–212. doi: 10.1016/j.neuropharm.2008.07.033

Kitchen, I., Slowe, S. J., Matthes, H. W. D., and Kieffer, B. L. (1997). Quantitative autoradiographic mapping of m -, d - and k -opioid receptors in knockout mice lacking the m -opioid receptor gene. *Brain Res.* 778, 73–88. doi: 10.1016/S0006-8993(97)00988-8

Kramer, P. F., Christensen, C. H., Hazelwood, L., Dobi, A., Bock, R., Sibley, D. R., et al. (2011). Dopamine D2 receptor overexpression alters behavior and physiology in Drd2-EGFP mice. *J. Neurosci.* 31, 126–132. doi: 10.1523/JNEUROSCI.4287-10.2011

Kumar, K., Sharma, S., Kumar, P., and Deshmukh, R. (2013). Therapeutic potential of GABA(B) receptor ligands in drug addiction, anxiety, depression and other CNS disorders. *Pharmacol. Biochem. Behav.* 110, 174–184. doi: 10.1016/j.pbb.2013.07.003

Lagerström, M. C., and Schiöth, H. B. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat. Rev. Drug Discov.* 7, 339–357. doi: 10.1038/nrd2518

Laurent, V., Wong, F. L., and Balleine, B. W. (2014). δ-Opioid receptors in the accumbens shell mediate the influence of both excitatory and inhibitory predictions on choice. *Br. J. Pharmacol.* doi: 10.1111/bph.12731 [Epub ahead of print].

Lee, K.-W., Kim, Y., Kim, A. M., Helmin, K., Nairn, A. C., and Greengard, P. (2006). Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing medium spiny neurons in nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3399–3404. doi: 10.1073/pnas.0511244103

Lee, M., Cahill, C., Vincent, J., and Beaudet, A. (2002). Internalization and trafficking of opioid receptor ligands in rat cortical neurons. *Synapse* 111, 102–111. doi: 10.1002/syn.10014

LeMerrer, J., Becker, J. A. J., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009

Lesscher, H. M. B., Bailey, A., Burbach, J. P. H., van Ree, J. M., Kitchen, I., and Gerrits, M. A. F. M. (2003). Receptor-selective changes in u-, delta- and kappa-opioid receptors after chronic naltrexone treatment in mice. *Eur. J. Neurosci.* 17, 1006–1012. doi: 10.1046/j.1460-9568.2003.02502.x

Liman, E. R., Zhang, Y. V., and Montell, C. (2014). Peripheral coding of taste. *Neuron* 81, 984–1000. doi: 10.1016/j.neuron.2014.02.022

Liu, Q., Sikand, P., Ma, C., Tang, Z., Han, L., Li, Z., et al. (2012). Mechanisms of itch evoked by β-alanine. *J. Neurosci.* 32, 14532–14537. doi: 10.1523/JNEUROSCI.3509-12.2012

Lutz, P.-E., and Kieffer, B. L. (2013). The multiple facets of opioid receptor function: implications for addiction. *Curr. Opin. Neurobiol.* 23, 473–479. doi: 10.1016/j.conb.2013.02.005

Madziva, M. T., and Edwardson, J. M. (2001). Trafficking of green fluorescent protein-tagged muscarinic M4 receptors in NG108-15 cells. *Eur. J. Pharmacol.* 428, 9–18. doi: 10.1016/S0014-2999(01)01266-3

Mailleux, P., and Vanderhaeghen, J. J. (1992). Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. *Neuroscience* 48, 655–668. doi: 10.1016/0306-4522(92)90409-U

Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U

Marder, E. (2012). Neuromodulation of neuronal circuits: back to the future. *Neuron* 76, 1–11. doi: 10.1016/j.neuron.2012.09.010

Marsicano, G., and Lutz, B. (1999). Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur. J. Neurosci.* 11, 4213–4225. doi: 10.1046/j.1460-9568.1999.00847.x

McDonald, N. A., Henstridge, C. M., Connolly, C. N., and Irving, A. J. (2007). Generation and functional characterization of fluorescent, N-terminally tagged CB1 receptor chimeras for live-cell imaging. *Mol. Cell. Neurosci.* 35, 237–248. doi: 10.1016/j.mcn.2007.02.016

McLean, A. J., and Milligan, G. (2000). Ligand regulation of green fluorescent protein-tagged forms of the human beta(1)- and beta(2)-adrenoceptors; comparisons with the unmodified receptors. *Br. J. Pharmacol.* 130, 1825–1832. doi: 10.1038/sj.bjp.0703506

Minnis, J. G., Patierno, S., Kohlmeier, S. E., Brecha, N. C., Tonini, M., and Sternini, C. (2003). Ligand-induced μ opioid receptor endocytosis and recycling in enteric neurons. *Neuroscience* 119, 33–42. doi: 10.1016/S0306-4522(03)00135-0

Mizutani, M., Pino, P. A., Saederup, N., Charo, I. F., Ransohoff, R. M., and Cardona, A. E. (2012). The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *J. Immunol.* 188, 29–36. doi: 10.4049/jimmunol.1100421

Moreira, I. S. (2014). Structural features of the G-protein/GPCR interactions. *Biochim. Biophys. Acta* 1840, 16–33. doi: 10.1016/j.bbagen.2013.08.027

Morinville, A., Cahill, C. M., Kieffer, B., Collier, B., and Beaudet, A. (2004). Mu-opioid receptor knockout prevents changes in delta-opioid receptor trafficking induced by chronic inflammatory pain. *Pain* 109, 266–273. doi: 10.1016/j.pain.2004.01.011

Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* 20, 87–90. doi: 10.1038/nbt0102-87

Nickols, H. H., and Conn, P. J. (2014). Development of allosteric modulators of GPCRs for treatment of CNS disorders. *Neurobiol. Dis.* 61, 55–71. doi: 10.1016/j.nbd.2013.09.013

Nienhaus, K., and Nienhaus, G. U. (2014). Fluorescent proteins for live-cell imaging with super-resolution. *Chem. Soc. Rev.* 43, 1088–1106. doi: 10.1039/c3cs60171d

Ostrom, R. S., and Insel, P. A. (2004). The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br. J. Pharmacol.* 143, 235–245. doi: 10.1038/sj.bjp.0705930

Overington, J. P., Al-Lazikani, B., and Hopkins, A. L. (2006). How many drug targets are there? *Nat. Rev. Drug Discov.* 5, 993–996. doi: 10.1038/nrd2199

Papay, R., Gaivin, R., McCune, D. F., Rorabaugh, B. R., Macklin, W. B., McGrath, J. C., et al. (2004). Mouse alpha1B-adrenergic receptor is expressed in neurons and NG2 oligodendrocytes. *J. Comp. Neurol.* 478, 1–10. doi: 10.1002/cne.20215

Pertwee, R. G. (2006). The pharmacology of cannabinoid receptors and their ligands: an overview. *Int. J. Obes.* (*Lond.*) 30(Suppl. 1), S13–8. doi: 10.1038/sj.ijo.0803272

Poole, D. P., Pelayo, J.-C., Scherrer, G., Evans, C. J., Kieffer, B. L., and Bunnett, N. W. (2011). Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice. *Gastroenterology* 141, 982–991.e1–8. doi: 10.1053/j.gastro.2011.05.042

Pradhan, A. A. A., Becker, J. A. J., Scherrer, G., Tryoen-Toth, P., Filliol, D., Matifas, A., et al. (2009). In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS ONE* 4:e5425. doi: 10.1371/journal.pone.0005425

Pradhan, A. A. A., and Clarke, P. B. S. (2005). Comparison between delta-opioid receptor functional response and autoradiographic labeling in rat brain and spinal cord. *J. Comp. Neurol.* 481, 416–426. doi: 10.1002/cne.20378

Pradhan, A. A. A., Walwyn, W., Nozaki, C., Filliol, D., Erbs, E., Matifas, A., et al. (2010). Ligand-directed trafficking of the δ-opioid receptor in vivo: two paths toward analgesic tolerance. *J. Neurosci.* 30, 16459–16468. doi: 10.1523/JNEUROSCI.3748-10.2010

Price, B. A., Sandoval, I. M., Chan, F., Simons, D. L., Wu, S. M., Wensel, T. G., et al. (2011). Mislocalization and degradation of human P23H-rhodopsin-GFP in a knockin mouse model of retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 52, 9728–9736. doi: 10.1167/iovs.11-8654

Rezai, X., Faget, L., Bednarek, E., Schwab, Y., Kieffer, B. L., and Massotte, D. (2012). Mouse δ opioid receptors are located on presynaptic afferents to hippocampal pyramidal cells. *Cell. Mol. Neurobiol.* 32, 509–516. doi: 10.1007/s10571-011-9791-1

Rezai, X., Kieffer, B. L., Roux, M. J., and Massotte, D. (2013). Delta opioid receptors regulate temporoammonic-activated feedforward inhibition to the mouse CA1 hippocampus. *PLoS ONE* 8:e79081. doi: 10.1371/journal.pone.0079081

Rigato, C., Swinnen, N., Buckinx, R., Couillin, I., Mangin, J.-M., Rigo, J.-M., et al. (2012). Microglia proliferation is controlled by P2X7 receptors in a Pannexin-1-independent manner during early embryonic spinal cord invasion. *J. Neurosci.* 32, 11559–11573. doi: 10.1523/JNEUROSCI.1042-12.2012

Rosenbaum, D. M., Rasmussen, S. G. F., and Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. *Nature* 459, 356–363. doi: 10.1038/nature08144

Roy, S., Rached, M., and Gallo-Payet, N. (2007). Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms alpha and beta in isogenic human embryonic kidney 293 cells. *Mol. Endocrinol.* 21, 1656–1669. doi: 10.1210/me.2007-0041

Rozenfeld, R., Bushlin, I., Gomes, I., Tzavaras, N., Gupta, A., Neves, S., et al. (2012). Receptor heteromerization expands the repertoire of cannabinoid signaling in rodent neurons. *PLoS ONE* 7:e29239. doi: 10.1371/journal.pone.0029239

Saederup, N., Cardona, A. E., Croft, K., Mizutani, M., Cotleur, A. C., Tsou, C.-L., et al. (2010). Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS ONE* 5:e13693. doi: 10.1371/journal.pone.0013693

Sandoval, I. M., Price, B. A., Gross, A. K., Chan, F., Sammons, J. D., Wilson, J. H., et al. (2014). Abrupt onset of mutations in a developmentally regulated gene during terminal differentiation of post-mitotic photoreceptor neurons in mice. *PLoS ONE* 9:e108135. doi: 10.1371/journal.pone.0108135

Scherrer, G., Imamachi, N., Cao, Y.-Q., Contet, C., Mennicken, F., O'Donnell, D., et al. (2009). Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* 137, 1148–1159. doi: 10.1016/j.cell.2009.04.019

Scherrer, G., Tryoen-to, P., Filliol, D., Matifas, A., Laustriat, D., Cao, Y. Q., et al. (2006). Knockin mice expressing fluorescent delta -opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9691–9696. doi: 10.1073/pnas.0603359103

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E., and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* 22, 1567–1572. doi: 10.1038/nbt1037

Shaner, N. C., Patterson, G. H., and Davidson, M. W. (2007). Advances in fluorescent protein technology. *J. Cell Sci.* 120, 4247–4260. doi: 10.1242/jcs.005801

Shimomura, O., Johnson, F., and Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell. Comp. Physiol.* 59, 223–239. doi: 10.1002/jcp.1030590302

Slowe, S. J., Simonin, F., Kieffer, B. L., and Kitchen, I. (1999). Quantitative autoradiography of mu, delta and kappa opioid receptors in kappa-opioid receptor knockout mice. *Brain Res.* 818, 335–345. doi: 10.1016/S0006-8993(98)01201-3

Stockton, S. D., and Devi, L. A. (2012). Functional relevance of μ-δ opioid receptor heteromerization: a role in novel signaling and implications for the treatment of addiction disorders: from a symposium on new concepts in mu-opioid pharmacology. *Drug Alcohol Depend.* 121, 167–172. doi: 10.1016/j.drugalcdep.2011.10.025

Stone, L. M., Barrows, J., Finger, T. E., and Kinnamon, S. C. (2007). Expression of T1Rs and gustducin in palatal taste buds of mice. *Chem. Senses* 32, 255–262. doi: 10.1093/chemse/bjl053

Stumm, R. K., Zhou, C., Schulz, S., and Höllt, V. (2004). Neuronal types expressing mu- and delta-opioid receptor mRNA in the rat hippocampal formation. *J. Comp. Neurol.* 469, 107–118. doi: 10.1002/cne.10997

Subach, F. V, Patterson, G. H., Manley, S., Gillette, J. M., Lippincott-Schwartz, J., and Verkhusha, V. V. (2009). Photoactivatable mCherry for high-resolution two-color fluorescence microscopy. *Nat. Methods* 6, 153–159. doi: 10.1038/nmeth.1298

Tan, H., Ahmad, T., Loureiro, M., Zunder, J., and Laviolette, S. R. (2014). The role of cannabinoid transmission in emotional memory formation: implications for addiction and schizophrenia. *Front. Psychiatry* 5:73. doi: 10.3389/fpsyt.2014.00073

Tempel, A., and Zukin, R. (1987). Neuroanatomical patterns of the mu, delta, and kappa opioid receptors of rat brain as determined by quantitative in vitro autoradiography. *Proc. Natl. Acad. Sci. U.S.A.* 84, 4308–4312. doi: 10.1073/pnas.84.12.4308

Tian, X., Kai, L., Hockberger, P. E., Wokosin, D. L., and Surmeier, D. J. (2010). MEF-2 regulates activity-dependent spine loss in striatopallidal medium spiny neurons. *Mol. Cell. Neurosci.* 44, 94–108. doi: 10.1016/j.mcn.2010.01.012

Trafton, J. A., Abbadie, C., Marek, K., and Basbaum, A. I. (2000). Postsynaptic signaling via the mu -opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J. Neurosci.* 20, 8578–8584.

Tsien, R. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* 67, 509–544. doi: 10.1146/annurev.biochem.67.1.509

Tsou, K., Brown, S., and Sanudo-Pena, M. (1997). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83, 393–411. doi: 10.1016/S0306-4522(97)00436-3

Vaccari, C., Lolait, S. J., and Ostrowski, N. L. (1998). Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. *Endocrinology* 139, 5015–5033. doi: 10.1210/endo.139.12.6382

Valjent, E., Bertran-Gonzalez, J., Hervé, D., Fisone, G., and Girault, J.-A. (2009). Looking BAC at striatal signaling: cell-specific analysis in new transgenic mice. *Trends Neurosci.* 32, 538–547. doi: 10.1016/j.tins.2009.06.005

van den Pol, A. N. (2012). Neuropeptide transmission in brain circuits. *Neuron* 76, 98–115. doi: 10.1016/j.neuron.2012.09.014

van Rijn, R. M., Whistler, J. L., and Waldhoer, M. (2010). Opioid-receptor-heteromer-specific trafficking and pharmacology. *Curr. Opin. Pharmacol.* 10, 73–79. doi: 10.1016/j.coph.2009.09.007

Virgone-Carlotta, A., Uhlrich, J., Akram, M. N., Ressnikoff, D., Chrétien, F., Domenget, C., et al. (2013). Mapping and kinetics of microglia/neuron cell-to-cell contacts in the 6-OHDA murine model of Parkinson's disease. *Glia* 61, 1645–1658. doi: 10.1002/glia.22546

Voigt, A., Hübner, S., Lossow, K., Hermans-Borgmeyer, I., Boehm, U., and Meyerhof, W. (2012). Genetic labeling of Tas1r1 and Tas2r131 taste receptor cells in mice. *Chem. Senses* 37, 897–911. doi: 10.1093/chemse/bjs082

von Zastrow, M., and Williams, J. T. (2012). Modulating neuromodulation by receptor membrane traffic in the endocytic pathway. *Neuron* 76, 22–32. doi: 10.1016/j.neuron.2012.09.022

Walther, C., and Ferguson, S. S. G. (2013). Arrestins: role in the desensitization, sequestration, and vesicular trafficking of G protein-coupled receptors. *Prog. Mol. Biol. Transl. Sci.* 118, 93–113. doi: 10.1016/B978-0-12-394440-5.00004-8

Walwyn, W. M., Miotto, K. A., and Evans, C. J. (2010). Opioid pharmaceuticals and addiction: the issues, and research directions seeking solutions. *Drug Alcohol Depend.* 108, 156–165. doi: 10.1016/j.drugalcdep.2010.01.001

Walwyn, W. M., Wei, W., Xie, C.-W., Chiu, K., Kieffer, B. L., Evans, C. J., et al. (2006). Mu opioid receptor-effector coupling and trafficking in dorsal root ganglia neurons. *Neuroscience* 142, 493–503. doi: 10.1016/j.neuroscience.2006.06.021

Wang, H.-B., Zhao, B., Zhong, Y.-Q., Li, K.-C., Li, Z.-Y., Wang, Q., et al. (2010). Coexpression of delta- and mu-opioid receptors in nociceptive sensory neurons. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13117–13122. doi: 10.1073/pnas.1008382107

Wensel, T. G., Gross, A. K., Chan, F., Sykoudis, K., and Wilson, J. H. (2005). Rhodopsin-EGFP knock-ins for imaging quantal gene alterations. *Vision Res.* 45, 3445–3453. doi: 10.1016/j.visres.2005.07.016

Winters, B. D., Krüger, J. M., Huang, X., Gallaher, Z. R., Ishikawa, M., Czaja, K., et al. (2012). Cannabinoid receptor 1-expressing neurons in the nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* 109, E2717–E2725. doi: 10.1073/pnas.1206303109

Wrobel, L., Schorscher-Petcu, A., Dupré, A., Yoshida, M., Nishimori, K., and Tribollet, E. (2011). Distribution and identity of neurons expressing the oxytocin receptor in the mouse spinal cord. *Neurosci. Lett.* 495, 49–54. doi: 10.1016/j.neulet.2011.03.033

Xie, W.-Y., He, Y., Yang, Y.-R., Li, Y.-F., Kang, K., Xing, B.-M., et al. (2009). Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the delta-opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. *J. Neurosci.* 29, 3551–3564. doi: 10.1523/JNEUROSCI.0415-09.2009

Yoshida, M., Takayanagi, Y., Inoue, K., Kimura, T., Young, L. J., Onaka, T., et al. (2009). Evidence that oxytocin exerts anxiolytic effects via oxytocin receptor expressed in serotonergic neurons in mice. *J. Neurosci.* 29, 2259–2271. doi: 10.1523/JNEUROSCI.5593-08.2009

Yoshimura, N., Seki, S., Novakovic, S. D., Tzoumaka, E., Erickson, V. L., Erickson, K. A., et al. (2001). The involvement of the tetrodotoxin-resistant sodium channel Na(v)1.8 (PN3/SNS) in a rat model of visceral pain. *J. Neurosci.* 21, 8690–8696.

Zylka, M. J., Rice, F. L., and Anderson, D. J. (2005). Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* 45, 17–25. doi: 10.1016/j.neuron.2004.12.015

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Interactions of the opioid and cannabinoid systems in reward: Insights from knockout studies

Katia Befort*

CNRS, Laboratoire de Neurosciences Cognitives et Adaptatives – UMR7364, Faculté de Psychologie, Neuropôle de Strasbourg – Université de Strasbourg, Strasbourg, France

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Fernando Berrendero, Universitat Pompeu Fabra, Spain
Krisztina Monory, University Medical Centre of the Johannes Gutenberg University, Germany

***Correspondence:**
Katia Befort, CNRS, Laboratoire de Neurosciences Cognitives et Adaptatives – UMR7364, Faculté de Psychologie, Neuropôle de Strasbourg – Université de Strasbourg, 12, rue Goethe, F -67000 Strasbourg, France
e-mail: katia.befort@unistra.fr

The opioid system consists of three receptors, mu, delta, and kappa, which are activated by endogenous opioid peptides (enkephalins, endorphins, and dynorphins). The endogenous cannabinoid system comprises lipid neuromodulators (endocannabinoids), enzymes for their synthesis and their degradation and two well-characterized receptors, cannabinoid receptors CB1 and CB2. These systems play a major role in the control of pain as well as in mood regulation, reward processing and the development of addiction. Both opioid and cannabinoid receptors are coupled to G proteins and are expressed throughout the brain reinforcement circuitry. Extending classical pharmacology, research using genetically modified mice has provided important progress in the identification of the specific contribution of each component of these endogenous systems *in vivo* on reward process. This review will summarize available genetic tools and our present knowledge on the consequences of gene knockout on reinforced behaviors in both systems, with a focus on their potential interactions. A better understanding of opioid–cannabinoid interactions may provide novel strategies for therapies in addicted individuals.

Keywords: opioid, cannabinoid, G protein-coupled receptors, reward, genetically modified mice

INTRODUCTION

Drug abuse often leads to a complex pharmaco-dependent state which is defined by the term addiction. Addiction is considered as a neuropsychiatric disease. It develops from an initial recreational drug use, evolves toward compulsive drug-seeking behavior and excessive drug-intake with the appearance of negative emotional states such as anxiety or irritability when the drug is not accessible, and uncontrolled intake reaching a stage where the drug interferes with daily activities, despite the emergence of adverse consequences (Leshner, 1997; Everitt and Robbins, 2005; Robinson and Berridge, 2008; Koob, 2009). This pathological process develops in 15–30% of casual drug users and several factors may explain individual's vulnerability to addiction, including genetic, psychological and environmental factors (Swendsen and Le Moal, 2011; Belin and Deroche-Gamonet, 2012; Pattij and De Vries, 2013; Saunders and Robinson, 2013). Addiction is a major threat to public health and represents a societal problem especially in developed countries and the economic cost it entails (investments in research, treatment and prevention) is considerable (Gustavsson et al., 2011).

Among illicit drugs, opiate and cannabinoid derivatives are highly abused in Europe. Morphine-like opiates are powerful analgesics and currently represent the major therapeutic remedies for the treatment of severe pain. They are also abused for their recreational euphoric effects. In Europe, 1.3 million people are addicted to heroin, the primary drug for which treatment requests are sought. Cannabis is the most worldwide consumed drug of abuse, with THC being the most abundant active constituent found in the various preparations of the drug. More than 73 million European citizens have used cannabis in the last year and it is estimated that about 7% of cannabis users has become dependent on this drug. There is also a high prevalence of users who seek treatment for dependence on it (http://www.emcdda.europa.eu/publications/edr/trends-developments/2014). Interestingly, new derivatives of these abused drugs are invading the market, notably through internet. Fentanyl derivatives as new opioid drugs and synthetic cannabimimetics, also known as "spices," are becoming more and more popular (Fattore and Fratta, 2011). These abusive substances interact with two neuromodulator sytems, the opioid and the endocannabinoid systems.

THE OPIOID SYSTEM

The opioid system consists of endogenous opioid peptides (enkephalins, endorphins, and dynorphins) from precursors (Penk, Pdyn, and Pomc) which activate three opioid receptors, namely mu, delta, and kappa (Kieffer, 1995). The three membrane receptors, cloned in the early nineties (Evans et al., 1992; Kieffer et al., 1992; Simonin et al., 1994, 1995; Mestek et al., 1995) are GPCR with coupling to Gi/Go proteins, of which the 3D structure was recently resolved (see Filizola and Devi, 2014).

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide, N-arachidonoylethanolamide; CB1, type 1 cannabinoid receptor; CB2, type 2 cannabinoid receptor; cKO, conditional knockout mice; CPA, conditioned place aversion; CP 55,940, (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; CPP, conditioned place preference; CPu, caudate putamen; DA, dopamine; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; G protein, guanine nucleotide binding protein; GABA, c-aminobutyric acid; GPCR, G protein coupled receptor; KO, knockout; MGL, monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; THC, Delta-9-tetrahydrocannabinol; WIN 55,212-2, 2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl-1-naphtalenylmethanone mesylate; WT, wild-type.

Opioid receptors and endogenous opioid peptides are largely expressed throughout the nervous system, noticeably within areas of the neurocircuitry of addiction associated with reward, motivation, or learning and stress (Mansour et al., 1995; Le Merrer et al., 2009; Koob and Volkow, 2010; Erbs et al., 2014). Besides its key role in many aspects of addition (Lutz and Kieffer, 2013a), the opioid system also plays a part in a diverse range of physiological functions including nociception, mood control, eating behavior, or cognitive processes (Contet et al., 2004; Pradhan et al., 2011; Stein, 2013; Bodnar, 2014; Nogueiras et al., 2014).

THE ENDOCANNABINOID SYSTEM

The endocannabinoid system is a neuromodulatory system consisting of two well characterized transmembrane receptors coupled to G protein (Gi/Go), CB1, and CB2 cloned in the 1990's (Matsuda et al., 1990; Munro et al., 1993). The endogenous ligands are lipid neuromodulators, the main ones being AEA and 2-AG. Both are synthesized from phospholipid precursors and act locally as retrograde regulators of synaptic transmission throughout the central nervous system. These lipids are released by postsynaptic neurons and mainly activate presynaptic cannabinoid receptors to transiently or persistently suppress transmitter release from both excitatory and inhibitory synapses (recently reviewed in Ohno-Shosaku and Kano, 2014). Multiple pathways are involved in AEA biosynthesis with several still not fully characterized enzymes. AEA can be generated from the membrane phospholipid precursor N-arachidonoyl phosphatidylethalonamine (NAPE) through a two-step process with first a calcium-dependent transacylase followed by a phospholipase D (NAPE-PLD) hydrolysis (Liu et al., 2008). Phospholipase C (PLC) and DAGL are involved in 2-AG synthesis (Ahn et al., 2008). Their degradation is conducted by two specific enzymatic systems, the FAAH (Cravatt et al., 1996) and the MGL (Dinh et al., 2002), for AEA and 2-AG, respectively (Ahn et al., 2008). The endocannabinoid system plays a key role in energy balance, modulation of pain response, with processing of central and peripheral pain signals, learning and memory, reward and emotions. It has also been shown to be involved in neurogenesis and would play a neuroprotective role in some pathological conditions (for recent reviews see Gardner, 2005; Solinas et al., 2008; Maldonado et al., 2011; Zanettini et al., 2011; Panagis et al., 2014; Piomelli, 2014). Distribution of the two receptors in the central and peripheral system is rather different (Pertwee, 2010). Indeed, CB1 is highly abundant in the central nervous system in areas involved in reward, regulation of appetite and nociception (see **Figure 1**) while CB2 was initially described as a peripheral receptor (Maldonado et al., 2006, 2011; Mackie, 2008). Recent studies have proposed a low but significant expression of this receptor in several brain structures including striatum, hippocampus, and thalamus (Wotherspoon et al., 2005; Gong et al., 2006; Onaivi et al., 2006) and more recently into ventral tegmental area neurons (Zhang et al., 2014). Only few data are therefore available for the CB2 receptor in central function but growing evidence suggest a role in addictive processes, with an implication in cocaine, nicotine, or ethanol effects (Xi et al., 2011; Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013; Ortega-Alvaro et al., 2013). To our knowledge, no data is available thus far concerning a potential role of CB2 in opioid mediated responses. Interestingly, other non-CB1 and non-CB2 receptors have been proposed to interact with endocannabinoids like the orphan GPCR GPR55 or a channel vanilloid TRPV1 recognizing capsaicin. These interactions could potentially explain some pharmacology of cannabis that cannot be accounted for by CB1 and CB2 activation, but further studies using KO approaches may help to provide a better understanding of this pharmacology (De Petrocellis and Di Marzo, 2010).

CROSS TALK BETWEEN THESE NEUROTRANSMITTER SYSTEMS

Many neurotransmitter systems are involved when addiction develops, and both opioid and endocannabinoid systems are

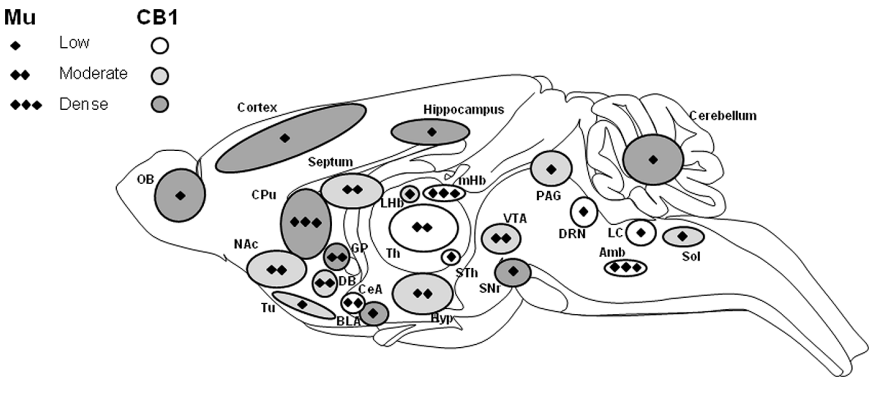

FIGURE 1 | Schematic representation of the distribution of mu opioid and CB1 cannabinoid receptors in the central nervous system. CB1 receptor distribution over the whole central nervous system is indicated by circle shapes with low (white), moderate (gray) and high (dark gray) expression. Major localization of CB1 receptor (mRNA and protein) is in cortical areas, amygdala, striatum, and cerebellum. Moderate and low expression levels are observed in thalamic, hypothalamic, and brainstem regions. Interestingly, the mu opioid receptor is also expressed in these CB1 expressing brain areas but at various levels, indicated by diamonds for low (one), moderate (two), and high (three) expression levels (adapted from Mackie, 2005; Erbs et al., 2014 and references therein). Amb, ambiguous nucleus; BLA, basolateral amygdala; CeA, central amygdala; CPu, caudate putamen; DB, diagonal band; DRN, dorsal raphe nucleus; GP, globus pallidus; Hyp, hypothalamus; LC, locus coeruleus; LHb, lateral habenular nucleus; mHb, medial habenular nucleus; NAc, nucleus accumbens; OB, olfactory bulb; PAG, periaqueductal gray; SNr, substantia nigra pars reticulate; Sol, nucleus of the solitary tract; STh, subthalamic nucleus (ventral thalamus); Th, dorsal thalamus; Tu, olfactory tubercle; VTA, ventral tegmental area.

major players in addictive disorders. In addition to their specific ligands, both systems have also been implicated in the action mechanism of several other addictive drugs, like ethanol, nicotine, or psychostimulants. Although the endocannabinoid system has been known to interact with other systems like hypocretin, dopaminergic, and adenosinergic systems (Fernandez-Ruiz et al., 2010; Ferre et al., 2010; Tebano et al., 2012), its interaction with the opioid system is now well established (Fattore et al., 2005; Vigano et al., 2005; Robledo et al., 2008; Trigo et al., 2010). These two systems share neuroanatomical, neurochemical, and pharmacological, characteristics, this phenomenon is yet less well documented for the CB2 receptor. **Figure 1** illustrates brain structures expressing CB1 receptors and depicts expression level of mu opioid receptors in these areas. The existence of a specific opioid–cannabinoid interaction in the modulation of neurochemical effects as well as behavioral responses associated with reward and relapse have been demonstrated by pharmacological and genetic approaches but experimental results remain controversial (Manzanares et al., 1999; Fattore et al., 2005; Maldonado et al., 2011). Furthermore, molecular interactions between receptors have been shown with colocalization or heterodimerization data mainly for CB1 and delta or mu opioid receptors within spinal cord, striatum, or locus coeruleus. This phenomenon may also account for specific responses at the cellular level (Scavone et al., 2013; Massotte, 2014). However, the physiological effects of these molecular interactions have had yet to be revealed.

AIM OF THE REVIEW

Pharmacological evidence for cross-talk with the synergetic effect of opioid and cannabinoid ligands in many functions related to addiction (mood, stress, learning process . . .) have been revealed and here we will review the implications of both systems regarding reward aspects. As several reviews have recently reported about these interactions (see above), we will focus our interest only on genetic studies using KO mice. We will first present the available genetic tools for both systems. We will then provide an update of results on reinforced behaviors to highlight insights into the particular role of the opioid system in responses to cannabinoids and the endocannabinoid system in responses to prototypical opiates like morphine. We will summarize the behavioral responses of KO mice to these drugs and propose a role for the potential interaction of these two endogenous systems in addictive processes.

REWARD MEASURES IN MICE

Opioid and cannabinoid derivatives induce dependence. To study rewarding effects mediated by specific brain circuits in preclinical research, several behavioral models have been developed in rodents. The most reliable model to evaluate the reinforcing properties of a psychoactive compound in rodents is the self-administration (SA) paradigm which is based on a voluntary procedure to obtain the drug, coupled with the association of a signal (Panlilio and Goldberg, 2007). This operant system allows measuring both rewarding as well as motivational effects of an abused drug. Several aspect of addictive behaviors can be evaluated with this paradigm, with acquisition (fixed ratio) and motivation (progressive ratio) for the drug as well as extinction (response rate when drug-delivery has stopped) and reinstatement induced by cues, context or stress (relapse to drug-seeking) which will reflect aspects of excessive consumption (Sanchis-Segura and Spanagel, 2006). Intravenous SA has been extensively developed for opiates but more difficult to establish for cannabinoid compounds. Adaptations including drug priming, low doses, food restriction, animal restraint, or use of various cannabinoid agonists were often necessary (Maldonado, 2002; Panlilio et al., 2010; Panagis et al., 2014). Nevertheless, iv SA of both THC and the synthetic cannabinoid WIN55,212-2 have been successfully described both in rats and mice, and extended to the study of KO mice (Martellotta et al., 1998; Fattore et al., 2001; Mendizabal et al., 2006; Flores et al., 2014). A very recent study demonstrated for the first time that 2-AG is self-administered by rats and stimulates DA transmission (De Luca et al., 2014).

In addition, a well-accepted model to study the reinforcement properties of abused drugs is the CPP which is a non-operant paradigm. The reinforcing properties are associated with environmental stimuli (cues), such as the context in which the drug is administered. If the drug or a combination of drugs is aversive, animals avoid the drug-paired compartment (CPA) (Tzschentke, 2007). These paradigms have been widely used to study opiates or cannabinoids effects in mutant mice. However, data reporting reinforcing properties for THC and other cannabinoids are rather controversial with a critical concern about experimental conditions, with dose or injection schedule as major parameters to reveal either positive CPP or negative CPA properties of cannabinoids (Panagis et al., 2014).

On top of these two main paradigms (SA and CPP) other tasks have been developed like intracranial self-stimulation (ICSS) as a model to measure reward-facilitating effect of an abused substance although it is rather difficult to set up in mice and therefore little data is available (Panagis et al., 2014). Furthermore, withdrawal signs appear after cessation of chronic drug exposure, either spontaneously or precipitated by an antagonist treatment, and these signs can be scored for providing an index of dependence (Maldonado et al., 1996). In order to make a meaningful comparison in the evaluation of the specific involvement of components of opioid or cannabinoid systems in reward process, it is crucial to compare, when possible, the different mutant lines with their WT littermates in the exact same procedure to avoid bias from technical or experimental variations. Interestingly, such direct comparison has been recently performed for the four components of the opioid system (mu, delta, Penk, and Pdyn) to demonstrate differential behavior in the acquisition and relapse of cocaine SA in the four mutant mice (Gutierrez-Cuesta et al., 2014).

GENERATION OF DEFICIENT MICE IN REGARDS TO COMPONENTS OF THE OPIOID OR CANNABINOID SYSTEMS

For each component of the opioid and the cannabinoid systems, various lines of genetically modified mice have been generated. **Table 1** presents a list for conventional KO mouse lines that have been described so far. The original papers describing the development of the constitutive deletion are presented with the targeted area of the suppressed gene.

Table 1 | Knockout mouse lines for the opioid and the cannabinoid systems.

Gene knockout	Targeted exon	Reference
Opioid system		
Oprm	Exon 2	Matthes et al. (1996)
	Exon 1	Sora et al. (2001)
	Exon 1	Tian et al. (1997)
	Exons 2 and 3	Loh et al. (1998)
	Exon 1	Schuller et al. (1999)
	Exons 2 and 3	van Rijn and Whistler (2009)
	Exon 11 (splice variant)	Pan et al. (2009)
Oprd	Exon 2	Zhu et al. (1999)
	Exon 1	Filliol et al. (2000)
	Exon 2	van Rijn and Whistler (2009)
Oprk	Exon 1	Simonin et al. (1998)
	Exon 3	Hough et al. (2000)
	Exon 3	Ansonoff et al. (2006)
	Exon 2	van Rijn and Whistler (2009)
	Exon 3	Van't Veer et al. (2013)
Oprm/oprd		Simonin et al. (2001)
Oprm/oprd/oprk		Simonin et al. (2001)
		Clarke et al. (2002)
Penk	Exon 3	Konig et al. (1996)
	Exon 3	Ragnauth et al. (2001)
Pdyn	Exon 3	Sharifi et al. (2001)
	Exon 3	Zimmer et al. (2001)
	Exon 3	Loacker et al. (2007)
Pomc	Exon 3	Rubinstein et al. (1996)
	Exon 3	Yaswen et al. (1999)
Penk/Pdyn		Clarke et al. (2003)
Cannabinoid system		
Cnr1	Exon 2	Zimmer et al. (1999)
	Exon 2	Ledent et al. (1999)
	Exon 2	Marsicano et al. (2002)
	Exon 2	Robbe et al. (2002)
Cnr2	Exon 2	Jarai et al. (1999), Buckley et al. (2000)
	Exon 2	Wotherspoon et al. (2005)
FAAH	Exon 1	Cravatt et al. (2001)
MGL	Exon 3	Uchigashima et al. (2011)
	Exons 3 and 4	Taschler et al. (2011)
	Intron 3–exon 4 (gene trapping)	Schlosburg et al. (2010)
	Exons 1 and 2	Chanda et al. (2010)
NAPE-PLD	Exon 4	Leung et al. (2006)
	Exon 3	Tsuboi et al. (2011)
DAGLalpha	Exon 1	Gao et al. (2010)
	Exons 3 and 4	Tanimura et al. (2010)
	Intron 4-Exon 1(gene trapping)	Yoshino et al. (2011)
DAGLbeta	Exon 1	Gao et al. (2010)
	Exons 10 and 11	Tanimura et al. (2010)
	Exon 1 (gene trapping)	Yoshino et al. (2011)
cnr1/cnr2		Jarai et al. (1999)
FAAH/cnr1		Sun et al. (2009)
FAAH/cnr2		Sun et al. (2009)
FAAH/cnr1		Wise et al. (2007)

This table summarizes the published report of KO mouse lines for the different partners of these two systems and combinatorial lines, with the original papers as reference. The area of the gene that has been targeted is indicated.

THE OPIOID SYSTEM

For components of the opioid system, the mu receptor drew the most attention with the description of six distinct genetically modified lines targeting the coding regions of the *oprm* gene, with either exon 1, exon 2 or both exons 2 and 3 targeted for the deletion (Matthes et al., 1996; Tian et al., 1997; Loh et al., 1998; Schuller et al., 1999; Sora et al., 2001; Pan et al., 2009; van Rijn and Whistler, 2009). Interestingly, the mu opioid receptor KO mice allowed to unambiguously demonstrate that the mu receptor was the molecular target for morphine, the prototype of opiate ligand widely used in clinics for its therapeutic effect in pain relief. Morphine had neither analgesic effects nor rewarding properties in these mutant mice (for reviews, see Contet et al., 2004; Gaveriaux-Ruff, 2013). An additional mutant line was constructed which targeted exon 11, a splice variant for the mu receptor, located upstream of exon 1. In these deficient mice, a 25% decrease of receptor expression was observed (Pan et al., 2009), leading to difficult interpretation of the KO effect on opiate pharmacology (Gaveriaux-Ruff, 2013). For deletion of the delta receptor, either exon 1 or 2 were targeted in the *oprd* gene (Zhu et al., 1999; Filliol et al., 2000; van Rijn and Whistler, 2009). These mice were characterized for behavioral responses related to mood and analgesia, but the contribution of delta receptor in reward processes was less clear (Pradhan et al., 2011; Charbogne et al., 2014). Five distinct constructions have been reported targeting either exon 1, 2, or 3 of the *oprk* gene to obtain KO mice for the kappa opioid receptor (Simonin et al., 1998; Hough et al., 2000; Ansonoff et al., 2006; van Rijn and Whistler, 2009; Van't Veer et al., 2013). The two most recent mutants were strategically obtained in order to generate a parallel conditional KO mice (see below) using a Cre-lox approach, with targeted exons floxed with loxP sites. The mutation impaired pharmacological actions of the selective kappa-agonist U-50,488H, and revealed a tonic implication of kappa receptors in the perception of visceral pain. Morphine-CPP was unchanged, but both morphine withdrawal signs as well as emotional responses during

opiate abstinence were reduced (Simonin et al., 1998; Lutz et al., 2014), suggesting an anti-reward role for kappa receptors.

Mice with deleted opioid peptide precursors were also generated. For proopiomelanocortin (*Pomc*), two lines were produced, one specifically deleting βendorphin (Rubinstein et al., 1996) while the second was targeting the whole coding region, deleting both opioid and non-opioid active petides (Yaswen et al., 1999). KO mice for *Penk* gene were generated by two distinct laboratories, both leading to deletion of the 5′ part of exon 3 (Konig et al., 1996; Ragnauth et al., 2001). For deleting dynorphin in mutant animals, exons 3 and 4 (Sharifi et al., 2001) or exon 3 with a part of exon 4 (Zimmer et al., 2001) of the *Pdyn* gene were targeted. Data from peptide KO mice in regards to opiate rewarding effect were more complex. The βendorphin KO mice showed increased (Skoubis et al., 2005) or unchanged (Niikura et al., 2008) morphine-CPP depending on the dose and paradigm used and it was invariable both in mice lacking Penk (Skoubis et al., 2005) or Pdyn (Zimmer et al., 2001; Mizoguchi et al., 2010).

THE CANNABINOID SYSTEM

Four independent KO lines have been generated for the CB1 receptor, encoded by a single large coding exon in the *cnr1* gene (exon 2). The first three lines were generated with the introduction of a PGK or neomycine resistance cassette in the coding region (Ledent et al., 1999; Zimmer et al., 1999; Robbe et al., 2002). For the fourth line, loxP sites were introduced flanking exon 2 and this floxed line was further crossed with a line constitutively expressing the Cre recombinase enzyme, therefore generating a full CB1 KO by deletion of the sequence between the two lox P sites (Marsicano et al., 2002). These mice were mostly unresponsive to cannabinoid ligands in mediating analgesia, reinforcement, hypothermia, hypolocomotion, and hypotension (Valverde and Torrens, 2012; Nadal et al., 2013). Two mouse lines were described for the deletion of the *cnr2* gene coding for CB2 receptor, one by Zimmer's team (Jarai et al., 1999; Buckley et al., 2000) and the other one by the company Deltagen (Wotherspoon et al., 2005). Both were developed by deleting part of the coding region (in exon 2), leaving the start codon with a portion of the amino terminus sequence and aminoacids coding for some transmembrane domains of the receptor. In these constructions expression of the amino-terminal part of the CB2 receptor could potentially occur, but in both cases, it was shown that the receptor was non-functional in the mutant mice (Dhopeshwarkar and Mackie, 2014). Two mutant lines have been described for the NAPE-PLD enzyme involved in AEA synthesis, targeting exon 3 (Tsuboi et al., 2011) or exon 4 (Leung et al., 2006). These KO mice have highlighted the complexity of AEA synthesis with both calcium-dependent and -independent mechanisms. Two isoforms of DAGLα and DAGLβ responsible for the synthesis of 2-AG have been described and KO lines have been generated for each of them with both homologous recombination and gene trapping approaches (Gao et al., 2010; Tanimura et al., 2010; Yoshino et al., 2011). The DAGLα KO animals showed a markedly reduced 2-AG brain content whereas levels were normal in brain regions of KO for the β isoform indicating a much greater contribution of DAGLα to 2-AG biosynthesis in the central nervous system. These mutant mice were particularly useful in the characterization of DAGL involvement in retrograde endocannabinoid signaling (Frazier, 2011). The endocannabinoid system is characterized by a rapid catabolism of the endogenous ligands. Among the degrading enzymes of endocannabinoids, FAAH is the major enzyme responsible for the degradation of AEA and one KO line was generated targeting exon 1 of the *Faah* gene (Cravatt et al., 2001). These mutant mice exhibited more than 15-fold higher brain levels of AEA than WT animals and displayed reduced pain sensitivity. The major degrading enzyme of the endocannabinoid 2-AG is MGL and four KO lines were generated. Three KO lines targeting *mgll* gene exons 1 and 2 (Chanda et al., 2010), exon 3, or exons 3 and 4 were recently generated with a Cre/lox approach (Taschler et al., 2011; Uchigashima et al., 2011). Another line was obtained by gene trapping technology (Texas Institute of Genomic Medicine) with a gene trap cassette inserted into the *mgll* intron 3, upstream of the catalytic exon 4 (Schlosburg et al., 2010). Genetic deletion of MGL leads to alteration in endocannabinoid signaling with increased brain 2-AG levels by ∼10-fold. These animals were mainly characterized by behavioral consequences of the gene deletion for pain perception (Schlosburg et al., 2010; Uchigashima et al., 2011; Petrenko et al., 2014).

COMBINATORIAL MOUSE LINES

Interbreeding of mutant mouse lines allowed generating combinatorial mutant mice both within the opioid and the cannabinoid systems (see references in **Table 1**). These combinatorial lines constituted useful tools to clarify the specific role of particular components of both systems in reward and analgesia, as well as to evaluate *in vivo* selectivity for specific ligands and receptor subtype identification (Kieffer and Gaveriaux-Ruff, 2002; Nadal et al., 2013). Data for reward responses obtained using multiple mutants for cannabinoid or opioid components are detailed below.

COMPENSATORY EFFECTS OF THE NULL MUTATION

Globally, a normal development was described for the various mutant lines, with KO mice being fertile, caring for their offspring, and not showing any major behavioral abnormalities. A higher mortality rate was described for one of the CB1 KO line (Zimmer et al., 1999) but not reported for the two others. Interestingly, among the combinatorial mice, the triple mutant of the opioid receptors present a striking increase in body weight and size, but this obese-like phenotype needs further characterization (Befort and Gaveriaux-Ruff, personal communication). Compensatory mechanisms may have developed in some KO animals, but no systematic studies are available. Deletion of opioid receptors did not markedly modify the expression or activity of the other opioid receptors or the expression of opioid peptides as described by the initial characterizations of the distinct mutants lines (see references in **Table 1** and Kitchen et al., 1997; Slowe et al., 1999; Oakley et al., 2003). A complete autoradiographic mapping of the delta KO mice indicated decreased binding levels of mu and kappa ligands in specific brain areas (Goody et al., 2002). Deletion of opioid peptides modified other partners of the opioid system, with a region-dependent increased of both mu and delta receptor expression levels observed in the Penk KO line (Brady et al., 1999; Clarke et al., 2003) and for the three opioid receptors in the Pdyn and the double Pdyn/Penk mutant line with no additive effects (Clarke

et al., 2003). Interestingly, specific changes of CB1 receptor expression or activity were reported in mu and delta opioid receptor mutant lines (Berrendero et al., 2003). In the mu KO brain, there was no difference in CB1 expression but a decreased efficacy of the classical cannabinoid agonist WIN 55,212-2 was observed specifically in the CPu while both density of CB1 receptor and activation by WIN 55,212-2 increased in substantia nigra of delta KO animals.

Compensatory effects in KO animals concerning the cannabinoid system have been described both for receptor or catabolic enzyme KO mice. The invalidation of the CB1 receptor gene was associated with age-dependent adaptive changes of endocannabinoid metabolism, with increased FAAH and AEA membrane transporter activities in KO hippocampus and cortex, decreased AEA content in hippocampus but no change in 2-AG levels (Di Marzo et al., 2000; Maccarrone et al., 2001, 2002). In the FAAH KO mice, CB1 receptor mRNA decreased in CPu, nucleus accumbens (core), hippocampus (CA1), hypothalamic nucleus (VMN), and amygdala. Its functional activity was also markedly reduced in CPu, the core of nucleus accumbens, and CA3 region of the hippocampus (Vinod et al., 2008). Interestingly, reduction of CB1 receptor density and activity were also observed in MGL KO mouse brain, which may prevent the manifestation of the dramatically enhanced 2-AG behavioral effects in these mice (Chanda et al., 2010; Schlosburg et al., 2010). In DAGLα- and DAGLβ-KO, no difference was reported for CB1 mRNA (Gao et al., 2010) or protein (Tanimura et al., 2010) levels in comparison to WT mice. In these KO mice, CB1 brain functional signaling was unaltered (Aaltonen et al., 2014). To our knowledge, no data is available for any compensatory effect on CB2 expression or activity in the distinct cannabinoid KOs. However, some reports indicate modifications of the opioid system in CB1 KO animals. An increase of both enkephalin and dynorphin mRNA expression was observed in the striatum (Steiner et al., 1999; Gerald et al., 2006, 2008) as well as an increase in kappa and delta opioid receptor activities without changes in their binding (Uriguen et al., 2005). No compensatory changes of mRNA levels for the three opioid receptors were reported in dorsal root ganglia or spinal cord of the CB1 KO animals (Pol et al., 2006). In FAAH KO mice, Penk mRNA expression was decreased in both CPu and nucleus accumbens which paralleled a reduced mu opioid receptor functional activity (Vinod et al., 2008). Noteworthy, these compensatory alterations of opioid or cannabinoid components in specific regions of the mutant lines could account for interactions of the two systems which may be relevant for neuroadaptative processes involved in drug dependence.

CONDITIONAL APPROACHES

Knockout mice are very useful tools for understanding the contribution of each component of these systems in various conditions including pain, mood disorders or addiction (Valverde and Torrens, 2012; Gaveriaux-Ruff, 2013; Lutz and Kieffer, 2013b; Nadal et al., 2013; Charbogne et al., 2014). Recent approaches using gene manipulation in mice have been developed with the widely used Cre-loxP recombinase system to generate cKO (Fowler and Kenny, 2012; **Table 2**). It consists of crossing mice whose target genes are floxed (flanked with two loxP sites) together with mice expressing the Cre-recombinase under a specific promoter. This allows a time-, organ- or site-specific deletion of a target gene. This strategy allowed uncoupling the central and peripheral functions of CB1 receptors (Agarwal et al., 2007) and more recently of mu or delta opioid receptors (Gaveriaux-Ruff et al., 2011; Weibel et al., 2013) using the promoter of the channel Nav1.8 only expressed in DRGs, revealing a key role for these receptors expressed in primary nociceptive neurons in inflammatory pain. To investigate molecular mechanisms at the level of neuronal circuitry, selective deletion of a particular gene can also be achieved in specific neuronal types. For example, deletion of the delta opioid receptors specifically in forebrain GABAergic neurons was obtained by crossing a delta opioid floxed mouse line (Gaveriaux-Ruff et al., 2011) together with a dlx5-6-Cre mouse line, specifically expressing the Cre-recombinase in GABAergic forebrain neurons in order to investigate the role of these specific delta receptors in anxiety (Chu Sin Chung et al., 2014). This latter mouse line was previously crossed with the CB1 floxed mice to successfully obtain a GABA-CB1 conditional mutant (Monory et al., 2006). These mutants were also compared with several other cKO bearing a deletion of CB1 receptor in differing specific neuronal populations: forebrain glutamatergic neurons (CB1CamKIIa-Cre mice or CaMK-CB1KO), cortical glutamatergic neurons (CB1NEX-Cre mice or Glu-CB1KO), both glutamatergic and GABAergic neurons (Glu/GABA-CB1KO) or D1-dopaminergic neurons (CB1Drd1a-Cre mice) (Marsicano et al., 2003; Monory et al., 2006, 2007; Bellocchio et al., 2010) for studying the role of CB1 receptors as well as behavioral and autonomic effects of the agonist THC. For the opioid system, a recent study reported the generation of a conditional mutant for the kappa opioid receptor, selectively deleted in DA-expressing neurons. These kappa cKO mice showed reduced anxiety-like behavior as well as increased sensitivity to cocaine, consistent with a role for kappa receptors in negative regulation of DA function (Van't Veer et al., 2013). For the cannabinoid system, cKO lines were also generated for the CB1 receptor to study its specific implication in neurons (Maresz et al., 2007) or peripheral nerves (Pryce et al., 2014), in serotoninergic (Dubreucq et al., 2012b) or paraventricular (Dubreucq et al., 2012a) and ventromedial (Bellocchio et al., 2013) hypothalamic neurons. CB1 was also specifically deleted in astroglial cells to investigate its role in working memory and long-term hippocampal depression (Han et al., 2012). CB1 was deleted in specific cell types like hepatocytes to study its role in ethanol-induced fatty liver (Jeong et al., 2008), lymphocytes (Maresz et al., 2007) or epidermal keratinocytes (Gaffal et al., 2013) to investigate its potential role in regulation of inflammatory responses. Another strategy to generate a cKO mouse is by using viral mediated construct carrying the Cre-recombinase injected directly in the structure of interest of a target gene-floxed mouse. For example, the mu opioid receptor was selectively deleted in the dorsal raphe, the main serotoninergic brain area, and this deletion abolished the development of social withdrawal in a model of heroin abstinence (Lutz et al., 2014).

In opposition to the loss of function approach, recent studies used a rescue strategy where the target gene is re-expressed in a null mutant, in only a subset of cells (**Table 2**). This helps to provide information concerning the sufficient role of

Table 2 | Conditional knockout mouse lines for the opioid and the cannabinoid systems.

Target Gene	Targeted neurons or structures for selective deletion "loss of function"	Targeted neurons or structures for selective expression "rescue"	Reference
Opioid system			
Oprm	Primary sensory neurons expressing Nav1.8 channel (Nav1.8-Cre)		Weibel et al. (2013)
		Subpopulation of striatal medium spiny neurons	Cui et al. (2014)
Oprd	Primary sensory neurons expressing Nav1.8 channel (Nav1.8-Cre)		Gaveriaux-Ruff et al. (2011)
	Forebrain GABAergic neurons (Dlx5/6-Cre)		Chu Sin Chung et al. (2014)
Oprk	Dopamine containing neurons (DAT-Cre)		Van't Veer et al. (2013)
Cannabinoid system			
Cnr1	Principal forebrain neurons (CamKII-Cre)		Marsicano et al. (2003)
	Forebrain GABAergic neurons (Dlx5/6-Cre)		Monory et al. (2006)
	Cortical glutamatergic neurons (NEX-Cre)		Monory et al. (2006)
	Glutamatergic and GABAergic neurons (Glu/GABA)		Bellocchio et al. (2010)
	Primary sensory neurons expressing Nav1.8 channel (Nav1.8-Cre)		Agarwal et al. (2007)
	D1-dopaminergic neurons (Drd1a-Cre)		Monory et al. (2007)
	Serotoninergic neurons (TPH2-CreER^{T2})		Dubreucq et al. (2012b)
	Paraventricular hypothalamic neurons (Sim1-Cre)		Dubreucq et al. (2012a)
	Ventromedial hypothalamic neurons (SF1-cre)		Bellocchio et al. (2013)
	Neurons Nestin (Nes-Cre)		Maresz et al. (2007)
	Peripheral nerve (peripherin-Cre)		Pryce et al. (2014)
	Astrocytes (GFAP- CreER^{T2})		Han et al. (2012)
	Hepatocytes (Alb-Cre)		Jeong et al. (2008)
	Lymphocytes (lck-Cre)		Maresz et al. (2007)
	Keratinocytes (K14-Cre)		Gaffal et al. (2013)
		Dorsal telencephalic glutamatergic neurons (Glu-CB1-RS)	Ruehle et al. (2013)
FAAH		Nervous system (FAAH-NS)	Cravatt et al. (2004)

This table summarizes the recent published reports of cKO mouse lines for the different partners of opioid and cannabinoid systems using "loss of function" or "rescue" strategies.

the cell type expressing the target gene for a given function or establishing whether other cellular subtypes or circuits are necessary. When mu opioid receptor were re-expressed only in a subpopulation of striatal direct-pathway neurons, in a mu KO background, it restored opiate reward and opiate-induced striatal DA release, partially restored motivation to self-administer an opiate, but the rescued mice lacked opiate analgesia or withdrawal (Cui et al., 2014). In a similar genetic strategy, CB1 receptor expression was restored exclusively in dorsal telencephalic glutamatergic neurons and proved sufficient to control neuronal functions that are in large part hippocampus-dependent, while it was insufficient for proper amygdala functions (Ruehle et al., 2013). A conditional line where the expression of the FAAH enzyme has been restricted to the nervous system (FAAH-NS) was generated by crossing the FAAH KO line with a transgenic mouse, expressing FAAH under the neural specific enolase promoter (Cravatt et al., 2004). These mice exhibited a discrete subset of the biochemical and behavioral phenotypes observed in FAAH KO mice providing key insights into the distinct functions played by the central and peripheral lipids transmitter signaling systems *in vivo*.

In conclusion, despite potential limits such as developmental effects of the mutation or compensatory mechanisms to overcome consequences of the mutation, the use of mutants wherein a component of either opioid or cannabinoid system is selectively deleted from restricted neuronal populations provides essential tools for a comprehensive understanding of mechanisms underlying cannabinoid or opioid effects in reward circuitry. So far, these conditional lines for opioid and cannabinoid systems were mostly characterized for pain or emotional behavioral responses,

and few data is yet to become available for reward aspects (**Table 2**).

CANNABINOID REINFORCING EFFECTS IN OPIOID KNOCKOUT MICE

For evaluating the effect of cannabinoids in opioid mutant mice, THC-induced CPP was mostly used (**Table 3**). Interestingly, the same protocol was used for all tested opioid KO mice with 1 mg/kg ip dose with a priming injection in the home cage. In these conditions, no differences in place preference induced by THC was observed in delta or kappa KO mice while THC-CPP was abolished in mu KO mutants (Ghozland et al., 2002) as well as in the double mu-delta KO mice (Castane et al., 2003). These data support the hypothesis that mu receptors mediate rewarding properties of THC. A similar protocol was used to induce aversion, but with a higher dose of THC (5 mg/kg ip) wherein mu KO mice showed a decreased CPA (Ghozland et al., 2002). THC-induced CPA was abolished in similar conditions in both Pdyn (Zimmer et al., 2001) and kappa KO mice (Ghozland et al., 2002). Self-administration of the synthetic cannabinoid agonist WIN55,212-2 was successfully established in freely moving mice with a low priming dose (0.1 mg/kg i.p.,) and with this protocol, Pdyn KO mice showed facilitated SA (Mendizabal et al., 2006). Altogether, these data support the idea that the kappa/dynorphin system plays a key role in mediating cannabinoid dysphoric effects and therefore negatively modulates their rewarding effects (Mendizabal et al., 2006). Contribution of delta receptors in reward appears complex (Charbogne et al., 2014; Gutierrez-Cuesta et al., 2014) and it has

Table 3 | Rewarding and dependence responses for cannabinoids and opioids measured in KO mouse lines for both systems.

Gene knockout	Behavioral response	Genotype effect	Reference
Opioid system			
Oprm	CPP, THC (1 mg/kg, i.p.)	Abolished	Ghozland et al. (2002)
	CPA THC (5 mg/kg, i.p.)	Decreased	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Unchanged	
	WD, THC (10 mg/kg, s.c. 5d)	Unchanged	Lichtman et al. (2001)
	WD, THC (30 or 100 mg/kg, s.c. 5d)	Decreased	
Oprd	CPP, THC (1 mg/kg, i.p.)	Unchanged	Ghozland et al. (2002)
	CPA THC (5 mg/kg, i.p.)	Unchanged	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Unchanged	
Oprm/Oprd	CPP, THC (1 mg/kg, i.p.)	Decreased	Castane et al. (2003)
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Decreased	
Oprk	CPP, THC (1 mg/kg, i.p.)	Unchanged	Ghozland et al. (2002)
	CPP, THC (1 mg/kg, i.p.)w/o priming	Present, absent in WT	
	CPA, THC (5 mg/kg, i.p.)	Abolished	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Unchanged	
Penk	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Decreased	Valverde et al. (2000)
Pdyn	CPA, THC (5 mg/kg, i.p.)	Abolished	Ghozland et al. (2002)
	SA, WIN 55,212(6.25 mg/kg/inf, i.v.)	Increased	Mendizabal et al. (2006)
	SA, WIN 55,212(12.5 mg/kg/inf, i.v.)	Abolished	
	WD, THC (20 mg/kg, i.p. 2x/d, 6d)	Decreased	Zimmer et al. (2001)
Cannabinoid system			
Cnr1	CPP, morphine (5 mg/kg, s.c.)	Abolished	Martin et al. (2000)
	CPA, morphine + naloxone (20–100 mg/kg i.p. over 6d + 0.1 mg/kg s.c.)	Unchanged	
	CPP, morphine (4–8 mg/kg, i.p.)	Unchanged	Rice et al. (2002)
	CPA, morphine + naloxone (8 mg/kg + 5 mg/kg, i.p.)	Unchanged	
	SA, morphine (2 ug/kg.inf, i.v.)	Abolished	Cossu et al. (2001)
	SA, morphine (1, 2, 4 ug/kg/inf, i.v.)	Decreased	Ledent et al. (1999)
	WD, morphine (20 mg/kg to 100 mg/kg, 5d)	Decreased	
	WD, morphine (75 mg/kg pellet, 5d)	Decreased	Lichtman et al. (2001)
	CPA, U50,488H (1 mg/kg, s.c.)	Abolished	Ledent et al. (1999)

This table summarizes published reports of behavioral responses in reward and precipitated withdrawal for cannabinoids in opioid KO lines and opioids in cannabinoid KO mutants (CPA, conditioned place aversion; CPP, conditioned place preference; d, day; inf, infusion; i.p., intraperitoneal; s.c., subcutaneous; WD, withdrawal; w/o, without).

not yet been established for cannabinoid reward, neither pharmacologically nor genetically. A potential role of this particular receptor in cannabinoid reward awaits further studies investigating either cannabinoid SA (motivation aspects) or delta cKO mutant responses (deletion of specific subpopulation of receptors).

Another aspect that was explored in opioid KO mice is cannabinoid dependence. Upon chronic THC treatment, antagonist-induced withdrawal signs measured in WT animals were unchanged for Pdyn KO (Zimmer et al., 2001) or single mutant mice for mu, delta or kappa opioid receptors (Ghozland et al., 2002). Signs were attenuated in KO animals for Penk (Valverde et al., 2000), for the double mu-delta receptor mutant (Castane et al., 2003) as well as for mu receptor KO, at a high dose (Lichtman et al., 2001) (**Table 3**). No data are yet available for the other opioid peptide KO mice concerning cannabinoid physical dependence. Collectively, available data indicate the involvement of the enkephalinergic system, with a cooperative action of mu and delta receptors, in the expression of cannabinoid dependence.

OPIOID REINFORCING EFFECTS IN CANNABINOID KNOCKOUT MICE

Knockout approaches have greatly improved our knowledge on the role of CB1 receptors in addiction in general, even though contradictory data exist (Maldonado et al., 2006). In particular, for opiate responses (**Table 3**) induced by mu agonists, CB1 KO mice showed no morphine-induced place preference (5 mg/kg, s.c., 3 injections over 6 days) (Martin et al., 2000) and a diminished propensity to self-administer morphine (Ledent et al., 1999; Cossu et al., 2001). A microdialysis study revealed that morphine-induced increase of extracellular DA was not observed in CB1 KO mice (Mascia et al., 1999). Taken together, these data suggest a reduction in morphine's reinforcing activity in the absence of the CB1 receptor. Another study could not reveal any changes in place preference using a slightly more intensive conditioning paradigm and a different set up with two doses of morphine (4 or 8 mg/kg, four injections over 4 days) (Rice et al., 2002). Interestingly, no differences between WT and CB1 KO mice could be observed in a CPA paradigm where the opioid antagonist naloxone was used to induce withdrawal in morphine-treated mice via two distinct paradigms (Martin et al., 2000; Rice et al., 2002). Upon chronic morphine treatment, naloxone-induced withdrawal signs measured in WT animals were attenuated (Ledent et al., 1999; Lichtman et al., 2001). Together, these findings suggest that CB1 receptors are not involved in the disphoric effects of morphine withdrawal (CPA) but are noticeably required for the development of physical dependence or of somatic signs of opiate withdrawal. Surprisingly, other important effects of morphine, like acute induced analgesia and tolerance to chronic morphine-induced analgesia, were not altered in CB1 KO animals. These findings together with the data on mu opioid KO mice with cannabinoid treatments suggest a bidirectional influence of mu opioid and CB1 cannabinoid receptors on reward processes. Aversive effects of the kappa opioid agonist U50,488H were also blunted in CB1 receptor KO mice (Ledent et al., 1999). Together with the data of kappa opioid and Pdyn KO mice, it indicates that both cannabinoid and opioid systems modulate negative motivational drug effects. To our knowledge, no data concerning the specific effect of delta selective opioid agonists on reward in CB1 KO mice are available. Interestingly, it has been demonstrated that the absence of CB1 receptor also results in a reduction of the sensitivity to the rewarding properties of sucrose (Sanchis-Segura et al., 2004), as well as other reinforcers (for recent reviews, see (Lopez-Moreno et al., 2010; Maldonado et al., 2013). Together with pharmacological approaches (Maldonado et al., 2006), KO data therefore provide confirmatory support that CB1 receptor play a modulatory role in the reinforced behaviors maintained by sucrose and some other reinforcers with, in particular, a mutual interaction of opioid and cannabinoid systems.

For the other components of the endocannabinoid system, no specific data for genetically modified animals were reported for the investigation of opioid reward, although pharmacological inhibition of the endocannabinoid catabolic enzymes attenuates both naloxone-induced withdrawal as well as spontaneous withdrawal signs in morphine dependent mice (Ramesh et al., 2011, 2013), indicating a potential role of these enzymes in opioid dependence.

CONCLUSION AND PERSPECTIVES

Globally, despite some compensatory alterations at both opioid and cannabinoid levels in mutant lines, KO studies have provided insights into the mutual role of both opioid and cannabinoid systems on reward. In particular, these studies have highlighted the major role for both mu opioid and CB1 receptors in these processes. Clearly, the mu opioid receptor is a convergent molecular target mediating rewarding properties of opioid compounds but also of other drugs of abuse, including cannabinoids. CB1 receptor also appears as a modulator of opioid reward. On the other hand, KO approaches for endogenous opioid peptides or enzymes for synthesis or degradation of endocannabinoids have been very useful to clarify their specific role in both endogenous systems but less/no data are available for reward mechanisms. These mutants therefore need further investigations to clarify their potential implication in cannabinoid/opioid reward aspects.

Conventional genetically modified animals have strengthened our current knowledge of the interaction between these two systems, but further studies using conditional approaches will be necessary to clarify the potential crosstalk existing specifically in reward processes. Interaction between these two neuromodulator systems may be dependent on the brain area where it occurs, even inside the brain rewarding networks (Parolaro et al., 2010). Both mu opioid and CB1 receptors are highly expressed in these networks in similar brain structures and a potential interaction in areas where they are both strongly expressed is probable. Noticeably, opposite expression levels are observed in discrete areas like amygdala (BLA versus central amygdala) as well as habenula (medial versus lateral nuclei) and these differences may also account for a modulatory role of the two systems in reward processes (**Figure 1**). Approaches using double mutants for both receptors would be useful to further understand their mutual role in drug reward. Moreover, in this perspective, conditional approaches will surely provide invaluable insights into opioid and cannabinoid interaction at the circuitry level. The growing number of cKO mutant lines becoming available will help this side of

research. Likewise, the implication of the CB2 receptor in these interactions has not yet been explored and may be particularly relevant in specific brain structures. In fact, demonstration of CB2 expression in several brain structures has opened a field of investigation for a possible role in addiction that should help to reveal potential direct interaction between CB2 and the opioid system.

G protein coupled receptor can associate as heteromers and extended research is now directed toward elucidating the physiological role of such heteromers and finding therapeutical approaches targeting these entities (see recent reviews Fujita et al., 2014; Massotte, 2014). Several lines of evidence have suggested interactions between delta or mu opioid receptors and the CB1 receptors. Close vicinity of CB1 receptors with mu or delta opioid receptors has recently been established at the neuronal level, suggesting heteromeric formation *in vivo* and potential impact on both receptors signaling properties. A recent study demonstrated an important role for the heterodimer CB1-delta in neuropathic pain where cortical functions of delta receptor were altered (Bushlin et al., 2012). CB1 and mu receptors associate as heteromers in cultured cells and a recent study showed that bivalent ligands for both receptors are potent analgesic devoid of tolerance (Le Naour et al., 2013), suggesting potential functional heteromers in pain. Therefore, one can easily predict that similar mechanisms may occur in another pathological state like addiction and this opens up new prospects for pharmacological action of cannabinoid and opioid drugs. In this context, it will be critical to see whether CB2 also plays a role as a potential heteromeric interactor with opioid receptors.

No effective therapeutic approaches for cannabis dependence are currently available and opioid addiction therapies are not fully satisfying for all patients. Further studies are therefore needed to clarify the mechanistic basis of interaction of the two systems, which would aid in the development of drug therapies to reduce dependence and abuse. Antibodies or bivalent ligands as mentioned previously represent interesting therapeutic targets. In addition, dual enkephalinase inhibitors and cannabinoid catabolic enzyme inhibitors have been proposed as attractive therapeutic targets to treat pain (Roques et al., 2012) and such bifunctional compounds may also be relevant as promising strategies for alleviating dependence.

Substantial progress has been made in understanding the cellular and molecular mechanisms of prolonged use of cannabinoid or opioid drugs (Kreek et al., 2012; Fratta and Fattore, 2013). In addition to their direct role in reward, interaction between opioid and cannabinoid neuromodulator systems has been proposed to explain some aspects of vulnerability to addiction and, in this perspective, recent attention has been focused on yet another critical level, epigenetics. These molecular processes, including methylation of DNA, post-translational modifications of histones and regulation by microRNA, regulate gene expression and are crucial in long-term adaptations induced by drugs (Nestler, 2014). Recent studies have shown a direct association between THC-induced Penk upregulation through reduction of histone H3 lysine 9 pattern of methylation and increased heroin SA (Tomasiewicz et al., 2012). Adolescent THC-exposure also resulted in altered heroin SA in the subsequent generation of rats, an effect associated with changes in mRNA expression of cannabinoid, DA, and glutamatergic receptor genes in the striatum, suggesting adaptations to long-term drug effect and germline transmission, most likely involving epigenetic changes (Szutorisz et al., 2014). How these neuromodulator systems are dependent on various internal and external environmental factors, and therefore are involved in epigenetics and whether one system influences the epigenetic machinery to control the other system, are unresolved questions for upcoming studies (D'Addario et al., 2013). Future investigation in this field will be necessary to better delineate the neurobiological mechanisms underlying these neuroadaptations.

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REFERENCES

Aaltonen, N., Riera Ribas, C., Lehtonen, M., Savinainen, J. R., and Laitinen, J. T. (2014). Brain regional cannabinoid CB(1) receptor signalling and alternative enzymatic pathways for 2-arachidonoylglycerol generation in brain sections of diacylglycerol lipase deficient mice. *Eur. J. Pharm. Sci.* 51, 87–95. doi: 10.1016/j.ejps.2013.08.035

Agarwal, N., Pacher, P., Tegeder, I., Amaya, F., Constantin, C. E., Brenner, G. J., et al. (2007). Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat. Neurosci.* 10, 870–879. doi: 10.1038/nn1916

Ahn, K., Mckinney, M. K., and Cravatt, B. F. (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chem. Rev.* 108, 1687–1707. doi: 10.1021/cr0782067

Ansonoff, M. A., Zhang, J., Czyzyk, T., Rothman, R. B., Stewart, J., Xu, H., et al. (2006). Antinociceptive and hypothermic effects of salvinorin a are abolished in a novel strain of kappa-opioid receptor-1 knockout mice. *J. Pharmacol. Exp. Ther.* 318, 641–648. doi: 10.1124/jpet.106.101998

Belin, D., and Deroche-Gamonet, V. (2012). Responses to novelty and vulnerability to cocaine addiction: contribution of a multi-symptomatic animal model. *Cold Spring Harb. Perspect. Med.* 2:a011940. doi: 10.1101/cshperspect.a011940.

Bellocchio, L., Lafenetre, P., Cannich, A., Cota, D., Puente, N., Grandes, P., et al. (2010). Bimodal control of stimulated food intake by the endocannabinoid system. *Nat. Neurosci.* 13, 281–283. doi: 10.1038/nn.2494

Bellocchio, L., Soria-Gomez, E., Quarta, C., Metna-Laurent, M., Cardinal, P., Binder, E., et al. (2013). Activation of the sympathetic nervous system mediates hypophagic and anxiety-like effects of CB(1) receptor blockade. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4786–4791. doi: 10.1073/pnas.1218573110

Berrendero, F., Mendizabal, V., Murtra, P., Kieffer, B. L., and Maldonado, R. (2003). Cannabinoid receptor and WIN 55 212-2-stimulated [35S]-GTPgammaS binding in the brain of mu-, delta-, and kappa-opioid receptor knockout mice. *Eur. J. Neurosci.* 18, 2197–2202. doi: 10.1046/j.1460-9568.2003.02951.x

Bodnar, R. J. (2014). Endogenous opiates and behavior: 2013. *Peptides* 62C, 67–136. doi: 10.1016/j.peptides.2014.09.013

Brady, L. S., Herkenham, M., Rothman, R. B., Partilla, J. S., Konig, M., Zimmer, A. M., et al. (1999). Region-specific up-regulation of opioid receptor binding in enkephalin knockout mice. *Brain Res. Mol. Brain Res.* 68, 193–197. doi: 10.1016/S0169-328X(99)00090-X

Buckley, N. E., Mccoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., et al. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur. J. Pharmacol.* 396, 141–149. doi: 10.1016/S0014-2999(00)00211-9

Bushlin, I., Gupta, A., Stockton, S. D. Jr., Miller, L. K., and Devi, L. A. (2012). Dimerization with cannabinoid receptors allosterically modulates delta opioid receptor activity during neuropathic pain. *PLoS ONE* 7:e49789. doi: 10.1371/journal.pone.0049789

Castane, A., Robledo, P., Matifas, A., Kieffer, B. L., and Maldonado, R. (2003). Cannabinoid withdrawal syndrome is reduced in double mu and delta opioid receptor knockout mice. *Eur. J. Neurosci.* 17, 155–159. doi: 10.1046/j.1460-9568.2003.02409.x

Chanda, P. K., Gao, Y., Mark, L., Btesh, J., Strassle, B. W., Lu, P., et al. (2010). Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. *Mol. Pharmacol.* 78, 996–1003. doi: 10.1124/mol.110.068304

Charbogne, P., Kieffer, B. L., and Befort, K. (2014). 15 years of genetic approaches in vivo for addiction research: opioid receptor and peptide gene knockout in mouse models of drug abuse. *Neuropharmacology* 76(Pt B), 204–217. doi: 10.1016/j.neuropharm.2013.08.028

Chu Sin Chung, P., Keyworth, H. L., Martin-Garcia, E., Charbogne, P., Darcq, E., Bailey, A., et al. (2014). A novel anxiogenic role for the delta opioid receptor expressed in GABAergic forebrain neurons. *Biol. Psychiatry* doi: 10.1016/j.biopsych.2014.07.033 [Epub ahead of print].

Clarke, S., Czyzyk, T., Ansonoff, M., Nitsche, J. F., Hsu, M. S., Nilsson, L., et al. (2002). Autoradiography of opioid and ORL1 ligands in opioid receptor triple knockout mice. *Eur. J. Neurosci.* 16, 1705–1712. doi: 10.1046/j.1460-9568.2002.02239.x

Clarke, S., Zimmer, A., Zimmer, A. M., Hill, R. G., and Kitchen, I. (2003). Region selective up-regulation of micro-, delta- and kappa-opioid receptors but not opioid receptor-like 1 receptors in the brains of enkephalin and dynorphin knockout mice. *Neuroscience* 122, 479–489. doi: 10.1016/j.neuroscience.2003.07.011

Contet, C., Kieffer, B. L., and Befort, K. (2004). Mu opioid receptor: a gateway to drug addiction. *Curr. Opin. Neurobiol.* 14, 370–378. doi: 10.1016/j.conb.2004.05.005

Cossu, G., Ledent, C., Fattore, L., Imperato, A., Bohme, G. A., Parmentier, M., et al. (2001). Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. *Behav. Brain Res.* 118, 61–65. doi: 10.1016/S0166-4328(00)00311-9

Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., et al. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9371–9376. doi: 10.1073/pnas.161191698

Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384, 83–87. doi: 10.1038/384083a0

Cravatt, B. F., Saghatelian, A., Hawkins, E. G., Clement, A. B., Bracey, M. H., and Lichtman, A. H. (2004). Functional disassociation of the central and peripheral fatty acid amide signaling systems. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10821–10826. doi: 10.1073/pnas.0401292101

Cui, Y., Ostlund, S. B., James, A. S., Park, C. S., Ge, W., Roberts, K. W., et al. (2014). Targeted expression of mu-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nat. Neurosci.* 17, 254–261. doi: 10.1038/nn.3622

D'Addario, C., Di Francesco, A., Pucci, M., Finazzi Agro, A., and Maccarrone, M. (2013). Epigenetic mechanisms and endocannabinoid signalling. *FEBS J.* 280, 1905–1917. doi: 10.1111/febs.12125

De Luca, M. A., Valentini, V., Bimpisidis, Z., Cacciapaglia, F., Caboni, P., and Di Chiara, G. (2014). Endocannabinoid 2-arachidonoylglycerol self-administration by sprague-dawley rats and stimulation of in vivo dopamine transmission in the nucleus accumbens Shell. *Front. Psychiatry* 5:140. doi: 10.3389/fpsyt.2014.00140

De Petrocellis, L., and Di Marzo, V. (2010). Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. *J. Neuroimmune Pharmacol.* 5, 103–121. doi: 10.1007/s11481-009-9177-z

Dhopeshwarkar, A., and Mackie, K. (2014). CB2 cannabinoid receptors as a therapeutic target-what does the future hold? *Mol. Pharmacol.* 86, 430–437. doi: 10.1124/mol.114.094649

Di Marzo, V., Breivogel, C. S., Tao, Q., Bridgen, D. T., Razdan, R. K., Zimmer, A. M., et al. (2000). Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. *J. Neurochem.* 75, 2434–2444. doi: 10.1046/j.1471-4159.2000.0752434.x

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., et al. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10819–10824. doi: 10.1073/pnas.152334899

Dubreucq, S., Kambire, S., Conforzi, M., Metna-Laurent, M., Cannich, A., Soria-Gomez, E., et al. (2012a). Cannabinoid type 1 receptors located on single-minded 1-expressing neurons control emotional behaviors. *Neuroscience* 204, 230–244. doi: 10.1016/j.neuroscience.2011.08.049

Dubreucq, S., Matias, I., Cardinal, P., Haring, M., Lutz, B., Marsicano, G., et al. (2012b). Genetic dissection of the role of cannabinoid type-1 receptors in the emotional consequences of repeated social stress in mice. *Neuropsychopharmacology* 37, 1885–1900. doi: 10.1038/npp.2012.36

Erbs, E., Faget, L., Scherrer, G., Matifas, A., Filliol, D., Vonesch, J. L., et al. (2014). A mu-delta opioid receptor brain atlas reveals neuronal co-occurrence in subcortical networks. *Brain Struct. Funct.* doi: 10.1007/s00429-014-0717-9 [Epub ahead of print].

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

Everitt, B. J., and Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat. Neurosci.* 8, 1481–1489. doi: 10.1038/nn1579

Fattore, L., Cossu, G., Martellotta, C. M., and Fratta, W. (2001). Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212-2 in rats. *Psychopharmacology (Berl)* 156, 410–416. doi: 10.1007/s002130100734

Fattore, L., Deiana, S., Spano, S. M., Cossu, G., Fadda, P., Scherma, M., et al. (2005). Endocannabinoid system and opioid addiction: behavioural aspects. *Pharmacol. Biochem. Behav.* 81, 343–359. doi: 10.1016/j.pbb.2005.01.031

Fattore, L., and Fratta, W. (2011). Beyond THC: the new generation of cannabinoid designer drugs. *Front. Behav. Neurosci.* 5:60. doi: 10.3389/fnbeh.2011.00060

Fernandez-Ruiz, J., Hernandez, M., and Ramos, J. A. (2010). Cannabinoid-dopamine interaction in the pathophysiology and treatment of CNS disorders. *CNS Neurosci. Ther.* 16, e72–e91. doi: 10.1111/j.1755-5949.2010.00144.x

Ferre, S., Lluis, C., Justinova, Z., Quiroz, C., Orru, M., Navarro, G., et al. (2010). Adenosine-cannabinoid receptor interactions. Implications for striatal function. *Br. J. Pharmacol.* 160, 443–453. doi: 10.1111/j.1476-5381.2010.00723.x

Filizola, M., and Devi, L. A. (2014). Grand opening of structure-guided design for novel opioids. *Trends Pharmacol. Sci.* 34, 6–12. doi: 10.1016/j.tips.2012.10.002

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H. W., Simonin, F., et al. (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat. Genet.* 25, 195–200. doi: 10.1038/76061

Flores, A., Maldonado, R., and Berrendero, F. (2014). The hypocretin/orexin receptor-1 as a novel target to modulate cannabinoid reward. *Biol. Psychiatry* 75, 499–507. doi: 10.1016/j.biopsych.2013.06.012

Fowler, C. D., and Kenny, P. J. (2012). Utility of genetically modified mice for understanding the neurobiology of substance use disorders. *Hum. Genet.* 131, 941–957. doi: 10.1007/s00439-011-1129-z

Fratta, W., and Fattore, L. (2013). Molecular mechanisms of cannabinoid addiction. *Curr. Opin. Neurobiol.* 23, 487–492. doi: 10.1016/j.conb.2013.02.002

Frazier, C. J. (2011). Key questions of endocannabinoid signalling in the CNS: which, where and when? *J. Physiol.* 589, 4807–4808. doi: 10.1113/jphysiol.2011.219493

Fujita, W., Gomes, I., and Devi, L. A. (2014). Revolution in GPCR signalling: opioid receptor heteromers as novel therapeutic targets: IUPHAR review 10. *Br. J. Pharmacol.* 171, 4155–4176. doi: 10.1111/bph.12798

Gaffal, E., Cron, M., Glodde, N., Bald, T., Kuner, R., Zimmer, A., et al. (2013). Cannabinoid 1 receptors in keratinocytes modulate proinflammatory chemokine secretion and attenuate contact allergic inflammation. *J. Immunol.* 190, 4929–4936. doi: 10.4049/jimmunol.1201777

Gao, Y., Vasilyev, D. V., Goncalves, M. B., Howell, F. V., Hobbs, C., Reisenberg, M., et al. (2010). Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. *J. Neurosci.* 30, 2017–2024. doi: 10.1523/JNEUROSCI.5693-09.2010

Gardner, E. L. (2005). Endocannabinoid signaling system and brain reward: emphasis on dopamine. *Pharmacol. Biochem. Behav.* 81, 263–284. doi: 10.1016/j.pbb.2005.01.032

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

Gaveriaux-Ruff, C., Nozaki, C., Nadal, X., Hever, X. C., Weibel, R., Matifas, A., et al. (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain* 152, 1238–1248. doi: 10.1016/j.pain.2010.12.031

Gerald, T. M., Howlett, A. C., Ward, G. R., Ho, C., and Franklin, S. O. (2008). Gene expression of opioid and dopamine systems in mouse striatum: effects of CB1 receptors, age and sex. *Psychopharmacology (Berl.)* 198, 497–508. doi: 10.1007/s00213-008-1141–1148

Gerald, T. M., Ward, G. R., Howlett, A. C., and Franklin, S. O. (2006). CB1 knockout mice display significant changes in striatal opioid peptide and D4 dopamine receptor gene expression. *Brain Res.* 1093, 20–24. doi: 10.1016/j.brainres.2006.03.088

Ghozland, S., Matthes, H. W., Simonin, F., Filliol, D., Kieffer, B. L., and Maldonado, R. (2002). Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. *J. Neurosci.* 22, 1146–1154.

Gong, J. P., Onaivi, E. S., Ishiguro, H., Liu, Q. R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* 1071, 10–23. doi: 10.1016/j.brainres.2005.11.035

Goody, R. J., Oakley, S. M., Filliol, D., Kieffer, B. L., and Kitchen, I. (2002). Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice. *Brain Res.* 945, 9–19. doi: 10.1016/S0006-8993(02)02452-6

Gustavsson, A., Svensson, M., Jacobi, F., Allgulander, C., Alonso, J., Beghi, E., et al. (2011). Cost of disorders of the brain in Europe 2010. *Eur. Neuropsychopharmacol.* 21, 718–779. doi: 10.1016/j.euroneuro.2011.08.008

Gutierrez-Cuesta, J., Burokas, A., Mancino, S., Kummer, S., Martin-Garcia, E., and Maldonado, R. (2014). Effects of genetic deletion of endogenous opioid system components on the reinstatement of cocaine-seeking behavior in mice. *Neuropsychopharmacology.* doi: 10.1038/npp.2014.149

Han, J., Kesner, P., Metna-Laurent, M., Duan, T., Xu, L., Georges, F., et al. (2012). Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. *Cell* 148, 1039–1050. doi: 10.1016/j.cell.2012.01.037

Hough, L. B., Nalwalk, J. W., Chen, Y., Schuller, A., Zhu, Y., Zhang, J., et al. (2000). Improgan, a cimetidine analog, induces morphine-like antinociception in opioid receptor-knockout mice. *Brain Res.* 880, 102–108. doi: 10.1016/S0006-8993(00)02776-1

Ignatowska-Jankowska, B. M., Muldoon, P. P., Lichtman, A. H., and Damaj, M. I. (2013). The cannabinoid CB2 receptor is necessary for nicotine-conditioned place preference, but not other behavioral effects of nicotine in mice. *Psychopharmacology (Berl)* 229, 591–601. doi: 10.1007/s00213-013-3117-6

Jarai, Z., Wagner, J. A., Varga, K., Lake, K. D., Compton, D. R., Martin, B. R., et al. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14136–14141. doi: 10.1073/pnas.96.24.14136

Jeong, W. I., Osei-Hyiaman, D., Park, O., Liu, J., Batkai, S., Mukhopadhyay, P., et al. (2008). Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. *Cell Metab.* 7, 227–235. doi: 10.1016/j.cmet.2007.12.007

Kieffer, B. L. (1995). Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell Mol. Neurobiol.* 15, 615–635. doi: 10.1007/BF02071128

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

Kieffer, B. L., and Gaveriaux-Ruff, C. (2002). Exploring the opioid system by gene knockout. *Prog. Neurobiol.* 66, 285–306. doi: 10.1016/S0301-0082(02)00008-4

Kitchen, I., Slowe, S. J., Matthes, H. W., and Kieffer, B. (1997). Quantitative autoradiographic mapping of mu-, delta-, and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res.* 778, 73–88. doi: 10.1016/S0006-8993(97)00988-8

Konig, M., Zimmer, A. M., Steiner, H., Holmes, P. V., Crawley, J. N., Brownstein, M. J., et al. (1996). Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* 383, 535–538. doi: 10.1038/383535a0

Koob, G. F. (2009). Neurobiological substrates for the dark side of compulsivity in addiction. *Neuropharmacology* 56(Suppl. 1), 18–31. doi: 10.1016/j.neuropharm.2008.07.043

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

Kreek, M. J., Levran, O., Reed, B., Schlussman, S. D., Zhou, Y., and Butelman, E. R. (2012). Opiate addiction and cocaine addiction: underlying molecular neurobiology and genetics. *J. Clin. Invest.* 122, 3387–3393. doi: 10.1172/JCI60390

Le Merrer, J., Becker, J. A., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009

Le Naour, M., Akgun, E., Yekkirala, A., Lunzer, M. M., Powers, M. D., Kalyuzhny, A. E., et al. (2013). Bivalent ligands that target mu opioid (MOP) and cannabinoid1 (CB1) receptors are potent analgesics devoid of tolerance. *J. Med. Chem.* 56, 5505–5513. doi: 10.1021/jm4005219

Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J. F., Beslot, F., et al. (1999). Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* 283, 401–404. doi: 10.1126/science.283.5400.401

Leshner, A. I. (1997). Addiction is a brain disease, and it matters. *Science* 278, 45–47. doi: 10.1126/science.278.5335.45

Leung, D., Saghatelian, A., Simon, G. M., and Cravatt, B. F. (2006). Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry* 45, 4720–4726. doi: 10.1021/bi060163l

Lichtman, A. H., Sheikh, S. M., Loh, H. H., and Martin, B. R. (2001). Opioid and cannabinoid modulation of precipitated withdrawal in delta(9)-tetrahydrocannabinol and morphine-dependent mice. *J. Pharmacol. Exp. Ther.* 298, 1007–1014.

Liu, J., Wang, L., Harvey-White, J., Huang, B. X., Kim, H. Y., Luquet, S., et al. (2008). Multiple pathways involved in the biosynthesis of anandamide. *Neuropharmacology* 54, 1–7. doi: 10.1016/j.neuropharm.2007.05.020

Loacker, S., Sayyah, M., Wittmann, W., Herzog, H., and Schwarzer, C. (2007). Endogenous dynorphin in epileptogenesis and epilepsy: anticonvulsant net effect via kappa opioid receptors. *Brain* 130, 1017–1028. doi: 10.1093/brain/awl384

Loh, H. H., Liu, H. C., Cavalli, A., Yang, W., Chen, Y. F., and Wei, L. N. (1998). mu Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res. Mol. Brain Res.* 54, 321–326. doi: 10.1016/S0169-328X(97)00353-7

Lopez-Moreno, J. A., Lopez-Jimenez, A., Gorriti, M. A., and De Fonseca, F. R. (2010). Functional interactions between endogenous cannabinoid and opioid systems: focus on alcohol, genetics and drug-addicted behaviors. *Curr. Drug Targets* 11, 406–428. doi: 10.2174/138945010790980312

Lutz, P. E., Ayranci, G., Chu-Sin-Chung, P., Matifas, A., Koebel, P., Filliol, D., et al. (2014). Distinct mu, delta, and kappa opioid receptor mechanisms underlie low sociability and depressive-like behaviors during heroin abstinence. *Neuropsychopharmacology* 39, 2694–2705. doi: 10.1038/npp.2014.126

Lutz, P. E., and Kieffer, B. L. (2013a). The multiple facets of opioid receptor function: implications for addiction. *Curr. Opin. Neurobiol.* 23, 473–479. doi: 10.1016/j.conb.2013.02.005

Lutz, P. E., and Kieffer, B. L. (2013b). Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* 36, 195–206. doi: 10.1016/j.tins.2012.11.002

Maccarrone, M., Attina, M., Bari, M., Cartoni, A., Ledent, C., and Finazzi-Agro, A. (2001). Anandamide degradation and N-acylethanolamines level in wild-type and CB1 cannabinoid receptor knockout mice of different ages. *J. Neurochem.* 78, 339–348. doi: 10.1046/j.1471-4159.2001.00413.x

Maccarrone, M., Valverde, O., Barbaccia, M. L., Castane, A., Maldonado, R., Ledent, C., et al. (2002). Age-related changes of anandamide metabolism in CB1 cannabinoid receptor knockout mice: correlation with behaviour. *Eur. J. Neurosci.* 15, 1178–1186. doi: 10.1046/j.1460-9568.2002.01957.x

Mackie, K. (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb. Exp. Pharmacol.* 299–325.

Mackie, K. (2008). Cannabinoid receptors: where they are and what they do. *J. Neuroendocrinol.* 20(Suppl. 1), 10–14. doi: 10.1111/j.1365-2826.2008.01671.x

Maldonado, R. (2002). Study of cannabinoid dependence in animals. *Pharmacol. Ther.* 95, 153–164. doi: 10.1016/S0163-7258(02)00254-1

Maldonado, R., Berrendero, F., Ozaita, A., and Robledo, P. (2011). Neurochemical basis of cannabis addiction. *Neuroscience* 181, 1–17. doi: 10.1016/j.neuroscience.2011.02.035

Maldonado, R., Blendy, J. A., Tzavara, E., Gass, P., Roques, B. P., Hanoune, J., et al. (1996). Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 273, 657–659. doi: 10.1126/science.273.5275.657

Maldonado, R., Robledo, P., and Berrendero, F. (2013). Endocannabinoid system and drug addiction: new insights from mutant mice approaches. *Curr. Opin. Neurobiol.* 23, 480–486. doi: 10.1016/j.conb.2013.02.004

Maldonado, R., Valverde, O., and Berrendero, F. (2006). Involvement of the endocannabinoid system in drug addiction. *Trends Neurosci.* 29, 225–232. doi: 10.1016/j.tins.2006.01.008

Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U

Manzanares, J., Corchero, J., Romero, J., Fernandez-Ruiz, J. J., Ramos, J. A., and Fuentes, J. A. (1999). Pharmacological and biochemical interactions between opioids and cannabinoids. *Trends Pharmacol. Sci.* 20, 287–294. doi: 10.1016/S0165-6147(99)01339-5

Maresz, K., Pryce, G., Ponomarev, E. D., Marsicano, G., Croxford, J. L., Shriver, L. P., et al. (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat. Med.* 13, 492–497. doi: 10.1038/nm1561

Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., et al. (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88. doi: 10.1126/science.1088208

Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 418, 530–534. doi: 10.1038/nature00839

Martellotta, M. C., Cossu, G., Fattore, L., Gessa, G. L., and Fratta, W. (1998). Self-administration of the cannabinoid receptor agonist WIN 55,212-2 in drug-naive mice. *Neuroscience* 85, 327–330. doi: 10.1016/S0306-4522(98)00052-9

Martin, M., Ledent, C., Parmentier, M., Maldonado, R., and Valverde, O. (2000). Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. *Eur. J. Neurosci.* 12, 4038–4046. doi: 10.1046/j.1460-9568.2000.00287.x

Mascia, M. S., Obinu, M. C., Ledent, C., Parmentier, M., Bohme, G. A., Imperato, A., et al. (1999). Lack of morphine-induced dopamine release in the nucleus accumbens of cannabinoid CB(1) receptor knockout mice. *Eur. J. Pharmacol* 383, R1–R2. doi: 10.1016/S0014-2999(99)00656-1

Massotte, D. (2014). In vivo opioid receptor heteromerization: where do we stand? *Br. J. Pharmacol.* 172, 420–434. doi: 10.1111/bph.12702

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561–564. doi: 10.1038/346561a0

Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383, 819–823. doi: 10.1038/383819a0

Mendizabal, V., Zimmer, A., and Maldonado, R. (2006). Involvement of kappa/dynorphin system in WIN 55,212-2 self-administration in mice. *Neuropsychopharmacology* 31, 1957–1966. doi: 10.1038/sj.npp.1300957

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

Mizoguchi, H., Watanabe, C., Osada, S., Yoshioka, M., Aoki, Y., Natsui, S., et al. (2010). Lack of a rewarding effect and a locomotor-enhancing effect of the selective mu-opioid receptor agonist amidino-TAPA. *Psychopharmacology (Berl)* 212, 215–225. doi: 10.1007/s00213-010-1946-0

Monory, K., Blaudzun, H., Massa, F., Kaiser, N., Lemberger, T., Schutz, G., et al. (2007). Genetic dissection of behavioural and autonomic effects of Delta(9)-tetrahydrocannabinol in mice. *PLoS Biol.* 5:e269. doi: 10.1371/journal.pbio.0050269

Monory, K., Massa, F., Egertova, M., Eder, M., Blaudzun, H., Westenbroek, R., et al. (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* 51, 455–466. doi: 10.1016/j.neuron.2006.07.006

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365, 61–65. doi: 10.1038/365061a0

Nadal, X., La Porta, C., Andreea Bura, S., and Maldonado, R. (2013). Involvement of the opioid and cannabinoid systems in pain control: new insights from knockout studies. *Eur. J. Pharmacol.* 716, 142–157. doi: 10.1016/j.ejphar.2013.01.077

Navarrete, F., Rodriguez-Arias, M., Martin-Garcia, E., Navarro, D., Garcia-Gutierrez, M. S., Aguilar, M. A., et al. (2013). Role of CB2 cannabinoid receptors in the rewarding, reinforcing, and physical effects of nicotine. *Neuropsychopharmacology* 38, 2515–2524. doi: 10.1038/npp.2013.157

Nestler, E. J. (2014). Epigenetic mechanisms of drug addiction. *Neuropharmacology* 76 Pt B, 259–268. doi: 10.1016/j.neuropharm.2013.04.004

Niikura, K., Narita, M., Okutsu, D., Tsurukawa, Y., Nanjo, K., Kurahashi, K., et al. (2008). Implication of endogenous beta-endorphin in the inhibition of the morphine-induced rewarding effect by the direct activation of spinal protein kinase C in mice. *Neurosci. Lett.* 433, 54–58. doi: 10.1016/j.neulet.2007.12.042

Nogueiras, R., Romero-Pico, A., Vazquez, M. J., Novelle, M. G., Lopez, M., and Dieguez, C. (2014). The opioid system and food intake: homeostatic and hedonic mechanisms. *Obes. Facts* 5, 196–207. doi: 10.1159/000338163

Oakley, S. M., Toth, G., Borsodi, A., Kieffer, B. L., and Kitchen, I. (2003). G-protein coupling of delta-opioid receptors in brains of mu-opioid receptor knockout mice. *Eur. J. Pharmacol.* 466, 91–98. doi: 10.1016/S0014-2999(03)01531-0

Ohno-Shosaku, T., and Kano, M. (2014). Endocannabinoid-mediated retrograde modulation of synaptic transmission. *Curr. Opin. Neurobiol.* 29C, 1–8. doi: 10.1016/j.conb.2014.03.017

Onaivi, E. S., Ishiguro, H., Gong, J. P., Patel, S., Perchuk, A., Meozzi, P. A., et al. (2006). Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann. N. Y. Acad. Sci.* 1074, 514–536. doi: 10.1196/annals.1369.052

Ortega-Alvaro, A., Ternianov, A., Aracil-Fernandez, A., Navarrete, F., Garcia-Gutierrez, M. S., and Manzanares, J. (2013). Role of cannabinoid CB receptor in the reinforcing actions of ethanol. *Addict. Biol. doi:* 10.1111/adb.12076

Pan, Y. X., Xu, J., Xu, M., Rossi, G. C., Matulonis, J. E., and Pasternak, G. W. (2009). Involvement of exon 11-associated variants of the mu opioid receptor MOR-1 in heroin, but not morphine, actions. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4917–4922. doi: 10.1073/pnas.0811586106

Panagis, G., Mackey, B., and Vlachou, S. (2014). Cannabinoid regulation of brain reward processing with an emphasis on the role of CB1 receptors: a step back into the future. *Front. Psychiatry* 5:92. doi: 10.3389/fpsyt.2014.00092

Panlilio, L. V., and Goldberg, S. R. (2007). Self-administration of drugs in animals and humans as a model and an investigative tool. *Addiction* 102, 1863–1870. doi: 10.1111/j.1360-0443.2007.02011.x

Panlilio, L. V., Justinova, Z., and Goldberg, S. R. (2010). Animal models of cannabinoid reward. *Br. J. Pharmacol.* 160, 499–510. doi: 10.1111/j.1476-5381.2010.00775.x

Parolaro, D., Rubino, T., Vigano, D., Massi, P., Guidali, C., and Realini, N. (2010). Cellular mechanisms underlying the interaction between cannabinoid and opioid system. *Curr. Drug Targets* 11, 393–405. doi: 10.2174/138945010790980367

Pattij, T., and De Vries, T. J. (2013). The role of impulsivity in relapse vulnerability. *Curr. Opin. Neurobiol.* 23, 700–705. doi: 10.1016/j.conb.2013.01.023

Pertwee, R. G. (2010). Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Curr. Med. Chem* 17, 1360–1381. doi: 10.2174/092986710790980050

Petrenko, A. B., Yamazaki, M., Sakimura, K., Kano, M., and Baba, H. (2014). Augmented tonic pain-related behavior in knockout mice lacking monoacylglycerol lipase, a major degrading enzyme for the endocannabinoid 2-arachidonoylglycerol. *Behav. Brain Res.* 271, 51–58. doi: 10.1016/j.bbr.2014.05.063

Piomelli, D. (2014). More surprises lying ahead. The endocannabinoids keep us guessing. *Neuropharmacology* 76 Pt B, 228–234. doi: 10.1016/j.neuropharm.2013.07.026

Pol, O., Murtra, P., Caracuel, L., Valverde, O., Puig, M. M., and Maldonado, R. (2006). Expression of opioid receptors and c-fos in CB1 knockout mice exposed to neuropathic pain. *Neuropharmacology* 50, 123–132. doi: 10.1016/j.neuropharm.2005.11.002

Pradhan, A. A., Befort, K., Nozaki, C., Gaveriaux-Ruff, C., and Kieffer, B. L. (2011). The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol. Sci.* 32, 581–590. doi: 10.1016/j.tips.2011.06.008

Pryce, G., Visintin, C., Ramagopalan, S. V., Al-Izki, S., De Faveri, L. E., Nuamah, R. A., et al. (2014). Control of spasticity in a multiple sclerosis model using

central nervous system-excluded CB1 cannabinoid receptor agonists. *FASEB J.* 28, 117–130. doi: 10.1096/fj.13–239442

Ragnauth, A., Schuller, A., Morgan, M., Chan, J., Ogawa, S., Pintar, J., et al. (2001). Female preproenkephalin-knockout mice display altered emotional responses. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1958–1963. doi: 10.1073/pnas.041598498

Ramesh, D., Gamage, T. F., Vanuytsel, T., Owens, R. A., Abdullah, R. A., Niphakis, M. J., et al. (2013). Dual inhibition of endocannabinoid catabolic enzymes produces enhanced antiwithdrawal effects in morphine-dependent mice. *Neuropsychopharmacology* 38, 1039–1049. doi: 10.1038/npp.2012.269

Ramesh, D., Ross, G. R., Schlosburg, J. E., Owens, R. A., Abdullah, R. A., Kinsey, S. G., et al. (2011). Blockade of endocannabinoid hydrolytic enzymes attenuates precipitated opioid withdrawal symptoms in mice. *J. Pharmacol. Exp. Ther.* 339, 173–185. doi: 10.1124/jpet.111.181370

Rice, O. V., Gordon, N., and Gifford, A. N. (2002). Conditioned place preference to morphine in cannabinoid CB1 receptor knockout mice. *Brain Res.* 945, 135–138. doi: 10.1016/S0006-8993(02)02890-1

Robbe, D., Kopf, M., Remaury, A., Bockaert, J., and Manzoni, O. J. (2002). Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8384–8388. doi: 10.1073/pnas.122149199

Robinson, T. E., and Berridge, K. C. (2008). Review. The incentive sensitization theory of addiction: some current issues. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 3137–3146. doi: 10.1098/rstb.2008.0093

Robledo, P., Berrendero, F., Ozaita, A., and Maldonado, R. (2008). Advances in the field of cannabinoid–opioid cross-talk. *Addict. Biol.* 13, 213–224. doi: 10.1111/j.1369-1600.2008.00107.x

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

Rubinstein, M., Mogil, J. S., Japon, M., Chan, E. C., Allen, R. G., and Low, M. J. (1996). Absence of opioid stress-induced analgesia in mice lacking beta-endorphin by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3995–4000. doi: 10.1073/pnas.93.9.3995

Ruehle, S., Remmers, F., Romo-Parra, H., Massa, F., Wickert, M., Wortge, S., et al. (2013). Cannabinoid CB1 receptor in dorsal telencephalic glutamatergic neurons: distinctive sufficiency for hippocampus-dependent and amygdala-dependent synaptic and behavioral functions. *J. Neurosci.* 33, 10264–10277. doi: 10.1523/JNEUROSCI.4171-12.2013

Sanchis-Segura, C., Cline, B. H., Marsicano, G., Lutz, B., and Spanagel, R. (2004). Reduced sensitivity to reward in CB1 knockout mice. *Psychopharmacology (Berl)* 176, 223–232. doi: 10.1007/s00213-004-1877–1878

Sanchis-Segura, C., and Spanagel, R. (2006). Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addict. Biol.* 11, 2–38. doi: 10.1111/j.1369-1600.2006.00012.x

Saunders, B. T., and Robinson, T. E. (2013). Individual variation in resisting temptation: implications for addiction. *Neurosci. Biobehav. Rev.* 37, 1955–1975. doi: 10.1016/j.neubiorev.2013.02.008

Scavone, J. L., Sterling, R. C., and Van Bockstaele, E. J. (2013). Cannabinoid and opioid interactions: implications for opiate dependence and withdrawal. *Neuroscience* 248, 637–654. doi: 10.1016/j.neuroscience.2013.04.034

Schlosburg, J. E., Blankman, J. L., Long, J. Z., Nomura, D. K., Pan, B., Kinsey, S. G., et al. (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat. Neurosci.* 13, 1113–1119. doi: 10.1038/nn.2616

Schuller, A. G., King, M. A., Zhang, J., Bolan, E., Pan, Y. X., Morgan, D. J., et al. (1999). Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat. Neurosci.* 2, 151–156. doi: 10.1038/5706

Sharifi, N., Diehl, N., Yaswen, L., Brennan, M. B., and Hochgeschwender, U. (2001). Generation of dynorphin knockout mice. *Brain Res. Mol. Brain Res.* 86, 70–75. doi: 10.1016/S0169-328X(00)00264-3

Simonin, F., Befort, K., Gaveriaux-Ruff, C., Matthes, H., Nappey, V., Lannes, B., et al. (1994). The human delta-opioid receptor: genomic organization, cDNA cloning, functional expression, and distribution in human brain. *Mol. Pharmacol.* 46, 1015–1021.

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

Simonin, F., Slowe, S., Becker, J. A., Matthes, H. W., Filliol, D., Chluba, J., et al. (2001). Analysis of [3H]bremazocine binding in single and combinatorial opioid receptor knockout mice. *Eur. J. Pharmacol.* 414, 189–195. doi: 10.1016/S0014-2999(01)00822-6

Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., et al. (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J.* 17, 886–897. doi: 10.1093/emboj/17.4.886

Skoubis, P. D., Lam, H. A., Shoblock, J., Narayanan, S., and Maidment, N. T. (2005). Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur. J. Neurosci.* 21, 1379–1384. doi: 10.1111/j.1460-9568.2005.03956.x

Slowe, S. J., Simonin, F., Kieffer, B., and Kitchen, I. (1999). Quantitative autoradiography of mu-delta- and kappa1 opioid receptors in kappa-opioid receptor knockout mice. *Brain Res.* 818, 335–345. doi: 10.1016/S0006-8993(98)01201-3

Solinas, M., Goldberg, S. R., and Piomelli, D. (2008). The endocannabinoid system in brain reward processes. *Br. J. Pharmacol.* 154, 369–383. doi: 10.1038/bjp.2008.130

Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X. F., Hall, F. S., et al. (2001). Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu receptor reserve. *Neuropsychopharmacology* 25, 41–54. doi: 10.1016/S0893-133X(00)00252-9

Stein, C. (2013). Opioids, sensory systems and chronic pain. *Eur. J. Pharmacol.* 716, 179–187. doi: 10.1016/j.ejphar.2013.01.076

Steiner, H., Bonner, T. I., Zimmer, A. M., Kitai, S. T., and Zimmer, A. (1999). Altered gene expression in striatal projection neurons in CB1 cannabinoid receptor knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5786–5790. doi: 10.1073/pnas.96.10.5786

Sun, X., Wang, H., Okabe, M., Mackie, K., Kingsley, P. J., Marnett, L. J., et al. (2009). Genetic loss of faah compromises male fertility in mice. *Biol. Reprod.* 80, 235–242. doi: 10.1095/biolreprod.108.072736

Swendsen, J., and Le Moal, M. (2011). Individual vulnerability to addiction. *Ann. N. Y. Acad. Sci.* 1216, 73–85. doi: 10.1111/j.1749-6632.2010.05894.x

Szutorisz, H., Dinieri, J. A., Sweet, E., Egervari, G., Michaelides, M., Carter, J. M., et al. (2014). Parental THC exposure leads to compulsive heroin-seeking and altered striatal synaptic plasticity in the subsequent generation. *Neuropsychopharmacology* 39, 1315–1323. doi: 10.1038/npp.2013.352

Tanimura, A., Yamazaki, M., Hashimotodani, Y., Uchigashima, M., Kawata, S., Abe, M., et al. (2010). The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. *Neuron* 65, 320–327. doi: 10.1016/j.neuron.2010.01.021

Taschler, U., Radner, F. P., Heier, C., Schreiber, R., Schweiger, M., Schoiswohl, G., et al. (2011). Monoglyceride lipase deficiency in mice impairs lipolysis and attenuates diet-induced insulin resistance. *J. Biol. Chem.* 286, 17467–17477. doi: 10.1074/jbc.M110.215434

Tebano, M. T., Martire, A., and Popoli, P. (2012). Adenosine A(2A)-cannabinoid CB(1) receptor interaction: an integrative mechanism in striatal glutamatergic neurotransmission. *Brain Res.* 1476, 108–118. doi: 10.1016/j.brainres.2012.04.051

Tian, M., Broxmeyer, H. E., Fan, Y., Lai, Z., Zhang, S., Aronica, S., et al. (1997). Altered hematopoiesis, behavior, and sexual function in mu opioid receptor-deficient mice. *J. Exp. Med.* 185, 1517–1522. doi: 10.1084/jem.185.8.1517

Tomasiewicz, H. C., Jacobs, M. M., Wilkinson, M. B., Wilson, S. P., Nestler, E. J., and Hurd, Y. L. (2012). Proenkephalin mediates the enduring effects of adolescent cannabis exposure associated with adult opiate vulnerability. *Biol. Psychiatry* 72, 803–810. doi: 10.1016/j.biopsych.2012.04.026

Trigo, J. M., Martin-Garcia, E., Berrendero, F., Robledo, P., and Maldonado, R. (2010). The endogenous opioid system: a common substrate in drug addiction. *Drug Alcohol Depend.* 108, 183–194. doi: 10.1016/j.drugalcdep.2009.10.011

Tsuboi, K., Okamoto, Y., Ikematsu, N., Inoue, M., Shimizu, Y., Uyama, T., et al. (2011). Enzymatic formation of N-acylethanolamines from N-acylethanolamine plasmalogen through N-acylphosphatidylethanolamine-hydrolyzing phospholipase D-dependent and -independent pathways. *Biochim. Biophys. Acta* 1811, 565–577. doi: 10.1016/j.bbalip.2011.07.009

Tzschentke, T. M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.* 12, 227–462. doi: 10.1111/j.1369-1600.2007.00070.x

Uchigashima, M., Yamazaki, M., Yamasaki, M., Tanimura, A., Sakimura, K., Kano, M., et al. (2011). Molecular and morphological configuration for 2-arachidonoylglycerol-mediated retrograde signaling at mossy cell-granule cell synapses in the dentate gyrus. *J. Neurosci.* 31, 7700–7714. doi: 10.1523/JNEUROSCI.5665-10.2011

Uriguen, L., Berrendero, F., Ledent, C., Maldonado, R., and Manzanares, J. (2005). Kappa- and delta-opioid receptor functional activities are increased in the caudate putamen of cannabinoid CB1 receptor knockout mice. *Eur. J. Neurosci.* 22, 2106–2110. doi: 10.1111/j.1460-9568.2005.04372.x

Valverde, O., Maldonado, R., Valjent, E., Zimmer, A. M., and Zimmer, A. (2000). Cannabinoid withdrawal syndrome is reduced in pre-proenkephalin knock-out mice. *J. Neurosci.* 20, 9284–9289.

Valverde, O., and Torrens, M. (2012). CB1 receptor-deficient mice as a model for depression. *Neuroscience* 204, 193–206. doi: 10.1016/j.neuroscience.2011.09.031

Van't Veer, A., Bechtholt, A. J., Onvani, S., Potter, D., Wang, Y., Liu-Chen, L. Y., et al. (2013). Ablation of kappa-opioid receptors from brain dopamine neurons has anxiolytic-like effects and enhances cocaine-induced plasticity. *Neuropsychopharmacology* 38, 1585–1597. doi: 10.1038/npp.2013.58

van Rijn, R. M., and Whistler, J. L. (2009). The delta(1) opioid receptor is a heterodimer that opposes the actions of the delta(2) receptor on alcohol intake. *Biol. Psychiatry* 66, 777–784. doi: 10.1016/j.biopsych.2009.05.019

Vigano, D., Rubino, T., and Parolaro, D. (2005). Molecular and cellular basis of cannabinoid and opioid interactions. *Pharmacol. Biochem. Behav.* 81, 360–368. doi: 10.1016/j.pbb.2005.01.021

Vinod, K. Y., Sanguino, E., Yalamanchili, R., Manzanares, J., and Hungund, B. L. (2008). Manipulation of fatty acid amide hydrolase functional activity alters sensitivity and dependence to ethanol. *J. Neurochem.* 104, 233–243. doi: 10.1111/j.1471-4159.2007.04956.x

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

Wise, L. E., Shelton, C. C., Cravatt, B. F., Martin, B. R., and Lichtman, A. H. (2007). Assessment of anandamide's pharmacological effects in mice deficient of both fatty acid amide hydrolase and cannabinoid CB1 receptors. *Eur. J. Pharmacol.* 557, 44–48. doi: 10.1016/j.ejphar.2006.11.002

Wotherspoon, G., Fox, A., Mcintyre, P., Colley, S., Bevan, S., and Winter, J. (2005). Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* 135, 235–245. doi: 10.1016/j.neuroscience.2005.06.009

Xi, Z. X., Peng, X. Q., Li, X., Song, R., Zhang, H. Y., Liu, Q. R., et al. (2011). Brain cannabinoid CB(2) receptors modulate cocaine's actions in mice. *Nat. Neurosci.* 14, 1160–1166. doi: 10.1038/nn.2874

Yaswen, L., Diehl, N., Brennan, M. B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat. Med.* 5, 1066–1070. doi: 10.1038/12506

Yoshino, H., Miyamae, T., Hansen, G., Zambrowicz, B., Flynn, M., Pedicord, D., et al. (2011). Postsynaptic diacylglycerol lipase mediates retrograde endocannabinoid suppression of inhibition in mouse prefrontal cortex. *J. Physiol.* 589, 4857–4884. doi: 10.1113/jphysiol.2011.212225

Zanettini, C., Panlilio, L. V., Alicki, M., Goldberg, S. R., Haller, J., and Yasar, S. (2011). Effects of endocannabinoid system modulation on cognitive and emotional behavior. *Front. Behav. Neurosci.* 5:57. doi: 10.3389/fnbeh.2011.00057

Zhang, H. Y., Gao, M., Liu, Q. R., Bi, G. H., Li, X., Yang, H. J., et al. (2014). Cannabinoid CB2 receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice. *Proc. Natl. Acad. Sci. U.S.A.* doi: 10.1073/pnas.1413210111

Zhu, Y., King, M. A., Schuller, A. G., Nitsche, J. F., Reidl, M., Elde, R. P., et al. (1999). Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron* 24, 243–252. doi: 10.1016/S0896-6273(00)80836-3

Zimmer, A., Valjent, E., Konig, M., Zimmer, A. M., Robledo, P., Hahn, H., et al. (2001). Absence of delta-9-tetrahydrocannabinol dysphoric effects in dynorphin-deficient mice. *J. Neurosci.* 21, 9499–9505.

Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M., and Bonner, T. I. (1999). Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5780–5785. doi: 10.1073/pnas.96.10.5780

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Utilizing GCaMP transgenic mice to monitor endogenous $G_{q/11}$-coupled receptors

John G. Partridge [1,2] *

[1] Department of Pharmacology and Physiology, Georgetown University School of Medicine, Washington, DC, USA
[2] Interdisciplinary Program in Neuroscience, Georgetown University School of Medicine, Washington, DC, USA

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Andrzej Pilc, Polish Academy of Sciences, Poland
Loren L. Looger, Howard Hughes Medical Institute, USA

***Correspondence:**
John G. Partridge, Department of Pharmacology and Physiology, Georgetown University School of Medicine, Basic Science Building, Room 235, 3900 Reservoir Road, Washington, DC 20007, USA
e-mail: jp374@georgetown.edu

The family of GCaMPs are engineered proteins that contain Ca^{2+} binding motifs within a circularly permutated variant of the *Aequorea Victoria* green fluorescent protein (cp-GFP). The rapidly advancing field of utilizing GCaMP reporter constructs represents a major step forward in our ability to monitor intracellular Ca^{2+} dynamics. With the use of these genetically encoded Ca^{2+} sensors, investigators have studied activation of endogenous G_q types of G protein-coupled receptors (GPCRs) and subsequent rises in intracellular calcium. Escalations in intracellular Ca^{2+} from GPCR activation can be faithfully monitored in space and time as an increase in fluorescent emission from these proteins. Further, transgenic mice are now commercially available that express GCaMPs in a Cre recombinase dependent fashion. These GCaMP reporter mice can be bred to distinct Cre recombinase driver mice to direct expression of this sensor in unique populations of cells. Concerning the central nervous system (CNS), sources of calcium influx, including those arising from G_q activation can be observed in targeted cell types like neurons or astrocytes. This powerful genetic method allows simultaneous monitoring of the activity of dozens of cells upon activation of endogenous G_q-coupled GPCRs. Therefore, in combination with pharmacological tools, this strategy of monitoring GPCR activation is amenable to analysis of orthosteric and allosteric ligands of G_q-coupled receptors in their endogenous environments.

Keywords: GCaMP, Cre-loxP, G protein-coupled receptor, Ca^{2+} measurement

INTRODUCTION

Guanosine nucleotide-binding proteins (G proteins) are intracellular proteins involved in transmitting signals from outside a cell to the inside of the cell (Oldham and Hamm, 2008). Since their initial detection in the 1960's by Nobel laureates Martin Rodbell and Alfred Gilman, heterotrimeric forms of G proteins (G_α, G_β and G_γ) have received much consideration in the general fields of pharmacology, biochemistry and neuroscience. This is justified as their interacting, coupled receptors have been an established source of clinically active therapeutics. Several G proteins contain lipid modifications on one or more of their subunits to enable targeting to the plasma membrane while facilitating protein interactions. The precise arrangement and targeting of subunits in heterotrimeric G proteins affects not only which receptor with which it can interact, but also the downstream effector target. This general scheme of extracellular signal transduction has been selected for across evolution and has been repeated in nature abundantly. Built-in flexibilities originating from unique receptors, G-proteins and effectors provide the means to distribute distinct physiological response pathways to external stimuli ranging from photons to complex protein hormones (Katritch et al., 2013).

There are ∼16 genes found in human that encode different forms of G_α which belong to a larger group of enzymes called GTPases. The G_α subunit of heterotrimeic G proteins is highly controlled by factors that influence its ability to bind to guanosine triphosphate (GTP). The GTPase activity of G_α proteins hydrolyze GTP to guanosine diphosphate (GDP). When bound by GTP, G_α is considered in an active state and when bound by GDP, G_α is in a less active state (Limbird, 2004). The time course of the G protein signal is controlled by the duration of the GTP-bound alpha subunit, which can be regulated by RGS (regulators of G protein signaling) proteins, GEFs (guanine nucleotide exchange factors) or by covalent modifications. G_α subunits mediate the signal transduction pathway that initiates from an agonist occupied receptor to numerous intracellular effector proteins. For example, $G_\alpha$subunits in the G_α family stimulate the production of 3′-5′-cyclic adenosine monophosphate (cAMP) by activation of adenylyl cyclase. Another prominent branch of this family of biological signaling tools includes $G_{\alpha q}$.

$G_{\alpha q}$ and a closely related gene $G_{\alpha 11}$, are broadly expressed and maintain homeostatic processes in digestive, urinary, cardiovascular and central nervous systems (CNS). It is critical to appreciate that activated $G_{q/11}$ results in several parallel signaling pathways that include mitogen activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)/AKT pathways. However, the pathway in which GTP bound $G_{q/11}$ (as well as some combinations of $G_{\beta\gamma}$) can stimulate the activity of the

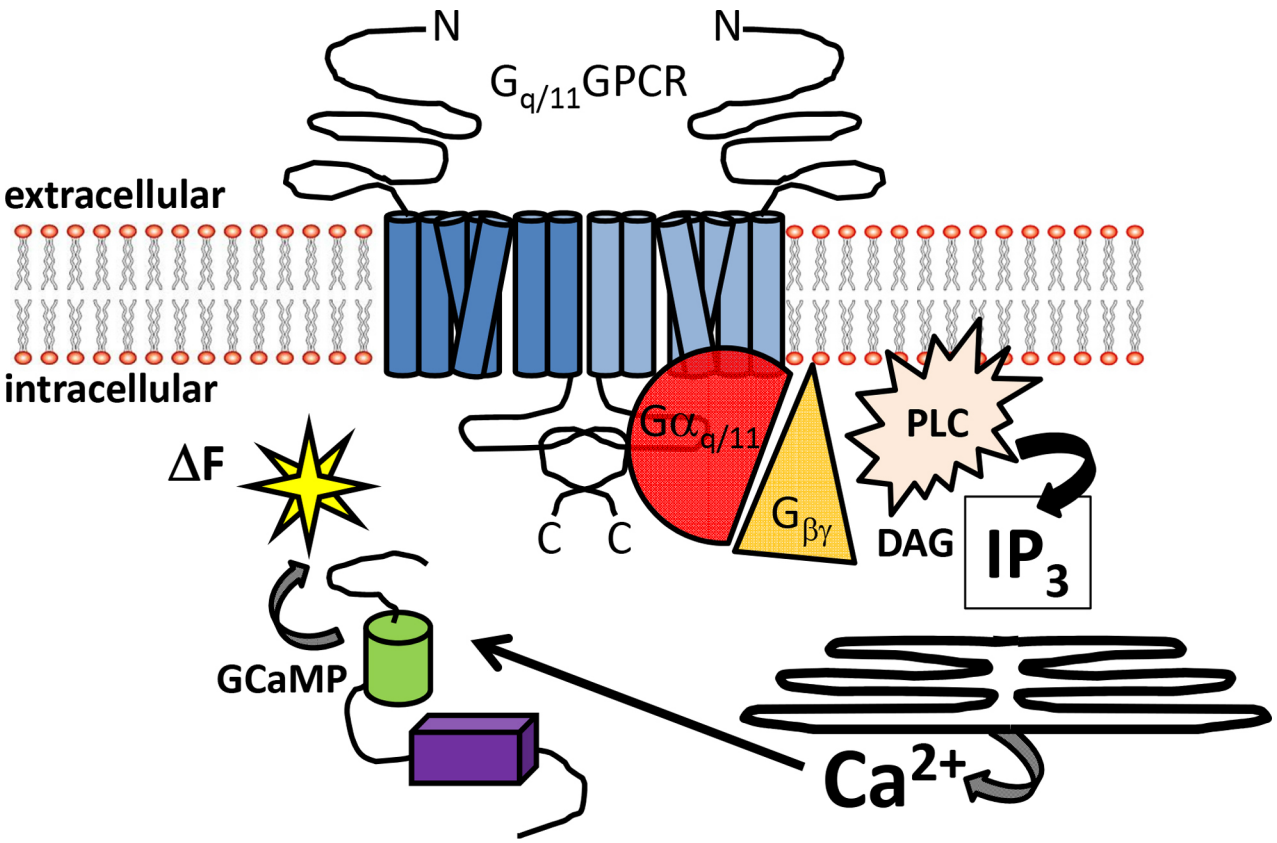

FIGURE 1 | Overview of $G_{q/11}$-coupled GPCR signal detection by GCaMP sensors. Schematic diagram illustrating the canonical signaling pathway of $G_{q/11}$-coupled GPCRs. Heptahelical dimeric receptors bound by agonist can activate the $G_{q/11}$ pathway to increase intracellular Ca^{2+}. The signaling intermediaries facilitating Ca^{2+} elevations include generation of IP_3 and DAG from PLC activation, gating of IP_3 receptors embedded in the ER and release of intracellular Ca^{2+} stores. Free Ca^{2+} can then bind to GCaMP sensors in the cytoplasm, resulting in enhanced fluorescence. Abbreviations: N: amino-terminus C: carboxy-terminus; PLC: phospholipase C; DAG: diacylglycerol, IP_3: inositol trisphosphate; ΔF: change in fluorescence.

effector protein phospholipase Cβ (PLCβ) is the most studied (Wettschureck and Offermanns, 2005). PLCβ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacyl glycerol (DAG) and inositol trisphosphate (IP_3). An increase in intracellular IP_3 rapidly gates ionotropic IP_3 receptors embedded in membranes of endoplasmic reticulum (ER). IP_3 receptor activation results in the flux of calcium ions (Ca^{2+}) from highly concentrated internal ER stores to low concentration intracellular cytoplasmic regions via channel gating (**Figure 1**). It is this branch of the canonical $G_{\alpha q/11}$ signal transduction pathway that will be the focal point of this review.

A vast amount of research has recognized an enormous array of extracellular and intracellular stimuli that dictate changes in the intracellular second messenger Ca^{2+}. The concentration over time profile of this divalent cation has variable functions in nearly every cell type throughout the animal kingdom (Berridge, 2006). Cells devote considerable energy in adjusting and maintaining a steep gradient between intracellular (<1 uM) and extracellular (>1 mM) Ca^{2+} concentrations. Intracellular calcium signals regulate processes that operate over time ranges varying from milliseconds to days. One general class of calcium mobilizing external stimuli includes agonists acting at $G_{q/11}$-coupled receptors. Examples of some G protein-coupled receptors (GPCRs) that preferentially interact with $G_{q/11}$ include the group I metabotropic glutamate; M1, M3, and M5 muscarinic acetylcholine; 5-HT_2 serotonergic, α1 adrenergic, vasopressin, angiotensin II and histamine H1 receptors among several others.

A great deal of our knowledge base of these receptor subtypes has its origins in the cloning era of these genes. The coding regions of many $G_{q/11}$-coupled GPCRs were inserted into recombinant, mammalian directed expression vectors and subsequently driven to be transcribed by strong promotors into a variety of host cells. Some of these expression studies have greatly contributed to our atomic level structural understanding and knowledge of these critical receptor subtypes. Expression strategies have served many other useful purposes including pharmacological profiling, detailed determination of signal transduction pathways as well as site-directed mutagenesis studies of critical amino acids involved in structure, function and ligand binding. However, one caveat with this general paradigm is the issue of over-expression of the receptor.

Does placing too many of the same receptor type bias a signal transduction pathway through mass action relationships? Can over-expression lead to too many spare receptors and lead

to aberrant constitutive activity? Questions like these have been addressed and will need to be monitored heading into the future utilizing this set of critical tools and methodologies. Nevertheless, methods examining $G_{\alpha q/11}$-coupled GPCRs in their endogenous states, which more closely reflect the natural environment, are becoming sharper and increasingly more available. The focus of this review article will address combining methods and paradigms from the fields of calcium imaging, mouse genetics and pharmacology to uncover endogenous $G_{\alpha q/11}$-coupled GPCRs and their responses to acute or sustained stimulation at the molecular and cellular levels.

GENETIC IDENTIFICATION OF CELLS WITH ENDOGENOUS GPCRs

Investigations into the role of endogenous $G_{q/11}$-coupled GPCRs in selective cells has become more prevalent using the power of transgenic animals. Temporally and spatially regulated genes can be monitored with fluorescent microscopy in mice by utilizing a DNA recombination system based on Cre recombinase. Cre based systems using P1 bacteriophage Cre recombinase which catalyze the excision of DNA located between flanking loxP sites, has been widely used since its first application in eukaryotic cells and transgenic mice (Sauer, 1987; Novak et al., 2000). Because recombination does not occur between the loxP sites until Cre is introduced, the modifications are termed conditional alterations. It is a conditional situation based on where and when the Cre recombinase gene is expressed. This powerful strategy permits the design of mouse lines with silent genetic manipulations (i.e., the flanking loxP sites) that can be un-silenced by Cre mediated recombination. In the beginning years of the twenty-first century, it became common in many laboratories to breed unique Cre recombinase "driver" mice to a mate carrying a silent flanking loxP mutation. Offspring could be produced that eliminated a coding region of a gene to generate tissue selective knock-out of a gene of interest. More recently, a variant of this general strategy has become popular by placing stop codons in between the loxP sites so as to "report" a gene, rather than eliminate it (Madisen et al., 2010). The usefulness of this technique is enhanced with distinct, commercially available reporter lines of mice (**Figure 2**). Illustrated in **Figure 2** is an example of this latter method that permits fluorescent identification of target cells or tissues that may express a $G_{q/11}$-coupled GPCR like neurons in layer five of cortex or striatal projection neurons.

A more straightforward genetic alternative to this approach includes the use of mice carrying bacterial artificial chromosome (BAC) transgenes. It is now routine to accurately drive the expression of genetically programmed fluorescent reporters (e.g., eGFP or tdTomato) or Cre recombinase in specific cellular populations with these large (150–350 kbp) transgenes. The GENSAT (Gene Expression Nervous System Atlas) project has used this technology to generate mouse lines with targeted cellular expression of eGFP or Cre recombinase (Gerfen et al., 2013). One advantage that the BAC insertion technology has over earlier transgenic methods is that the longer expanse of DNA encompasses much more non-coding regions containing information to direct the accurate expression of the reporter gene in time and space.

One critical assumption in using these methods is that the transgene insertion does not affect the normal physiology of an animal. However, the largely random chromosomal integration site of the BAC construct could have aberrant side effects on standard gene expression. Further, the large size of the insertion could contain unknown regulatory elements of other genes, again resulting in disruption of native genes. It is generally assumed that these are low probability events. However, in one important example in the recent literature, Kramer et al. (2011) described that Swiss Webster (SW) mice, homozygous for the *drd2*-eGFP BAC transgene had an altered phenotype (Kramer et al., 2011). However, subsequent manuscripts described the use of alternative background strains and/or reducing the copy number of BAC insertions to help control for possible affects that could lead to misinterpretations of data (Chan et al., 2012; Nelson et al., 2012). Together, all of these studies imply that BAC transgenic mice are extremely valuable tools that can be utilized to advance our understanding of endogenous GPCRs in defined cells. However, the data that results from these animals should be interpreted with the awareness of possible genetic misregulating elements contained in the BAC construct themselves or due to insertion site disruption of native genes.

DETECTION OF CALCIUM IONS WITH DYES OR "GCaMP" VARIANTS

As stated above, changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) can represent a fundamental change of state in many cell types. Biological processes ranging from cardiac muscle contraction, insulin secretion, cell adhesion, proliferation or cell death represent cellular and molecular reactions dependent upon $[Ca^{2+}]_i$. Notably, these signals vary with a time course of milliseconds in the case of muscle contraction, to minutes in the case of sustained insulin secretion, to hours or days in some cases of programmed cell death. Therefore, it is critical that the period of time in which $[Ca^{2+}]_i$ changes occur, can be reliably monitored in an endogenous environment. By measuring the kinetics of calcium transients, important information can be inferred such as properties of ligand kinetics, receptor reserve and amplification of signaling (Charlton and Vauquelin, 2010).

The rich history of $[Ca^{2+}]_i$ detection which dates back to the 1960's has recently been well reviewed (Grienberger and Konnerth, 2012). Briefly, bioluminescent calcium binding proteins like aequorin, or synthetic compounds like arsenazo III that changed absorbance spectrum with increasing calcium gave way to covalently modified hybrids of calcium chelating agents like EGTA or BAPTA (Shimomura et al., 1962; Brown et al., 1975; Tsien, 1980). This latter group of calcium indicator dyes, including the popular Fura-2, contains a fluorescent chromophore that can be monitored with light detection hardware. Fura-2 can be interchangeably excited with ultraviolet light at 340 nm or 380 nm in wavelength, and the ratio of the emitted light intensity at those two variable excitation wavelengths is directly correlated to the amount of intracellular calcium (Grynkiewicz et al., 1985).

Over the course of the past few decades, improvements in several variants of these fluorescent calcium indicators have been

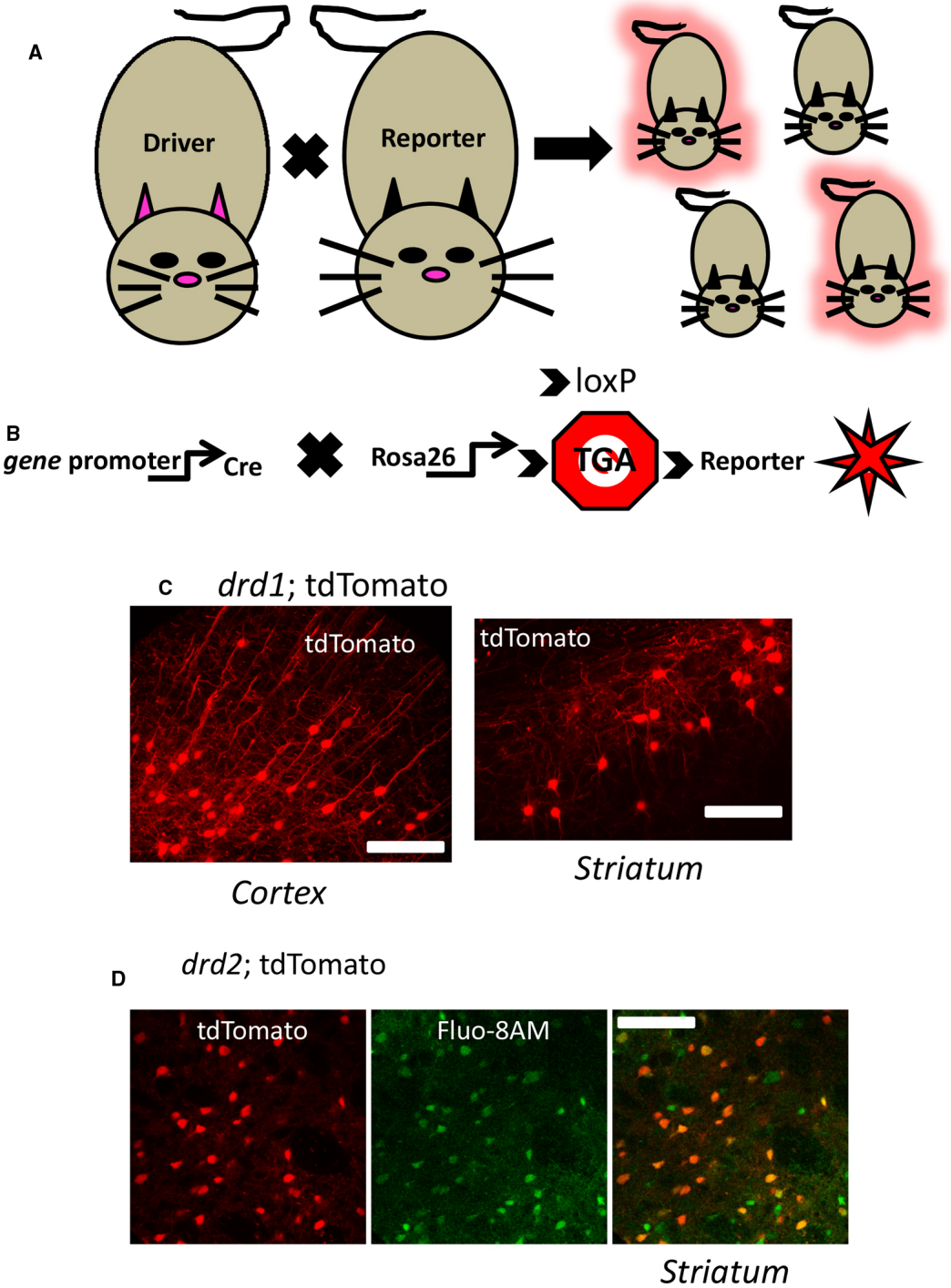

FIGURE 2 | Genetic identification of cells using cre-lox driver: reporter methods. (A) Mating a gene promotor containing "*driver*" mouse directed to express cre in a selective fashion to a cre-dependent "*reporter*" mouse yields offspring which may inherit the driver-reporter combination (*red* offspring) of genes. Those offspring which do not inherit the combination will not express the identification marker (*non-fluorescent* offspring). **(B)** A currently used strategy is to cross a cre driver mouse to a mouse expressing a cre-sensitive element at a ubiquitously expressed locus like *rosa26*. At this locus is a Stop codon (*Stop sign with TGA*) flanked by loxP sites (indicated by *arrows*) designed to be excised by cre recombinase activity allowing expression of genetically encoded fluorescent reporters (*red star* symbol) in the target cell population. **(C)** Illustrates a confocal image of a coronal mouse brain section from an animal inheriting the *drd1*; cre and *rosa26*; tdTomato genes described above, permitting fluorescent detection of dopamine D1 cells in cortex (*left*) and striatum (*right*). **(D)** Live confocal image of an acute striatal brain slice showing *drd2*: tdTomato neurons (red, *left*) and the same section bulk loaded with the calcium sensitive dye Flou-8 (green, *center*), allowing genetic identification of striatal neurons while imaging calcium (superimposed images, *right*). See Partridge et al. (2014) for methods. Scale bars in **(C)** and **(D)**: 100 μm.

developed that exhibit an increase in fluorescence upon binding Ca^{2+}. Cells can readily absorb membrane permeant acetyloxymethyl (AM) ester forms of these compounds by adding the dissolved indicator to various types of cell preparations. Endogenous, ubiquitous esterases cleave ester bonds and "trap" the now membrane impermeant Ca^{2+} sensitive dye intracellularly. The Ca^{2+} dependent amount of light emitted from these cells is generally measured using fluorescence microscopy, fluorescence microplate assays, or flow cytometry in combination with photon detection. The pharmacological and biophysical properties of these organic dyes have been reviewed (Paredes et al., 2008). One disadvantage of using these synthetic dyes is that they label tissue indiscriminately. For example, if you wish to study astrocytes in the CNS, application of the dye to the tissue will also label neighboring neurons. Further, many synthetic organic Ca^{2+} probes distribute into the cytosol, mitochondria, and other organelles making the measurements of "intracellular" Ca^{2+} more difficult to interpret.

During the same time period as synthetic dyes were improving, attempts to develop a genetically encoded $[Ca^{2+}]_i$ sensor were being performed. One such attempt has its origins in the use of complementary DNA from the *Aequorea victoria* green fluorescent protein (GFP) gene (Chalfie et al., 1994). GFP is a ∼27 kD protein that emits photons that fall within the visible spectrum when expressed in prokaryotic or eukaryotic cells upon proper excitation. GFP expression has become routine and widely used to examine an extensive range of biological questions ranging from gene expression to protein localization in living organisms. Over the past 20 years, GFP has been a major foundation for "directed evolution" into hundreds if not thousands of variations of the original wtGFP, many of which are currently being used as tools in fluorescent microscopy (Datta and Patterson, 2012). One genetic variant that developed about 7 years after GFP's initial cloning was designed by Nakai et al. (2001). This research group genetically engineered a chimeric protein termed G"CaM"P as it was created from a fusion of circularly permutated GFP, calmodulin (CaM), and M13, a peptide sequence from myosin light chain kinase. Upon elevation of intracellular Ca^{2+}, a conformational change occurred in GCaMP, enhancing fluorescent emission. Not surprisingly, due to the initial success of GCaMP as a Ca^{2+} sensor, it has been subsequently modified into increasingly higher numerical variants. More recent genetic versions of GCaMP are currently improving the signal-to-noise ratio of the fluorescence indicator, show improved kinetic responses, have variable Ca^{2+} binding affinities and other biophysical attributes that provide great flexibility in detection capacity (Akerboom et al., 2012; Sun et al., 2013). Additionally, "red shifted" genetically encoded calcium sensors have been generated that increase the spectral flexibility for imaging $[Ca^{2+}]_i$ (Yamada and Mikoshiba, 2012).

GENETICALLY BASED EXPRESSION OF Ca^{2+} SENSORS

The combination of the mouse genetic strategies described above and the use of improved GCaMPs to monitor $[Ca^{2+}]_i$ in different cell types has been accomplished (Fletcher et al., 2009; Chen et al., 2012; Zariwala et al., 2012). These studies and others report in genetically identifiable cell types, changes in $[Ca^{2+}]_i$ with enhanced fluorescence as a function of various stimuli. Frequently in these studies, the increased signal in GCaMP fluorescence derives from the underlying mechanisms of neuronal action potentials and/or excitatory synaptic transmission. More broadly, the literature focuses on extracellular calcium entry as the source for increased cytosolic calcium signals. Among these mechanisms include the opening of voltage-gated calcium channels, NMDA-type glutamate receptors and calcium permeable AMPA-type glutamate receptors. The biophysical and pharmacological properties of evolving GCaMPs have improved to the detection level of single action potentials (Tian et al., 2009; Akerboom et al., 2012; Chen et al., 2013). This improving sensitivity has allowed investigators to correlate to a given rise in fluorescence with an accurate estimation of the number of action potentials while simultaneously detecting fluorescence in dozens of distinct cell types (Wachowiak et al., 2013).

However, what seems to be under-utilized by GCaMP functionality in the literature to date is the versatility to monitor increases in intracellular calcium from *extracellular independent* sources. As described above, there are critical sources of calcium which do not originate from the extracellular pool of calcium and contribute to microdomains of Ca^{2+} signaling (Berridge, 2006). It is now clear that cytosolic calcium signaling originating from extracellular or intracellular sources is capable of influencing different domains or compartments within a cell. The importance of these localized domains of Ca^{2+} is that they control distinct spatial actions in different regions of the cell. For example, the ER is an organelle whereby calcium is pumped against its natural concentration gradient by proteins like the sarco-/endoplasmic reticulum calcium ATPase (SERCA). Mitochondria are other vital intracellular organelles that can serve as critical sources of calcium upon proper stimulation. These two examples represent significant reservoirs of calcium that facilitate a local rise in $[Ca^{2+}]_i$ by a subcellular dependent fashion. As an example of the advancing technology integrating genetics and $[Ca^{2+}]_i$ imaging, Li et al. (2014) recently measured changes in Ca^{2+} from mitochondria ($[Ca^{2+}]_m$) in astrocytes using improved and compartmentalized GCaMP probes while Bengtson et al. (2010) monitored calcium changes within the nucleus of CA1 pyramidal neurons. While these studies used more traditional DNA vector transfection or viral infection methods to introduce the designed GCaMP into selected cell types, it highlights that mitochondrial (Rizzuto et al., 1995) or nuclear localization signal sequences can be added to the GCaMP sensors to direct the sensor to subcellular organelles or compartments. Other examples of clever genetic manipulations include membrane tethering sequences fused in frame as done with MARCKS-GCaMP2 (Mao et al., 2008) or Lck-GCaMP3 (Shigetomi et al., 2010). These latter two examples could be important starting points to more rigorously screen $G_{q/11}$ calcium mobilization systems.

One advantage of the genetic techniques described above is that endogenous DNA recombination does a great deal of the work for the investigators without any requirements for survival surgery based methods. However, more invasive techniques, including stereotactic viral delivery or *in utero* electroporation (Yamada and Mikoshiba, 2012) can be used to extend the biological question posed.

MONITORING ENDOGENOUS $G_{q/11}$-COUPLED METABOTROPIC GLUTAMATE RECEPTORS USING ACUTE BRAIN SLICE PREPARATIONS

L-glutamate is the key excitatory neurotransmitter at the majority of synapses in the mammalian CNS. The initial detection of a distinct "metabolic" neuromodulatory glutamate receptor capable of generating IP_3 occurred almost three decades ago (Nicoletti et al., 1986). It was also discovered during this time period that activation of unique glutamate receptors could elevate intracellular Ca^{2+} in a "spike like" fashion upon receptor stimulation in the absence of extracellular Ca^{2+} (Murphy and Miller, 1988). The cloning era was able to make great strides in our understanding of the glutamate receptor family structure and function. Two main divisions of L-glutamate binding proteins include the ionotropic (AMPA, NMDA, and kainate) and metabotropic glutamate receptors (mGluRs). Of the eight mGluRs, it is now apparent that Group I mGluRs: mGlu1 and mGlu5 preferentially couple to the synthesis of DAG and IP_3 via $G_{q/11}$. The widespread yet tissue specific expression of group I mGluRs suggests that these modulatory receptors have the ability to affect various functions in the CNS. Since their detection, mGluRs have been a focal point of various therapeutic aims to assist in alleviating symptoms of disease states ranging from Parkinson's disease, diabetic neuropathy, melanoma, Autism spectrum disorders and generalized anxiety disorder (Niswender and Conn, 2010). For these reasons, GCaMP monitoring of $G_{q/11}$-mediated rises in cytosolic calcium can deepen our knowledge of a vital receptor class.

The Group I mGluRs are currently endowed with a rich array of pharmacological tools to dissect out particular branches and their role in signaling (Conn et al., 2014; Rook et al., 2015). One frequently used tool includes the compound (*S*)-3,5-dihydroxyphenylglycine (DHPG). DHPG has been utilized as one of the most selective Group I mGluR orthosteric agonists. Recently, brain slice preparations of the striatum detected rises in $[Ca^{2+}]_i$ from genetically identified neurons following acute DHPG application using either organic dye loading methods (Chen et al., 2011; Plotkin et al., 2013) or GCaMP3 transgene expression (Partridge et al., 2014). In the latter case, confocal imaging combined with mouse genetics using dopamine D1 (*drd1*) or D2 (*drd2*) gene driven Cre recombinase provided a scaffold to monitor DHPG mediated changes in $[Ca^{2+}]_i$. The acute application of DHPG did not affect the basal fluorescence of GCaMP3 in most of the imaged $D1^+$ or $D2^+$ striatal neurons (**Figure 3**). However, in cells which were depolarized by either chemical or electrical means, a robust Ca^{2+} signal resulted when slices were acutely exposed to the $G_{q/11}$-coupled GPCR agonist. These events were blocked by pretreatment with allosteric antagonists acting at mGluR1 and mGluR5. Further, the DHPG-mediated increase in GCaMP3 fluorescence was blocked by thapsigargin pre-treatment, an inhibitor of SERCA, strongly supporting a role for an intracellular source of calcium. The DHPG-mediated activation of native mGluRs as detected by GCaMP3, was fast and exhibited desensitization in the continued presence of this agonist. Further, in simultaneously current-clamped and GCaMP labeled neurons, the DHPG- mediated enhanced fluorescent signal was not associated with a change in membrane potential. This strongly supports the feasibility of these methods to detect active, endogenous GPCRs with GCaMP in an action-potential independent fashion.

Together, the data from that study indicate that striatal $D1^+$ and $D2^+$ projection neurons in acute brain slices express $G_{q/11}$-coupled mGluRs that can be observed with good time resolution by calcium sensors. The ability to detect increases in GCaMP3 fluorescence was clearly enhanced by presumably "pre-filling" the intracellular stores with calcium. However, this combination of methods can clearly be useful to monitor dozens of distinct neurons simultaneously while probing the native state of receptors with pharmacological tools.

Within that same study, the flexibility of the method was shown as GCaMP3 expression was directed to more sparse interneurons by crossing somatostatin (*sst*; Taniguchi et al., 2011) or tyrosine hydroxylase (*th*; Lindeberg et al., 2004) gene-driven Cre recombination. In these striatal GABAergic interneuron subtypes, DHPG application produced robust increases in GCaMP3 fluorescence that differed significantly in the duration of fluorescent signal compared to those elicited in the *drd1* or *drd2* driven strains. Electrical recordings from the various GCaMP3 expressing interneuron subtypes indicated that DHPG did evoke action potentials in the two interneuron populations in this brain region. A recent study utilizing uncaging of IP_3 came to a similar conclusion (Clements et al., 2013). Taken together, the data suggest a more classical type of $G_{q/11}$-mediated change in intracellular calcium in projection type *drd1* or *drd2* expressing neurons. In contrast, the actions of DHPG acting upon interneuron populations could be utilizing the ability of $G_{q/11}$ to couple to various TRP type channels (Gee et al., 2003; Ramsey et al., 2006). TRP channels were initially found to mediate phototransduction in fruit flies and are non-selection cation channels. The open probability of several types of TRP channels can be enhanced upon activation of $G_{q/11}$-coupled GPCRs. While more pharmacological evidence is necessary to validate this alternate pathway in striatal interneurons, this highlights the importance of the interpretation of the data. These studies and certainly others represent multidisciplinary techniques with rapidly evolving tools in which GPCRs can be assayed in natural states with relatively high temporal precision. This can greatly contribute to a deeper understanding of GPCR pharmacology while investigating the enormous heterogeneity of CNS cell types.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

G protein-coupled receptor signaling is a fundamental membrane-bound mechanism to detect selective changes in the local environment of animal cells. Because of the universal instrumentation of GPCRs across the animal kingdom, it is essential to understand the basic mechanisms on which they operate in an endogenous environment. Despite tremendous progress in our understanding of GPCR physiology and pharmacology, wide gaps remain in bridging the use of molecules that target these pathways to alleviate symptoms of disease and to develop clinically useful therapeutics. While this review has focused on one branch of the GPCR superfamily signaling pathway, opportunities to explore other canonical pathways like cAMP generation are being developed with luciferase based methods (Binkowski et al., 2011; DiRaddo et al., 2014). However,

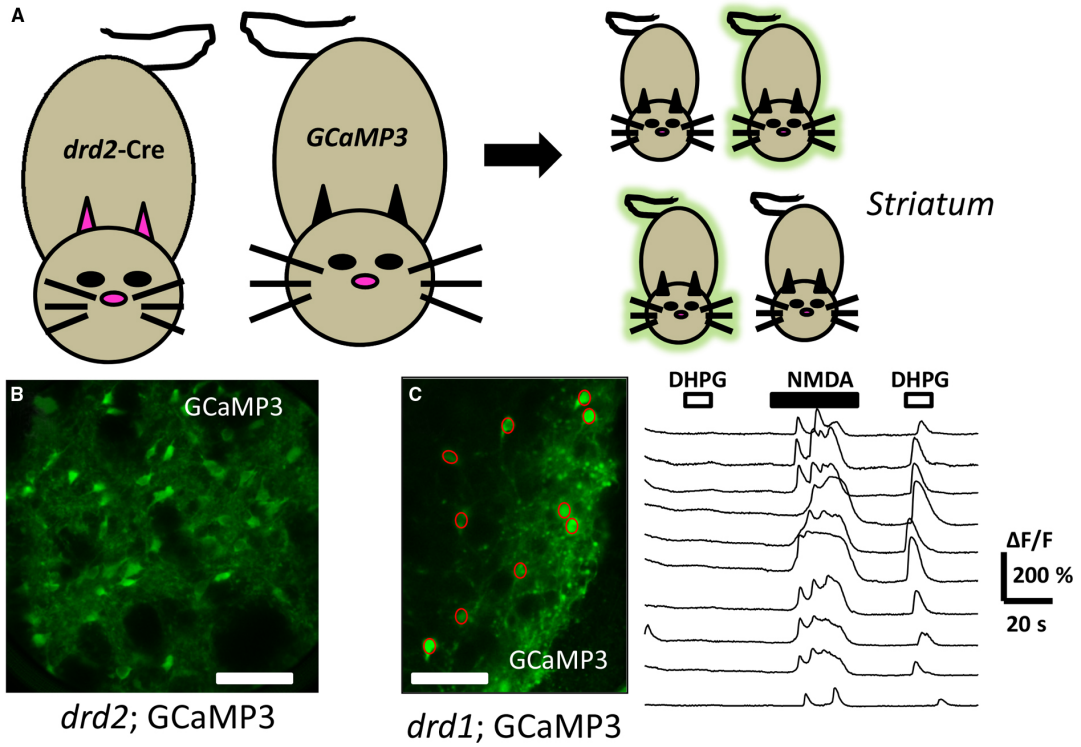

FIGURE 3 | Directed expression of genetically encoded calcium sensors to detect $G_{q/11}$-GPCR activation. (A) Mating a "*driver*" mouse directed to express cre in a selective dopamine D2 (*drd2*) fashion to a cre-dependent "*reporter*" GCaMP 3 mouse yields offspring which may inherit the driver-reporter combination of genes (*green* offspring). The offspring which do not inherit this combination will not express the calcium detector (*non-fluorescent* offspring). **(B)** Illustrates a confocal image of a coronal mouse brain section from an animal inheriting the *drd2*; cre and *rosa26*; GCaMP3 genes, permitting fluorescent detection of calcium changes in striatal dopamine D2 cells. **(C)** Live confocal image of an acute striatal brain slice showing *drd1*: GCaMP3 expressing neurons (green, *left*) with superimposed regions of interest (ROI, *red circles*) allowing genetically targeted calcium imaging. To the *right* are shown the time course of changes in $[Ca^{2+}]_i$ ($\Delta F/F$) in the ROIs corresponding to cell bodies. Each trace represents a different ROI or cell body. The top horizontal bars above the traces represent the time duration that the drugs DHPG (10 μM, *open bars*), or NMDA (20 μM, *filled bars*) were acutely applied. Note the ability to detect GPCR activation following activation of the cells. Scale bars in **(B)** and **(C)**: 100 μm.

fluorescent protein-based cAMP indicators have lagged behind Ca^{2+} sensors and require further development with improved dynamic range and brightness.

Another goal moving forward in the GPCR field is to develop a "universal" detector of endogenous GPCR activation. The detection of protein–protein interactions (e.g., receptor-G-proteins, liberation of $G_{\beta\gamma}$) would be one requirement of such a sensor. In fact, GPCR activation has been observed with several imaging probe techniques including intramolecular and intermolecular Förster resonance energy transfer (FRET)-based genetically encoded indicators (Lohse et al., 2012). However, an apparent constraint of this technique is that the introduction of dual fluorescent proteins (i.e., one acceptor and one donor), likely introduces steric hindrance and obstruction of essential protein–protein interactions necessary for energy transfer and the study of GPCRs in their endogenous states (Partridge et al., 2006). By combining the fields of fluorescent microscopy, mouse genetics and pharmacology we can enhance our understanding of GPCRs in their native state. Unanswered questions like the formation of various GPCR heteromers, altered pharmacology of heteromic receptors and cellular specificity can be answered with clever combinations of the methods mentioned in this review.

Improving GCaMP fluorescent signals have been detected using *in vivo* preparations (Hinckley and Pfaff, 2013; Dana et al., 2014), even in subcortical areas like the striatum (Cui et al., 2013). These elegant studies relied on the firing of action potentials to infer neuronal signaling. The challenge moving forward is to utilize fluorescent signals originating from the activation of GPCRs *in vivo*. This appears to be a reasonable challenge moving forward as detection of small but reliable Ca^{2+} increases can be detected in very fine mouse astrocyte processes both *in vitro* (Shigetomi et al., 2013) and *in vivo* (Otsu et al., 2015). These observations represent examples of calcium mobilization processes dependent upon acute GPCR activation.

REFERENCES

Akerboom, J., Chen, T. W., Wardill, T. J., Tian, L., Marvin, J. S., Mutlu, S., et al. (2012). Optimization of a GCaMP calcium indicator for neural activity imaging. *J. Neurosci.* 32, 13819–13840. doi: 10.1523/JNEUROSCI.2601-12.2012

Bengtson, C. P., Freitag, H. E., Weislogel, J. M., and Bading, H. (2010). Nuclear calcium sensors reveal that repetition of trains of synaptic stimuli boosts nuclear calcium signaling in CA1 pyramidal neurons. *Biophys. J.* 99, 4066–4077. doi: 10.1016/j.bpj.2010.10.044

Berridge, M. J. (2006). Calcium microdomains: organization and function. *Cell Calcium* 40, 405–412. doi: 10.1016/j.ceca.2006.09.002

Binkowski, B. F., Fan, F., and Wood, K. V. (2011). Luminescent biosensors for real-time monitoring of intracellular cAMP. *Methods Mol. Biol.* 756, 263–271. doi: 10.1007/978-1-61779-160-4_14

Brown, J. E., Cohen, L. B., De Weer, P., Pinto, L. H., Ross, W. N., and Salzberg, B. M. (1975). Rapid changes in intracellular free calcium concentration. Detection by metallochromic indicator dyes in squid giant axon. *Biophys. J.* 15, 1155–1160. doi: 10.1016/S0006-3495(75)85891-7

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805. doi: 10.1126/science.8303295

Chan, C. S., Peterson, J. D., Gertler, T. S., Glajch, K. E., Quintana, R. E., Cui, Q., et al. (2012). Strain-specific regulation of striatal phenotype in Drd2-eGFP BAC transgenic mice. *J. Neurosci.* 32, 9124–9132. doi: 10.1523/JNEUROSCI.0229-12.2012

Charlton, S. J., and Vauquelin, G. (2010). Elusive equilibrium: the challenge of interpreting receptor pharmacology using calcium assays. *Br. J. Pharmacol.* 161, 1250–1265. doi: 10.1111/j.1476-5381.2010.00863.x

Chen, M., Wan, Y., Ade, K., Ting, J., Feng, G., and Calakos, N. (2011). Sapap3 deletion anomalously activates short-term endocannabinoid-mediated synaptic plasticity. *J. Neurosci.* 31, 9563–9573. doi: 10.1523/JNEUROSCI.1701-11.2011

Chen, Q., Cichon, J., Wang, W., Qiu, L., Lee, S. J., Campbell, N. R., et al. (2012). Imaging neural activity using Thy1-GCaMP transgenic mice. *Neuron* 76, 297–308. doi: 10.1016/j.neuron.2012.07.011

Chen, T. W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., et al. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499, 295–300. doi: 10.1038/nature12354

Clements, M. A., Swapna, I., and Morikawa, H. (2013). Inositol 1,4,5-triphosphate drives glutamatergic and cholinergic inhibition selectively in spiny projection neurons in the striatum. *J. Neurosci.* 33, 2697–2708. doi: 10.1523/JNEUROSCI.4759-12.2013

Conn, P. J., Lindsley, C. W., Meiler, J., and Niswender, C. M. (2014). Opportunities and challenges in the discovery of allosteric modulators of GPCRs for treating CNS disorders. *Nat. Rev. Drug Discov.* 13, 692–708. doi: 10.1038/nrd4308

Cui, G., Jun, S. B., Jin, X., Pham, M. D., Vogel, S. S., Lovinger, D. M., et al. (2013). Concurrent activation of striatal direct and indirect pathways during action initiation. *Nature* 494, 238–242. doi: 10.1038/nature11846

Dana, H., Chen, T. W., Hu, A., Shields, B. C., Guo, C., Looger, L. L., et al. (2014). Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. *PLoS ONE* 9:e108697. doi: 10.1371/journal.pone.0108697

Datta, S. R., and Patterson, G. H. (2012). Optical highlighter molecules in neurobiology. *Curr. Opin. Neurobiol.* 22, 111–120. doi: 10.1016/j.conb.2011.11.007

DiRaddo, J. O., Miller, E. J., Hathaway, H. A., Grajkowska, E., Wroblewska, B., Wolfe, B. B., et al. (2014). A real-time method for measuring cAMP production modulated by Galphai/o-coupled metabotropic glutamate receptors. *J. Pharmacol. Exp. Ther.* 349, 373–382. doi: 10.1124/jpet.113.211532

Fletcher, M. L., Masurkar, A. V., Xing, J., Imamura, F., Xiong, W., Nagayama, S., et al. (2009). Optical imaging of postsynaptic odor representation in the glomerular layer of the mouse olfactory bulb. *J. Neurophysiol.* 102, 817–830. doi: 10.1152/jn.00020.2009

Gee, C. E., Benquet, P., and Gerber, U. (2003). Group I metabotropic glutamate receptors activate a calcium-sensitive transient receptor potential-like conductance in rat hippocampus. *J. Physiol.* 546, 655–664. doi: 10.1113/jphysiol.2002.032961

Gerfen, C. R., Paletzki, R., and Heintz, N. (2013). GENSAT BAC cre-recombinase driver lines to study the functional organization of cerebral cortical and basal ganglia circuits. *Neuron* 80, 1368–1383. doi: 10.1016/j.neuron.2013.10.016

Grienberger, C., and Konnerth, A. (2012). Imaging calcium in neurons. *Neuron* 73, 862–885. doi: 10.1016/j.neuron.2012.02.011

Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.

Hinckley, C. A., and Pfaff, S. L. (2013). Imaging spinal neuron ensembles active during locomotion with genetically encoded calcium indicators. *Ann. N. Y. Acad. Sci.* 1279, 71–79. doi: 10.1111/nyas.12092

Katritch, V., Cherezov, V., and Stevens, R. C. (2013). Structure-function of the G protein-coupled receptor superfamily. *Annu. Rev. Pharmacol. Toxicol.* 53, 531–556. doi: 10.1146/annurev-pharmtox-032112-135923

Kramer, P. F., Christensen, C. H., Hazelwood, L. A., Dobi, A., Bock, R., Sibley, D. R., et al. (2011). Dopamine D2 receptor overexpression alters behavior and physiology in Drd2-EGFP mice. *J. Neurosci.* 31, 126–132. doi: 10.1523/JNEUROSCI.4287-10.2011

Li, H., Wang, X., Zhang, N., Gottipati, M. K., Parpura, V., and Ding, S. (2014). Imaging of mitochondrial Ca dynamics in astrocytes using cell-specific mitochondria-targeted GCaMP5G/6s: Mitochondrial Ca uptake and cytosolic Ca availability via the endoplasmic reticulum store. *Cell Calcium* 56, 457–466. doi: 10.1016/j.ceca.2014.09.008

Limbird, L. E. (2004). The receptor concept: a continuing evolution. *Mol. Interv.* 4, 326–336. doi: 10.1124/mi.4.6.6

Lindeberg, J., Usoskin, D., Bengtsson, H., Gustafsson, A., Kylberg, A., Soderstrom, S., et al. (2004). Transgenic expression of Cre recombinase from the tyrosine hydroxylase locus. *Genesis* 40, 67–73. doi: 10.1002/gene.20065

Lohse, M. J., Nuber, S., and Hoffmann, C. (2012). Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol. Rev.* 64, 299–336. doi: 10.1124/pr.110.004309

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* 13, 133–140. doi: 10.1038/nn.2467

Mao, T., O'Connor, D. H., Scheuss, V., Nakai, J., and Svoboda, K. (2008). Characterization and subcellular targeting of GCaMP-type genetically-encoded calcium indicators. *PLoS ONE* 3:e1796. doi: 10.1371/journal.pone.0001796

Murphy, S. N., and Miller, R. J. (1988). A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 85, 8737–8741. doi: 10.1073/pnas.85.22.8737

Nakai, J., Ohkura, M., and Imoto, K. (2001). A high signal-to-noise $Ca2^{+}$ probe composed of a single green fluorescent protein. *Nat. Biotechnol.* 19, 137–141. doi: 10.1038/84397

Nelson, A. B., Hang, G. B., Grueter, B. A., Pascoli, V., Luscher, C., Malenka, R. C., et al. (2012). A comparison of striatal-dependent behaviors in wild-type and hemizygous Drd1a and Drd2 BAC transgenic mice. *J. Neurosci.* 32, 9119–9123. doi: 10.1523/JNEUROSCI.0224-12.2012

Nicoletti, F., Wroblewski, J. T., Novelli, A., Alho, H., Guidotti, A., and Costa, E. (1986). The activation of inositol phospholipid metabolism as a signal-transducing system for excitatory amino acids in primary cultures of cerebellar granule cells. *J. Neurosci.* 6, 1905–1911.

Niswender, C. M., and Conn, P. J. (2010). Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu. Rev. Pharmacol. Toxicol.* 50, 295–322. doi: 10.1146/annurev.pharmtox.011008.145533

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28, 147–155. doi: 10.1002/1526-968X(200011/12)28:3/4<147::AID-GENE90>3.0.CO;2-G

Oldham, W. M., and Hamm, H. E. (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat. Rev. Mol. Cell Biol.* 9, 60–71. doi: 10.1038/nrm2299

Otsu, Y., Couchman, K., Lyons, D. G., Collot, M., Agarwal, A., Mallet, J. M., et al. (2015). Calcium dynamics in astrocyte processes during neurovascular coupling. *Nat. Neurosci.* 18, 210–218. doi: 10.1038/nn.3906

Paredes, R. M., Etzler, J. C., Watts, L. T., Zheng, W., and Lechleiter, J. D. (2008). Chemical calcium indicators. *Methods* 46, 143–151. doi: 10.1016/j.ymeth.2008.09.025

Partridge, J. G., Lewin, A. E., Yasko, J. R., and Vicini, S. (2014). Contrasting actions of group I metabotropic glutamate receptors in distinct mouse striatal neurones. *J. Physiol.* 592, 2721–2733. doi: 10.1113/jphysiol.2014.272773

Partridge, J. G., Puhl, H. L. III, and Ikeda, S. R. (2006). Phosducin and phosducin-like protein attenuate G-protein-coupled receptor-mediated inhibition of voltage-gated calcium channels in rat sympathetic neurons. *Mol. Pharmacol.* 70, 90–100. doi: 10.1124/mol.105.021394

Plotkin, J. L., Shen, W., Rafalovich, I., Sebel, L. E., Day, M., Chan, C. S., et al. (2013). Regulation of dendritic calcium release in striatal spiny projection neurons. *J. Neurophysiol.* 110, 2325–2336. doi: 10.1152/jn.00422.2013

Ramsey, I. S., Delling, M., and Clapham, D. E. (2006). An introduction to TRP channels. *Annu. Rev. Physiol.* 68, 619–647. doi: 10.1146/annurev.physiol.68.040204.100431

Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., and Pozzan, T. (1995). Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. *Curr. Biol.* 5, 635–642. doi: 10.1016/S0960-9822(95)00128-X

Rook, J. M., Tantawy, M. N., Ansari, M. S., Felts, A. S., Stauffer, S. R., Emmitte, K. A., et al. (2015). Relationship between in vivo receptor occupancy and efficacy of metabotropic glutamate receptor subtype 5 allosteric modulators with different in vitro binding profiles. *Neuropsychopharmacology* 40, 755–765. doi: 10.1038/npp.2014.245

Sauer, B. (1987). Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7, 2087–2096.

Shigetomi, E., Bushong, E. A., Haustein, M. D., Tong, X., Jackson-Weaver, O., Kracun, S., et al. (2013). Imaging calcium microdomains within entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses. *J. Gen. Physiol.* 141, 633–647. doi: 10.1085/jgp.201210949

Shigetomi, E., Kracun, S., Sofroniew, M. V., and Khakh, B. S. (2010). A genetically targeted optical sensor to monitor calcium signals in astrocyte processes. *Nat. Neurosci.* 13, 759–766. doi: 10.1038/nn.2557

Shimomura, O., Johnson, F. H., and Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* 59, 223–239. doi: 10.1002/jcp.1030590302

Sun, X. R., Badura, A., Pacheco, D. A., Lynch, L. A., Schneider, E. R., Taylor, M. P., et al. (2013). Fast GCaMPs for improved tracking of neuronal activity. *Nat. Commun.* 4, 2170. doi: 10.1038/ncomms3170

Taniguchi, H., He, M., Wu, P., Kim, S., Paik, R., Sugino, K., et al. (2011). A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron* 71, 995–1013. doi: 10.1016/j.neuron.2011.07.026

Tian, L., Hires, S. A., Mao, T., Huber, D., Chiappe, M. E., Chalasani, S. H., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* 6, 875–881. doi: 10.1038/nmeth.1398

Tsien, R. Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19, 2396–2404. doi: 10.1021/bi00552a018

Wachowiak, M., Economo, M. N., Diaz-Quesada, M., Brunert, D., Wesson, D. W., White, J. A., et al. (2013). Optical dissection of odor information processing in vivo using GCaMPs expressed in specified cell types of the olfactory bulb. *J. Neurosci.* 33, 5285–5300. doi: 10.1523/JNEUROSCI.4824-12.2013

Wettschureck, N., and Offermanns, S. (2005). Mammalian G proteins and their cell type specific functions. *Physiol. Rev.* 85, 1159–1204. doi: 10.1152/physrev.00003.2005

Yamada, Y., and Mikoshiba, K. (2012). Quantitative comparison of novel GCaMP-type genetically encoded $Ca2^{+}$ indicators in mammalian neurons. *Front. Cell. Neurosci.* 6:41. doi: 10.3389/fncel.2012.00041

Zariwala, H. A., Borghuis, B. G., Hoogland, T. M., Madisen, L., Tian, L., De Zeeuw, C. I., Zeng, H., et al. (2012). A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. *J. Neurosci.* 32, 3131–3141. doi: 10.1523/JNEUROSCI.4469-11.2012

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Opioid and $GABA_B$ receptors differentially couple to an adenylyl cyclase/protein kinase A downstream effector after chronic morphine treatment

Elena E. Bagley*

Discipline of Pharmacology, Sydney Medical School, University of Sydney, Sydney, NSW, Australia

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Sulev Kõks, University of Tartu, Estonia
Susan Ingram, Oregon Health & Science University, USA

****Correspondence:***
Elena E. Bagley, Discipline of Pharmacology, University of Sydney, Blackburn Building DO6, Sydney, NSW 2006, Australia
e-mail: elena.bagley@sydney.edu.au

Opioids are intensely addictive, and cessation of their chronic use is associated with a highly aversive withdrawal syndrome. A cellular hallmark of withdrawal is an opioid sensitive protein kinase A-dependent increase in GABA transporter-1 (GAT-1) currents in periaqueductal gray (PAG) neurons. Elevated GAT-1 activity directly increases GABAergic neuronal excitability and synaptic GABA release, which will enhance GABAergic inhibition of PAG output neurons. This reduced activity of PAG output neurons to several brain regions, including the hypothalamus and medulla, contributes to many of the PAG-mediated signs of opioid withdrawal. The $GABA_B$ receptor agonist baclofen reduces some of the PAG mediated signs of opioid withdrawal. Like the opioid receptors the $GABA_B$ receptor is a G_i/G_o coupled G-protein coupled receptor. This suggests it could be modulating GAT-1 activity in PAG neurons through its inhibition of the adenylyl cyclase/protein kinase A pathway. Opioid modulation of the GAT-1 activity can be detected by changes in the reversal potential of opioid membrane currents. We found that when opioids are reducing the GAT-1 cation conductance and increasing the GIRK conductance the opioid agonist reversal potential is much more negative than E_k. Using this approach for $GABA_B$ receptors we show that the $GABA_B$ receptor agonist, baclofen, does not couple to inhibition of GAT-1 currents during opioid withdrawal. It is possible this differential signaling of the two G_i/G_o coupled G-protein coupled receptors is due to the strong compartmentalization of the $GABA_B$ receptor that does not favor signaling to the adenylyl cyclase/protein kinase A/GAT-1 pathway. This highlights the importance of studying the effects of G-protein coupled receptors in native tissue with endogenous G-protein coupled receptors and the full complement of relevant proteins and signaling molecules. This study suggests that baclofen reduces opioid withdrawal symptoms through a non-GAT-1 effector.

Keywords: opioid, GAT-1, $GABA_B$ receptor, periaqueductal gray, withdrawal

INTRODUCTION

Opioids are intensely addictive, and cessation of their chronic use is associated with a withdrawal syndrome consisting of severe early physical symptoms and late features such as craving. Relapse into drug-taking behaviors often occurs as a result of this withdrawal syndrome (Mattick and Hall, 1996; Williams et al., 2001), which is thought to result from neuronal adaptations that develop to restore homeostasis during chronic opioid exposure (Himmelsbach, 1943). On cessation of opioid administration, persistent counteradaptations in critical brain regions are unmasked and cause the withdrawal syndrome. A rebound increase of adenylyl cyclase/protein kinase A (PKA) signaling is one counteradaptation. While opioid agonists acutely inhibit adenylyl cyclase activity in the brain (Collier and Roy, 1974) and specifically in the periaqueductal gray (PAG) (Fedynyshyn and Lee, 1989), there is a compensatory increase in adenylyl cyclase signaling during chronic treatment with morphine *in vitro* (Sharma et al., 1975; Avidor-Reiss et al., 1997) and *in vivo* (Terwilliger et al., 1991) resulting in rebound hyperactivity of this cascade during withdrawal. Microinjections of PKA inhibitors into the PAG attenuate a spectrum of opioid withdrawal behaviors similar to those induced by microinjections of opioid antagonists (Maldonado et al., 1995; Punch et al., 1997). Whilst the importance of upregulated adenylyl cyclase/PKA during opioid withdrawal has been appreciated for many years we have only recently found the cellular target of PKA that results in withdrawal symptoms (Bagley et al., 2005b, 2011). We found that elevated PKA activity during withdrawal increases GABA transporter 1 (GAT-1) currents in PAG neurons (Bagley et al., 2005b). Elevated GAT-1 activity directly depolarizes and thus hyperexcites GABAergic PAG neurons and nerve terminals, which presumably enhances GABAergic inhibition of PAG output neurons (Bagley et al., 2005b). This reduced activity of PAG output neurons to several brain regions, including the hypothalamus and medulla, results in opioid withdrawal signs (Bagley et al., 2011).

Opioid receptors are G_i/G_o coupled G-protein coupled receptors that inhibit adenylyl cyclase through their Gα subunit. The $GABA_B$ receptor is another G_i/G_o coupled G-protein coupled

receptor (Bettler et al., 2004) that inhibits adenylyl cyclase activity (Gerber and Gähwiler, 1994; Kuner et al., 1999; Bettler et al., 2004; Vanhoose et al., 2004; Connelly et al., 2013). $GABA_B$ receptors are expressed in almost all PAG neurons (Chieng and Christie, 1996; Margeta-Mitrovic et al., 1999; Bagley et al., 2005a). The $GABA_B$ receptor agonist baclofen reduces some PAG mediated signs of opioid withdrawal in humans (Ahmadi-Abhari et al., 2001; Tyacke et al., 2010) and animal models (Bexis et al., 2001; Tyacke et al., 2010) and is used in drug cocktails for treatment of opioid withdrawal (Collis, 2008). Given the similarities in coupling and the therapeutic utility of $GABA_B$ receptor agonists, in this study we ask whether $GABA_B$ receptor agonists act like opioids to reduce GAT-1 activity during opioid withdrawal.

MATERIALS AND METHODS

CHRONIC TREATMENT WITH MORPHINE

Morphine dependence was induced by a series of subcutaneous injections of sustained-release morphine suspension into male C57B16/J mice (300 mg/kg morphine base) as in previous experiments (Bagley et al., 2005b, 2011). Injections (0.1–0.2 ml) were made under light halothane anesthesia on days 1, 3, and 5, and mice were used for experiments on days 6 or 7. Morphine base was suspended in 0.1 ml mannide mono-oleate (Arlacel A, Sigma), 0.4 ml light liquid paraffin and 0.5 ml 0.9% w/v NaCl in water. Vehicle-treated mice were injected on the same schedule with morphine-free suspension.

TISSUE PREPARATION AND RECORDINGS

PAG slices (220–250 μm) were cut from 4- to 6-week-old mice and were maintained at 34°C in a submerged chamber containing physiological saline (ACSF) equilibrated with 95% O_2 and 5% CO_2 and were later transferred to a chamber superfused at 2 ml/min with ACSF (34°C) for recording. The standard ACSF contained 126 mM NaCl, 2.5 mM KCl, 1.4 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 11 mM glucose, and 25 mM $NaHCO_3$. Brain slices from both morphine-dependent and vehicle-treated mice were maintained *in vitro* in ACSF containing 5 μM morphine. Unless otherwise stated, slices were spontaneously withdrawn by incubation in morphine-free ACSF for at least 1 h before an experiment. CGP55845 was a gift from Ciba Ltd (Basel, Switzerland).

PAG neurons were visualized using infra-red Nomarski optics. Perforated patch recordings were made using patch electrodes (4–5 mΩ) filled with 120 mM K acetate, 40 mM HEPES, 10 mM EGTA, 5 mM $MgCl_2$, with 0.25 mg/ml Pluronic F-127, 0.12 mg/ml amphotericin B (pH 7.2, 290 mosmol/l). Liquid junction potentials for K acetate internal solution of −8 mV with ACSF were corrected. Series resistance (<25 MΩ) was compensated by 80% and continuously monitored. During perforated patch recordings, currents were recorded using a Axopatch 200A amplifier (Axon Instruments), digitized, filtered (at 2 kHz), and then acquired (sampling at 10 kHz) in pClamp (Axon Instruments) or using Axograph Acquisition software (Axon Instruments).

All pooled data are expressed as mean ± s.e.m. We tested for significance using the unpaired Student's *t*-test.

RESULTS

In PAG neurons, withdrawal from chronic morphine-treatment stimulates a protein kinase A-dependent increase of the GAT-1 cation conductance (Bagley et al., 2005b, 2011). The increased GAT-1 activity is sensitive to opioid inhibition and therefore during opioid withdrawal it can be detected by changes in the opioid agonist met-enkephalin (MENK) current reversal potential. When MENK is reducing the GAT-1 cation conductance and increasing the GIRK conductance the MENK reversal potential will be much more negative than E_k (Bagley et al., 2005b). Superfusion of (MENK) produced an outward current in 4 out of 6 PAG neurons voltage clamped at −56 mV in slices from vehicle-treated mice (30 ± 4 pA, $n = 4$, **Figure 1A**). In neurons from vehicle-treated mice the ME current reversed polarity at a potential of −109 ± 4 mV ($n = 4$, **Figures 1B,D**), close to the potassium reversal potential (E_k, −103 mV) in these conditions as we have previously reported in mice (Bagley et al., 2005b, 2011). In neurons from chronic morphine-treated mice, the MENK-induced current reversed in only 2 of 5 cells (**Figure 1C**). In the neurons where the MENK current did not reverse polarity at the most negative potential tested, the reversal potentials was assigned a value of −136 mV, a conservative approach we adopted in previous studies to deal with technical inability to determine extremely negative reversal potentials (Bagley et al., 2005b, 2011). The nominal reversal potential for the 5 cells was −1.8 ± 4 mV (**Figures 1C,D**) that is significantly more negative than neurons from vehicle mice ($p = 0.034$, Students *t*-test, **Figure 2E**). We have previously shown that in the presence of the GAT-1 inhibitor, NO-711, results in ME currents that reversed polarity close to the value for MENK currents in neurons from vehicle-treated mice (Bagley et al., 2005b, 2011).

Superfusion of the $GABA_B$ receptor agonist baclofen (10 μM) produced an outward current in all 8 PAG neurons voltage clamped at −56 mV in slices from vehicle-treated mice (31 ± 5 pA, $n = 14$, **Figure 2A**). In neurons from vehicle treated mice the baclofen current reversed polarity at a potential of −116 ± 5 mV ($n = 8$, **Figures 2B,D,E**), close to E_k in these conditions (−103 mV). In neurons from morphine-treated mice the baclofen-induced outward current (30 ± 5 pA, $n = 17$) reversed polarity (−117 ± 2 mV, $n = 17$, **Figures 2C–E**) close to E_k (−103 mV) and at a similar membrane potential to cells from vehicle-treated mice ($p = 0.71$ Students *t*-test).

DISCUSSION

A cellular hallmark of withdrawal in PAG is protein kinase A-dependent increases in GAT-1 currents (Bagley et al., 2005b, 2011). This study showed that even though the opioid receptors and $GABA_B$ receptor are both G_i/G_o coupled G-proteins coupled receptors occurring in the same neurons $GABA_B$ receptors are unable to couple via PKA to the additional GAT-1 conductance. The selectivity is not due to the $GABA_B$ receptor and opioid receptors being located in different neurons. Whilst only two thirds to three quarters of PAG neurons are opioid sensitive, as shown in this study and previously (Vaughan et al., 2003), almost all PAG neurons are sensitive to $GABA_B$ receptor agonists (97% in this study and Chieng and Christie, 1995). In this study we only included neurons that were sensitive to both agonists. $GABA_B$

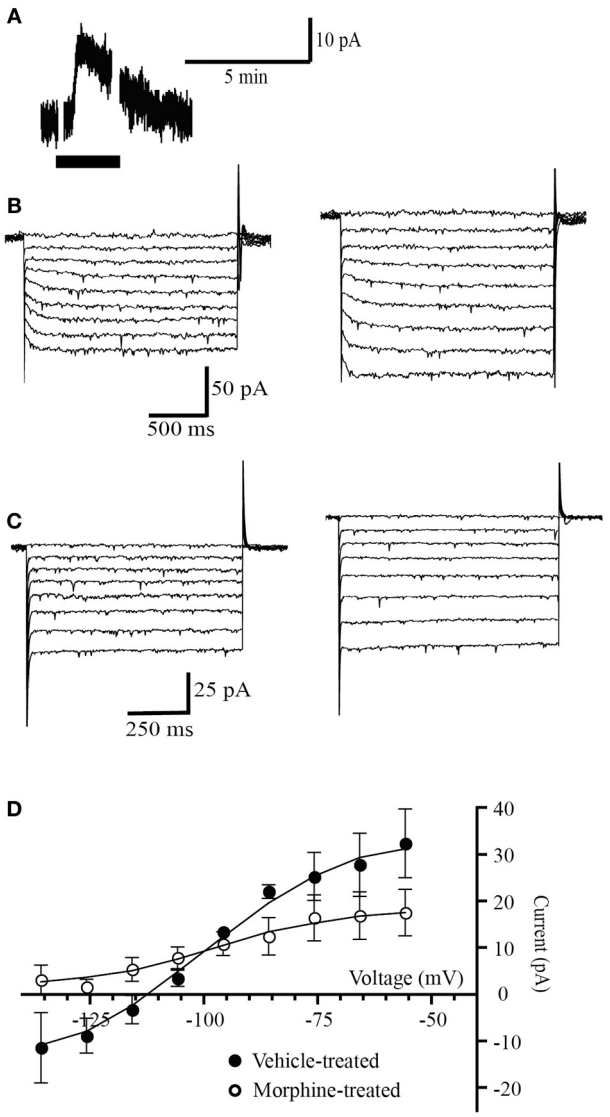

FIGURE 1 | Opioid receptors simultaneously couple to a potassium conductance and a GAT-1 conductance. (A) Example trace of currents from a neuron voltage clamped at −56 mV (drug superfusion shown by bars). MENK (30 μM) induced an outward current in neuron from a morphine-treated mouse. **(B,C)** Currents produced by voltage steps from −56 mV to −136 mV in −10 mV increments in a neuron from **(B)** a vehicle-treated mouse and **(C)** a morphine-treated mouse before (left) and during MENK (30 μM) application (right). **(D)** Subtracted current-voltage relationships for MENK (current in MENK—current during control conditions). Reversal potentials were determined at the point where they cross the abscissa. The MENK current reversed polarity near E_K in neurons from vehicle-treated ($n = 4$), but not in neurons from morphine-treated mice ($n = 5$).

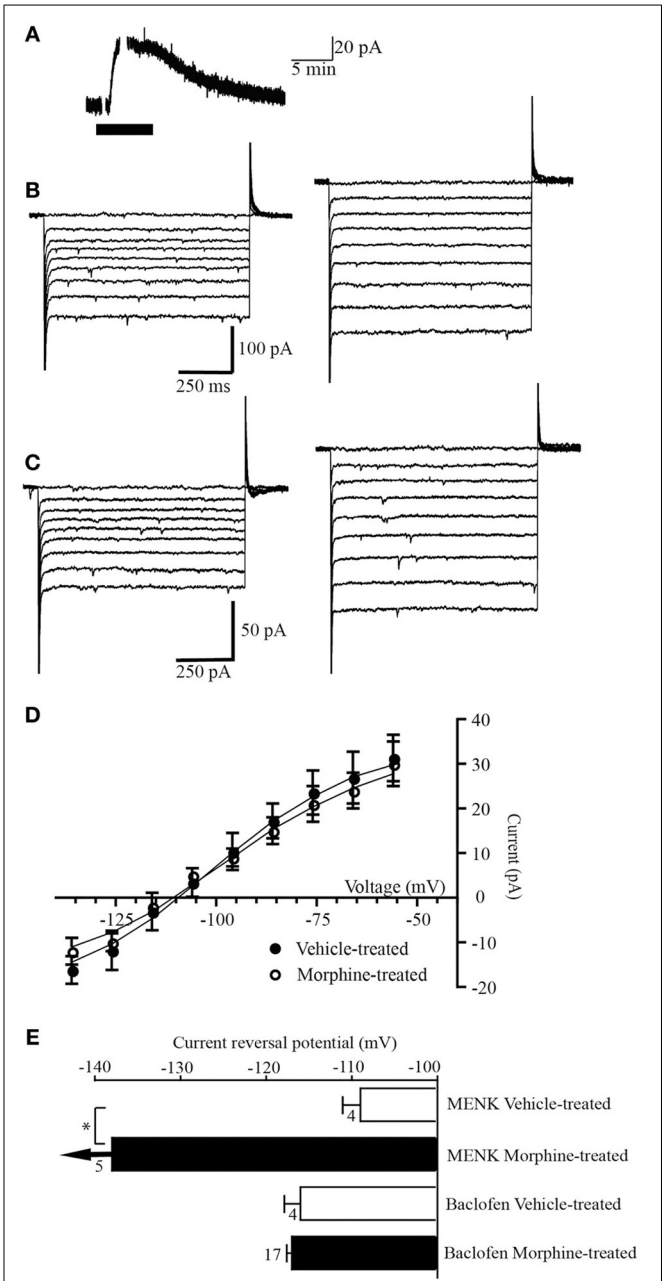

FIGURE 2 | $GABA_B$ receptors do not couple to a GAT-1 conductance. (A) Example trace of currents from a neuron voltage clamped at −56 mV (drug superfusion shown by bars). Baclofen (10 μM) induced an outward current in a neuron from a morphine-treated mouse. **(B,C)** Currents produced by voltage steps from −56 mV to −136 mV in −10 mV increments in a neuron from **(B)** a vehicle-treated mouse and **(C)** a morphine-treated mouse before (left) and during baclofen (10 μM) application (right). **(D)** Subtracted current-voltage relationships for baclofen (current in baclofen–current during control conditions). Reversal potentials were determined at the point where they cross the abscissa. The baclofen current reversed polarity near E_K in neurons from vehicle-treated ($n = 8$), and morphine-treated mice ($n = 17$). **(E)** Reversal potential of the MENK and baclofen-induced currents in cells from vehicle-treated and chronic morphine-treated mice. Arrows indicate that the average current did not reverse polarity at the most negative potential that could be tested (−136 mV). The number of neurons is shown beside the bar. *P-value of 0.034, Students t-test.

receptor agonists inhibit adenylyl cyclase after expression in cell lines (Kuner et al., 1999; Bettler et al., 2004) and in brain tissue (Gerber and Gähwiler, 1994; Vanhoose et al., 2004) and there are examples where this inhibition of adenylyl cyclase regulates ionic conductances in several different brain regions (Gerber and Gähwiler, 1994; Connelly et al., 2013). Therefore, if the receptors

are located on the same neurons and can couple to the adenylyl cyclase/PKA signaling pathway this suggests that there must be some sort of compartmentalization that prevents baclofen from regulating GAT-1 activity. Conversely, compartmentalization may favor opioid receptor regulation of GAT-1.

Like other G_i/G_o coupled G-proteins coupled receptors $GABA_B$ receptors can couple to several effectors, including calcium channels, GIRK and adenylyl cyclase. However, $GABA_B$ receptors appear to show greater segregation than other G-protein coupled receptor signaling and possibly stronger regulation into nano-signaling complexes. Opioids and $GABA_B$ receptors have previously been shown to differentially couple to their effectors. This occurs in GABAergic nerve terminals in the PAG. Opioid receptor activation reduces GABA release from nerve terminals in the PAG through modulation of a voltage dependent potassium channel where as $GABA_B$ receptors do not couple to GABA inhibition through this mechanism (Vaughan et al., 1997). It seems likely that, as in this study, both the $GABA_B$ receptors and opioid receptors are expressed on the same GABAergic terminals in the PAG again indicating compartmentalization. This also occurs outside the PAG. In locus coeruleus neurons both opioid and α2 receptors inhibit the same population of calcium channels in these neurons but $GABA_B$ receptors inhibit a separate population of calcium channels in the same cell (Chieng and Bekkers, 1999). Of all the G_i/G_o coupled effects there is evidence that $GABA_B$ receptor regulation of AC/PKA activity may be particularly affected by compartmentalization. $GABA_B$ receptor inhibition of adenylyl cyclase in the hippocampus was stimulation dependent whereas other G_i/G_o coupled receptors were able to inhibit adenylyl cyclase regardless of how it was stimulated (Vanhoose et al., 2004). These differences may be due to $GABA_B$ receptors being localized to nano-signaling complexes that influence their signaling to different effectors. The influence of nan-signaling complexes on $GABA_B$ receptors on signaling is evidenced by inclusion of $GABA_B$ receptors in nano-signaling complexes facilitating inhibition of calcium channels but not inhibition of adenylyl cyclase (Laviv et al., 2011). Further, $GABA_B$ receptors, and especially the splice variants of the $GABA_{B1}$ receptor, show differential subcellular localization and associations with protein clusters that alter their coupling to effectors (Vigot et al., 2006). Therefore, $GABA_B$ receptors may not modulate GAT-1 activity in this study because they are preferentially associated with proteins or located in regions of the cell that do not favor inhibition of adenylyl cyclase. Whilst the evidence for strong compartmentalization of $GABA_B$ receptor signaling is convincing it is also possible that it is the location/compartmentalization of the opioid receptors that allows their coupling to GAT-1 activity. Perhaps opioid receptors are more closely localized to the subcellular region or associated with the adenylyl cyclase/PKA/GAT-1 proteins upregulated by chronic opioid inhibition.

If we want to ask questions about processes occurring in the brain during particular disease states, as opposed to cell lines or cultured cells, it is critical that these experiments are conducted in native tissue. Experiments in native tissue study endogenous GPCRs with the full complement of relevant proteins and signaling molecules. In fact the opioid-AC-PKA modulation of GAT-1 was a surprise because the only consensus site for PKA phosphorylation is extracellular (Guastella et al., 1990) making it an unlikely candidate for regulation by PKA. Whilst the effect of PKA activation on the GAT-1 transporter has not been comprehensively studied, the subcellular location, enzymatic activity, and absolute level of GAT-1 is regulated in a complex inter-related manner by PKC activity, GABA concentration, ionic conditions, tyrosine kinase activity, and the release protein syntaxin 1A (Beckman and Quick, 1998; Beckman et al., 1998; Whitworth and Quick, 2001; Quick, 2002). Therefore, it is likely due to AC/PKA altering GAT-1 activity through an intermediary protein not expressed/or active in the cultured cells. Support for this proposal comes from another study of GAT-1 in brain tissue showing that AC/PKA activity facilitates GAT-1 transport by curbing tonic PKC-mediated inhibition of GAT-1 activity and cell surface expression (Cristóvão-Ferreira et al., 2009). Therefore, during opioid withdrawal the overshoot in AC/PKA activity in PAG neurons could be could indirectly increasing GAT-1 activity through reducing PKC restraints on GAT-1 activity. The results from this study also show how important it is to conduct experiments in native tissue. We would have predicted that another G_i/G_o coupled GPCR, such as $GABA_B$, that inhibits adenylyl cyclase (Gerber and Gähwiler, 1994; Kuner et al., 1999; Bettler et al., 2004; Vanhoose et al., 2004; Connelly et al., 2013) and is in the same cell as the opioid receptors would have modulated GAT-1 activity. Although $GABA_B$ receptors may modulate GAT-1 activity under other conditions or in different cells it does not occur in the PAG neurons important for opioid withdrawal.

The opioid sensitive GAT-1 activity in the PAG during withdrawal initiates the opioid withdrawal syndrome (Bagley et al., 2011). The $GABA_B$ agonist baclofen reduces some of the signs of opioid withdrawal (Ahmadi-Abhari et al., 2001; Bexis et al., 2001; Tyacke et al., 2010) but does not alter the GAT-1 activity. One possible explanation for this is that an important outcome of the elevated GAT-1 activity during withdrawal is depolarization of GABA neurons and a resultant increase in synaptic GABA release. The increased GABA release inhibits PAG output neurons, changes neurotransmitter release in their target brain regions and ultimately expression of the withdrawal signs. Whilst $GABA_B$ receptor activation can't reduce the GAT-1 activity that drives GABA release it could act to inhibit the excitability of GABAergic neurons through other mechanisms. Reduced GABA release could result from inhibition of GABA neuron excitability, through activation of GIRK, and inhibition of GABA release through non-GAT1 effectors. Through this alternative mechanism of inhibiting GABA release it would diminish inhibition of output neurons and thus withdrawal.

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REFERENCES

Ahmadi-Abhari, S., Akhondzadeh, S., Assadi, S., Shabestari, O., Farzanehgan, Z., and Kamlipour, A. (2001). Baclofen versus clonidine in the treatment of opiates withdrawal, side-effects aspect: a double-blind randomized controlled trial. *J. Clin. Pharm. Ther.* 26, 67–71. doi: 10.1046/j.1365-2710.2001.00325.x

Avidor-Reiss, T., Nevo, I., Saya, D., Bayewitch, M., and Vogel, Z. (1997). Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J. Biol. Chem.* 272, 5040–5047. doi: 10.1074/jbc.272.8.5040

Bagley, E., Chieng, B., Christie, M., and Connor, M. (2005a). Opioid tolerance in periaqueductal gray neurons isolated from mice chronically treated with morphine. *Br. J. Pharmacol.* 146, 68–76. doi: 10.1038/sj.bjp.0706315

Bagley, E., Gerke, M., Vaughan, C., Hack, S., and Christie, M. (2005b). GABA transporter currents activated by protein kinase A excite midbrain neurons during opioid withdrawal. *Neuron* 45, 433–445. doi: 10.1016/j.neuron.2004.12.049

Bagley, E., Hacker, J., Chefer, V., Mallet, C., McNally, G., Chieng, B., et al. (2011). Drug-induced GABA transporter currents enhance GABA release to induce opioid withdrawal behaviours. *Nat. Neurosci.* 14, 1548–1554. doi: 10.1038/nn.2940

Beckman, M. L., Bernstein, E. M., and Quick, M. W. (1998). Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. *J. Neurosci.* 18, 6103–6112.

Beckman, M. L., and Quick, M. W. (1998). Neurotransmitter transporters: Regulators of function and functional regulation. *J. Membr. Biol.* 164, 1–10. doi: 10.1007/s002329900388

Bettler, B., Kaupmann, K., Mosbacher, J., and Gassmann, M. (2004). Molecular structure and physiological functions of GABAB receptors. *Physiol. Rev.* 84, 835–867. doi: 10.1152/physrev.00036.2003

Bexis, S., Ong, J., and White, J. (2001). Attenuation of morphine withdrawal signs by the GABA(B) receptor agonist baclofen. *Life Sci.* 70, 395–401. doi: 10.1016/S0024-3205(01)01485-0

Chieng, B., and Bekkers, J. (1999). GABA(B), opioid and alpha2 receptor inhibition of calcium channels in acutely-dissociated locus coeruleus neurones. *Br. J. Pharmacol.* 127, 1533–1538. doi: 10.1038/sj.bjp.0702693

Chieng, B., and Christie, M. (1996). Local opioid withdrawal in rat single periaqueductal gray neurons *in vitro*. *J. Neurosci.* 16,7128–7136.

Chieng, B., and Christie, M. J. (1995). Hyperpolarization by GABAB receptor agonists in mid-brain periaqueductal gray neurones *in vitro*. *Br. J. Pharmacol.* 116, 1583–1588. doi: 10.1111/j.1476-5381.1995.tb16376.x

Collier, H. O., and Roy, A. C. (1974). Morphine-like drugs inhibit the stimulation by E prostaglandins of cyclic AMP formation by rat brain homogenate. *Nature* 248, 24–27. doi: 10.1038/248024a0

Collis, L. (2008). *NSW Drug and Alcohol Withdrawal Clinical Practice Guidelines*. Sydney, NSW: Better Health Centre Publications Warehouse.

Connelly, W., Fyson, S., Errington, A., McCafferty, C., Cope, D., Di Giovanni, G., et al. (2013). GABAB receptors regulate extrasynaptic GABAA receptors. *J. Neurosci.* 33,3780–3785. doi: 10.1523/JNEUROSCI.4989-12.2013

Cristóvão-Ferreira, S., Vaz, S., Ribeiro, J., and Sebastião, A. (2009). Adenosine A2A receptors enhance GABA transport into nerve terminals by restraining PKC inhibition of GAT-1. *J. Neurochem.* 109, 336–347. doi: 10.1111/j.1471-4159.2009.05963.x

Fedynyshyn, J. P., and Lee, N. M. (1989). μ type opioid receptors in rat periaqueductal gray-enriched P2 membrane are coupled to G-protein-mediated inhibition of adenylyl cyclase. *FEBS Lett.* 253, 23–27. doi: 10.1016/0014-5793(89)80921-4

Gerber, U., and Gähwiler, B. (1994). GABAB and adenosine receptors mediate enhancement of the K+ current, IAHP, by reducing adenylyl cyclase activity in rat CA3 hippocampal neurons. *J. Neurophysiol.* 72, 2360–2367.

Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., et al. (1990). Cloning and expression of a rat brain GABA transporter. *Nature*. 249, 1303–1306.

Himmelsbach, C. K. (1943). With reference to physical dependence. *Fed. Proc*. 2, 201–203.

Kuner, R., Köhr, G., Grünewald, S., Eisenhardt, G., Bach, A., and Kornau, H. (1999). Role of heteromer formation in GABAB receptor function. *Science* 283, 74–77. doi: 10.1126/science.283.5398.74

Laviv, T., Vertkin, I., Berdichevsky, Y., Fogel, H., Riven, I., Bettler, B., et al. (2011). Compartmentalization of the GABAB receptor signaling complex is required for presynaptic inhibition at hippocampal synapses. *J. Neurosci.* 31, 12523–12532. doi: 10.1523/JNEUROSCI.1527-11.2011

Maldonado, R., Valverde, O., Garbay, C., and Roques, B. P. (1995). Protein kinases in the locus coeruleus and periaqueductal gray matter are involved in the expression of opiate withdrawal. *Naunyn Schmiedebergs Arch. Pharmacol.* 352, 565–575. doi: 10.1007/BF00169392

Margeta-Mitrovic, M., Mitrovic, I., Riley, R. C., Jan, L. Y., and Basbaum, A. I. (1999). Immunohistochemical localization of $GABA_B$ receptors in the rat central nervous system *J. Comp. Neurol.* 405, 299–321.

Mattick, R. P., and Hall, W. (1996). Are detoxification programs effective? *Lancet* 347, 97–100. doi: 10.1016/S0140-6736(96)90215-9

Punch, L. J., Self, D. W., Nestler, E. J., and Taylor, J. R. (1997). Opposite modulation of opiate withdrawal behaviors on microinfusion of a protein kinase A inhibitor versus activator into the locus coeruleus or periaqueductal gray. *J. Neurosci.* 17, 8520–8527.

Quick, M. W. (2002). Substrates regulate γ-aminobutyric acid transporters in a syntaxin 1A-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5686–5691. doi: 10.1073/pnas.082712899

Sharma, S. K., Klee, W. A., and Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 72, 3092–3096. doi: 10.1073/pnas.72.8.3092

Terwilliger, R. Z., Beitner-Johnson, D., Sevarino, K. A., Crain, S. M., and Nestler, E. J. (1991). A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res.* 548, 100–110. doi: 10.1016/0006-8993(91)91111-D

Tyacke, R. J., Lingford-Hughes, A., Reed, L. J., and Nutt, D. J. (2010). $GABA_B$ receptors in addiction and its treatment. *Adv. Pharmacol.* 58, 373–396 doi: 10.1016/S1054-3589(10)58014-1

Vanhoose, A., Ritchie, M., and Winder, D. (2004). Regulation of cAMP levels in area CA1 of hippocampus by Gi/o-coupled receptors is stimulus dependent in mice. *Neurosci. Lett.* 370, 80–83. doi: 10.1016/j.neulet.2004.07.093

Vaughan, C., Bagley, E., Drew, G., Schuller, A., Pintar, J., Hack, S., et al. (2003). Cellular actions of opioids on periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. *Br. J. Pharmacol.* 139, 362–367. doi: 10.1038/sj.bjp.0705261

Vaughan, C., Ingram, S., Connor, M., and Christie, M. (1997). How opioids inhibit GABA-mediated neurotransmission. *Nature* 390, 611–614. doi: 10.1038/37610

Vigot, R., Barbieri, S., Bräuner-Osborne, H., Turecek, R., Shigemoto, R., Zhang, Y., et al. (2006). Differential compartmentalization and distinct functions of GABAB receptor variants. *Neuron* 50, 589–601. doi: 10.1016/j.neuron.2006.04.014

Whitworth, T. L., and Quick, M. W. (2001). Substrate induced regulation of γ-aminobutyric acid transporter trafficking requires tyrosine phosphorylation. *J. Biol. Chem.* 276, 42932–42937. doi: 10.1074/jbc.M107638200

Williams, J. T., Christie, M. J., and Manzoni, O. (2001). Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev*. 81, 299–343.

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A PKA activity sensor for quantitative analysis of endogenous GPCR signaling *via* 2-photon FRET-FLIM imaging

Yao Chen*[1,2], *Jessica L. Saulnier*[1,2], *Gary Yellen*[2] *and Bernardo L. Sabatini*[1,2]

[1] *Howard Hughes Medical Institute, Boston, MA, USA*
[2] *Department of Neurobiology, Harvard Medical School, Boston, MA, USA*

Edited by:
Dominique Massotte, Institut Des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Alfredo Meneses, Center for Research and Advanced Studies, Mexico
Thierry Durroux, Centre National de la Recherche Scientifique, France

***Correspondence:**
Bernardo L. Sabatini, Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115, USA
e-mail: bernardo_sabatini@hms.harvard.edu

Neuromodulators have profound effects on behavior, but the dynamics of their intracellular effectors has remained unclear. Most neuromodulators exert their function *via* G-protein-coupled receptors (GPCRs). One major challenge for understanding neuromodulator action is the lack of dynamic readouts of the biochemical signals produced by GPCR activation. The adenylate cyclase/cyclic AMP/protein kinase A (PKA) module is a central component of such biochemical signaling. This module is regulated by several behaviorally important neuromodulator receptors. Furthermore, PKA activity is necessary for the induction of many forms of synaptic plasticity as well as for the formation of long-term memory. In order to monitor PKA activity in brain tissue, we have developed a 2-photon fluorescence lifetime imaging microscopy (2pFLIM) compatible PKA sensor termed FLIM-AKAR, which is based on the ratiometric FRET sensor AKAR3. FLIM-AKAR shows a large dynamic range and little pH sensitivity. In addition, it is a rapidly diffusible cytoplasmic protein that specifically reports net PKA activity *in situ*. FLIM-AKAR expresses robustly in various brain regions with multiple transfection methods, can be targeted to genetically identified cell types, and responds to activation of both endogenous GPCRs and spatial-temporally specific delivery of glutamate. Initial experiments reveal differential regulation of PKA activity across subcellular compartments in response to neuromodulator inputs. Therefore, the reporter FLIM-AKAR, coupled with 2pFLIM, enables the study of PKA activity in response to neuromodulator inputs in genetically identified neurons in the brain, and sheds light on the intracellular dynamics of endogenous GPCR activation.

Keywords: PKA, FLIM, neuromodulation, cAMP, FLIM-AKAR, GPCR, glutamate, dendritic spine

INTRODUCTION

Neuromodulators such as dopamine, serotonin and opioids have profound effects on neurons, circuits and behavior (Hikosaka et al., 2008; Kreitzer and Malenka, 2008; Le Merrer et al., 2009). Perturbations in neuromodulator function have been linked to diseases such as Parkinson's, and neuromodulator therapy has been used to treat diseases such as depression and schizophrenia (Albin et al., 1989; Nemeroff and Owens, 2002; Le Merrer et al., 2009).

Extensive biochemical characterization has identified protein kinase A (PKA) as a convergent site of action for many neuromodulators and neurotransmitters (Greengard, 2001). Most neuromodulators exert their function *via* G-protein-coupled receptor (GPCRs); neurotransmitters including glutamate and GABA can also act *via* metabotropic receptors that are GPCRs. GPCRs coupled to Gαs and Gαi produce up- and down-regulation of adenylate cyclase (AC) activity, respectively. Activated AC produces cAMP whose accumulation activates PKA. Thus, Gαs- and Gαi-coupled GPCRs bidirectionally change PKA activity (Greengard, 2001). PKA, in turn, modulates synaptic transmission, long-term plasticity, learning and memory, and has been implicated in a number of neurodegenerative and psychiatric diseases (Brunelli et al., 1976; Kandel and Abel, 1995; Davis, 1996; Brandon et al., 1997; Tzounopoulos et al., 1998; Shaywitz and Greenberg, 1999; Baxter, 2003; Skeberdis et al., 2006; Tronson et al., 2006; Shen et al., 2008; Zhong et al., 2009; Higley and Sabatini, 2010). Therefore, PKA can act as a potential integrator of diverse cellular inputs to mediate synaptic and cellular changes.

The neurotransmitter and neuromodulator inputs that activate PKA carry important timing information—for example, dopamine release in the striatum is thought to modulate glutamatergic synapses that are active near the time of release and hence reinforce recently executed behaviors (Schultz, 1998; Berke and Hyman, 2000). In addition, the activity of PKA in different subcellular compartments, such as dendritic spines, the cytoplasm, and the nucleus, phosphorylates different substrates and triggers different cellular responses. Therefore, in order to understand how PKA dynamically integrates ongoing inputs to affect cellular and synaptic function, it is necessary to measure both the timing and subcellular location of PKA activity in response to endogenous GPCR activation. A Förster Resonance Energy Transfer (FRET)-based PKA activity reporter, AKAR3, was developed for ratiometric imaging (Allen and Zhang, 2006). AKAR3 consists of a fusion of a donor fluorophore (truncated CFP),

a phosphopeptide binding domain (FHA1), a consensus region of PKA substrates, and an acceptor fluorophore (circularly permuted Venus) (**Figure 1A**). When PKA is inactive, the donor and acceptor fluorophores are far apart, resulting in low FRET. Upon phosphorylation by PKA, the substrate region binds the phosphopeptide binding domain FHA1, bringing the donor and acceptor fluorophores together and resulting in high FRET. Conversely, dephosphorylation by phosphatases reverses the process. Thus, AKAR3 serves as a PKA substrate to report the balance between PKA and phosphatases, which we here refer to as net PKA activity.

Despite the success of AKAR3 and its derivatives as a ratiometric FRET reporter of PKA activity (Allen and Zhang, 2006; Vincent et al., 2008; Depry et al., 2011; Lam et al., 2012), it poses challenges for quantifying FRET in brain tissue, notably the difficulty to use AKAR3 with two photon (2p) microscopy. An alternative to ratiometric imaging for FRET measurement is Fluorescence Lifetime Imaging Microscopy (FLIM). FLIM only measures the donor, and not acceptor fluorescence, and the fluorescence lifetime of the donor reflects the FRET interaction between the donor and acceptor: increased FRET from donor to acceptor is directly reflected as a reduced fluorescence lifetime of the donor (**Supplementary Figure 1**). A FLIM reporter can potentially alleviate the challenge of 2p ratiometric imaging such as spectral bleedthrough and wavelength-dependent scattering, and allows us to monitor the spatiotemporal dynamics of net PKA activity in brain tissue.

Here, we report the development of a 2pFLIM compatible sensor FLIM-AKAR that reports the balance of PKA and phosphatase activity. The new reporter shows a large dynamic range, little pH sensitivity, and is specific for PKA. In addition, it acts as a rapidly diffusible cytoplasmic protein. The reporter can be introduced into neurons *via* biolistic transfection, *in utero* electroporation, or viral infection, and can report net PKA activity in subcellular compartments including the nucleus and dendritic spines. Furthermore, we engineered a Cre recombinase (Cre)-dependent FLIM-AKAR plasmid which can be packaged into adeno–associated viruses (AAV), allowing expression in genetically identified neurons. FLIM-AKAR signals robustly in response to AC activation, endogenous GPCR activation and induction of long-term potentiation at individual dendritic spines, and it shows differential kinetics of PKA signaling across subcellular compartments. Therefore, FLIM-AKAR, combined with 2pFLIM, provides an essential tool to quantitatively monitor the intracellular dynamics of signaling of a large class of GPCRs with high spatial temporal resolution.

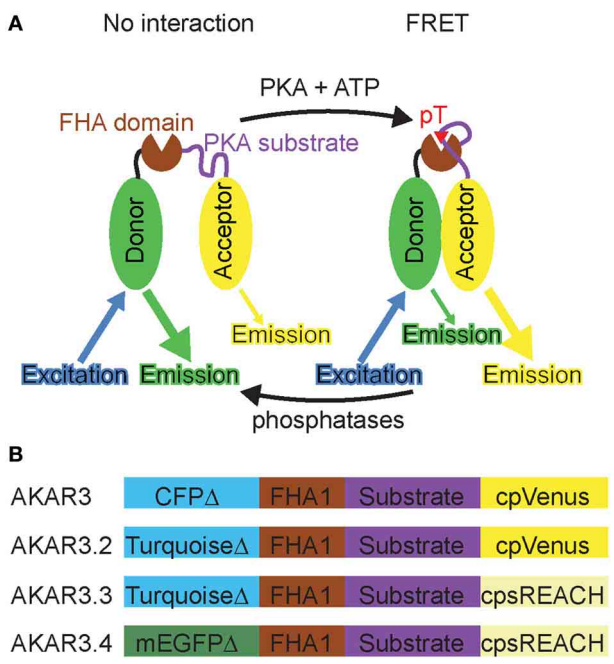

FIGURE 1 | Development of a PKA sensor compatible with 2-photon Fluorescence Lifetime Imaging Microscopy (2pFLIM). (A) Diagram illustrating how PKA activity induces FRET in the reporter. Upon phosophrylation by PKA, the substrate region binds FHA domain, bringing the donor and acceptor together and resulting in FRET. The phosphorylated reporter leads to an increase of acceptor:donor emission ratio, as well as a decrease in donor fluorescence lifetime because of an additional energy transfer pathway. **(B)** Schematic of the original PKA reporter AKAR3 (Allen and Zhang, 2006) and three new PKA reporters.

RESULTS

GENERATION AND COMPARISON OF CONSTRUCTS FOR 2pFLIM IMAGING OF NET PKA ACTIVITY

Although AKAR3 and its derivatives were successfully used for ratiometric imaging of PKA activity (Allen and Zhang, 2006; Vincent et al., 2008; Depry et al., 2011; Lam et al., 2012), they are not suitable for imaging in brain tissue with 2pFLIM due to spectral bleedthrough and the properties of the donor fluorophore. Therefore, we changed the donor-acceptor pair of AKAR3 in order to make a 2pFLIM reporter of PKA activity with the following characteristics: (1) brighter donor fluorescence to improve the signal-to-noise ratio; (2) darker acceptor fluorescence to minimize contamination into the donor channel; (3) donor fluorophore with less lifetime rundown; and (4) free donor fluorophore with lifetime distribution well fit by a single exponential, which makes curve fitting easy so that we can calculate FRET to free donor ratio (**Supplementary Figure 1**). The considerations for choosing the donor-acceptor pair are detailed in **Supplementary Table 1**.

We made three constructs to meet the above criteria (**Figure 1B**) and determined empirically which gave the best dynamic range. All of the constructs showed good expression in HEK293T cells (**Figure 2A** and data not shown). Following addition of the AC activator forskolin to drive PKA activity, all of the constructs showed decreased fluorescence lifetimes and increased FRET fractions of photons, consistent with higher FRET upon reporter phosophorylation (**Figures 2B–D**). Subsequent application of the PKA inhibitor H89 reversed these changes. Of the three new reporters, AKAR3.4 showed the largest amplitude change upon AC activation, as measured by both lifetime changes (Δlifetime) (**Figure 2E**) and changes in the FRET fractions of photons (**Figure 2F**).

Since intracellular pH can respond to electrical and biochemical signals in neurons (Berg et al., 2009; Tantama et al., 2011; Raimondo et al., 2012; Rathje et al., 2013) and can also affect

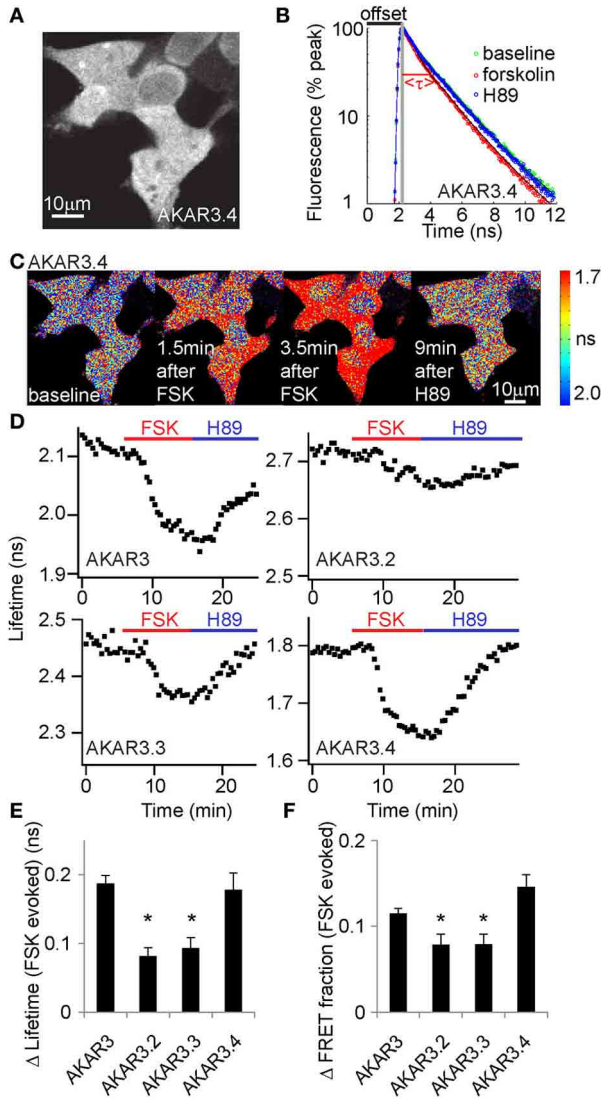

FIGURE 2 | AKAR3.4 reports PKA activity with the largest dynamic range in 2pFLIM measurements. (A) Image showing AKAR3.4 expression in Human Embryonic Kidney (HEK) cells 1 day after transfection. **(B)** Fluorescence decay curves following pulsed excitation. Application of the adenylate cyclase activator forskolin (50 μM) in AKAR3.4-transfected HEK cells results in faster decay and a decrease in lifetime, whereas application of the PKA inhibitor H89 (10 μM) reverses the decay curve to baseline. The offset arrival time, the time from the detection of photon excitation from the laser to the detection of the same pulse from the photomultiplier tube, is labeled in black, and the actual mean fluorescent lifetime τ is labeled in red. **(C)** Lifetime heat map of HEK cells transfected with AKAR3.4, showing lifetime changes induced by forskolin (FSK, 50 μM) followed by H89 (10 μM) treatment. **(D)** Example plots showing lifetime responses of the four different reporters to forskolin (50 μM, red bar) followed by H89 (10 μM, blue bar) treatment. Experiments were done in HEK cells transfected with the respective reporters. **(E)** Amplitudes of Δlifetime between baseline and forskolin (50 μM) treatments. **(F)** Change in the FRET fraction of photons (P_{FRET} based on the annotation in **Supplementary Figure 1**) between baseline and forskolin (50 μM) treatments. For **(E,F)**, $n = 12$, 10, 14, and 12 cells for AKAR, AKAR3.2, AKAR3.3, and AKAR3.4 respectively. $^*p < 0.003$ when the amplitudes of the reporter and AKAR3.4 were compared ($\alpha = 0.017$ for a familywise error rate of 0.05).

protein fluorescence, we characterized how the different constructs responded to changes in pH. To manipulate intracellular pH, the K^+/H^+ ionophore nigericin was used to permeabilize the plasma membrane to protons (Thomas et al., 1979), and extracellular solutions buffered to different pH values were applied. In addition, the cell-permeable PKA inhibitor H89 was included to eliminate any potential pH-induced change in PKA activity. Of the four constructs, AKAR3 and AKAR3.2 showed large changes with varying pH, whereas AKAR3.3 and AKAR3.4 showed little pH sensitivity (**Figure 3**). AKAR3.4 contains truncated monomeric eGFP (meGFPΔ) as the donor and circularly permuted dark YFP (cpsREACH) as the acceptor. Therefore, in addition to the largest amplitude of Δlifetime in response to AC activation and little pH sensitivity, AKAR3.4 also fulfilled the criteria outlined above, showing brighter donor fluorescence (Piston et al.; Shaner et al., 2007), darker acceptor fluorescence (Ganesan et al., 2006; Murakoshi et al., 2008), and less donor lifetime rundown than the original AKAR3 (**Figure 2D**). meGFP also shows a single exponential decay (Murakoshi et al., 2008), whereas CFP in AKAR3 shows multi-exponential decay. Thus, AKAR3.4 is the most suitable PKA activity reporter for quantitative 2pFLIM imaging. We termed it FLIM-AKAR and used it for the remainder of the study.

DIFFUSION AND SPECIFICITY OF FLIM-AKAR

FLIM-AKAR is a substrate for PKA phosphorylation and is not targeted to any subcellular compartments. Therefore, in order to understand if spatial patterns of the FLIM-AKAR response represent subcellular spatial differences in net PKA activity in real time, it is necessary to characterize its diffusional properties. To do so, we performed fluorescence recovery after photobleaching (FRAP) in CA1 pyramidal neurons to measure the replenishment of FLIM-AKAR after bleaching (**Figures 4A–C**). FRAP in aspiny regions of dendrites revealed a time constant of recovery of 323 ± 42 ms (mean ± standard error of the mean (SEM), $n = 16$ regions of dendrites, **Figure 4E**). FRAP in the heads of mushroom-like spines revealed time constants of recovery of 544 ± 49 ms (mean ± SEM, $n = 22$ spines, **Figure 4D**). This is comparable to time constants of diffusional equilibration across spine head for other diffusible proteins of similar size (Pologruto et al., 2004; Bloodgood and Sabatini, 2005; Harvey et al., 2008; Lee et al., 2009; Yasuda and Murakoshi, 2011). Therefore, we conclude that FLIM-AKAR behaves as a rapidly diffusible cytoplasmic protein. Thus, if a more persistent lifetime change is observed in the spine in a given experiment compared with the diffusion time constant, it is attributable to active PKA or phosphatase activity in the spine (see below).

Previous versions of AKAR do not respond to CamKII or PKC. In addition, in the presence of the PKA inhibitor H89 or the PKA inhibitor peptide (PKI), they do not respond to isoproterenol activation of the Gαs-coupled β-adrenergic receptors (Zhang et al., 2001, 2005; Allen and Zhang, 2006), confirming their specificity for PKA. We also tested the specificity of FLIM-AKAR to report changes in net PKA activity. First, we introduced a point mutation at the phosphorylation site in the substrate region. This mutant reporter (FLIM-AKAR^{T391A}) did not respond to forskolin or H89 application (**Figures 5A–C**), indicating that this

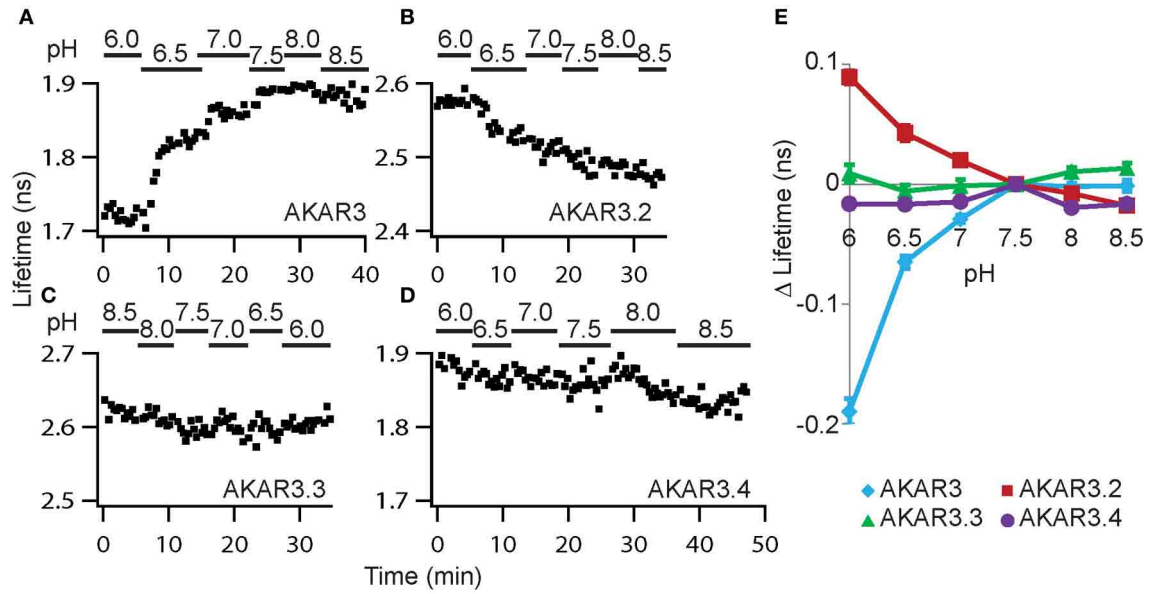

FIGURE 3 | AKAR3.4 shows little sensitivity to pH. (A–D) Example plots showing responses of the four reporters to pH changes. Experiments were performed in HEK cells at room temperature in the presence of nigericin (5 μM) and the PKA inhibitor H89 (10 μM). Extracellular solutions buffered to different pH values were applied. **(E)** Summary graph showing Δlifetime in response to different pH in HEK cells. Δlifetime was measured relative to that at pH 7.5. $n = 8$, 8, 7, and 7 cells for AKAR3, AKAR3.2, AKAR3.3, and AKAR3.4 respectively. Graphs show mean and SEMs.

phosphorylatable residue is required for FLIM-AKAR response to AC activation. Second, FLIM-AKAR did not respond to activation of PKC by phorbol 12, 13-dibutyrate (PDBu), confirming that the reporter is not sensitive to PKC activation ($n = 9$ cells). Third, addition of H89 to inhibit PKA largely reversed forskolin-induced Δlifetime (**Figures 5B,E**). Finally, in the presence of the PKA inhibitor peptide PKI (Ashby and Walsh, 1972, 1973; Dalton and Dewey, 2006), FLIM-AKAR did not respond to addition of forskolin or H89 (**Figures 5D–F**). Taken together, these results demonstrate that FLIM-AKAR does not respond to PKC, and is specific for PKA following AC activation by forskolin.

In vivo EXPRESSION AND RESPONSE TO ENDOGENOUS RECEPTOR ACTIVATION

We tested if FLIM-AKAR shows sufficiently high expression *in vivo* and adequate characteristics to report changes in PKA activity following glutamate or endogenous neuromodulator receptor activation in thick brain tissue. We introduced FLIM-AKAR with three different methods into brain tissue. First, we transfected FLIM-AKAR biolistically in organotypic slices (**Figure 6A**). Second, we introduced FLIM-AKAR by *in utero* electroporation into the cortex (data not shown) or hippocampus (**Figure 6B**). Third, we generated an adeno-associated virus (AAV) that expresses FLIM-AKAR in a Cre-recombinase (Cre) dependent manner, and injected the virus into mice expressing Cre in specific cell populations (**Figures 7A–C**). In all three cases, FLIM-AKAR showed robust expression, and can be seen in the soma and dendrites, including dendritic spines. Thus, FLIM-AKAR is a versatile reporter that can be introduced by a variety of methods to achieve expression *in vivo*.

In order to test if FLIM-AKAR has the sensitivity to respond to spatiotemporally precise and physiologically relevant stimuli, we delivered glutamate by 2-photon photolysis of photoactivatable glutamate (MNI-glutamate) adjacent to individual dendritic spines. The stimulated spine enlarged in response to a structural plasticity protocol previously reported in multiple studies to induce potentiation of the associated postsynaptic terminal (Matsuzaki et al., 2004; Steiner et al., 2008; Murakoshi et al., 2011). Strikingly, this resulted in a decrease in fluorescence lifetime during and after photoactivation to release glutamate (**Figure 6C**). Thus, 2-photon photolysis of photoactivatable glutamate leads to a net increase of PKA activity in the stimulated spine, and FLIM-AKAR can report net PKA activity in small volumes such as the spine in response to spatiotemporally precise stimuli.

To test if FLM-AKAR can respond to endogenous GPCR activation, we used isoproterenol to activate the Gαs-coupled β-adrenergic receptors in hippocampal CA1 pyramidal neurons (**Figure 6D**). Fluorescence lifetime decreased in both soma and dendrites, indicating an increase in net PKA activity. Subsequent application of forskolin resulted in a further lifetime decrease in somatic cytoplasm but not apical dendrites, indicating that isoproterenol induced maximal reporter phosphorylation in apical dendrites and partial AC activation in somatic cytoplasm.

Finally, we targeted FLIM-AKAR to genetically defined cell types and examined FLIM-AKAR response to endogenous GPCR activation in distinct cell types. For this purpose, we tested FLIM-AKAR in the striatum, a subcortical brain region that receives a large number of neuromodulator inputs (Kreitzer and Malenka, 2008; Lerner and Kreitzer, 2011). In the striatum, different neuromodulator receptors are preferentially expressed in different types of striatal spiny projection neurons (SPNs). Indirect pathway SPNs (iSPNs) preferentially express the Gαs-coupled adenosine

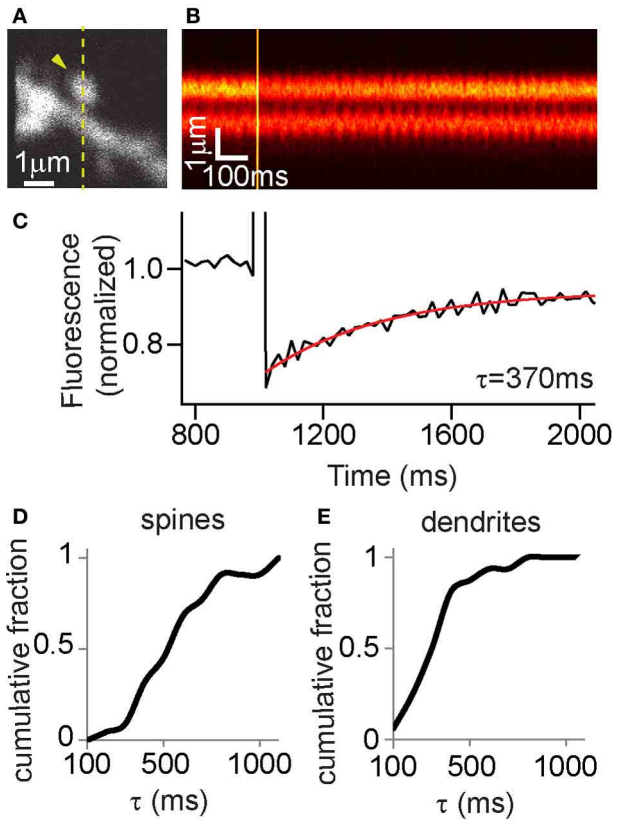

FIGURE 4 | FLIM-AKAR shows similar diffusion properties to a rapidly diffusing cytoplasmic protein. (A–E) Fluorescence Recovery After Photobleaching (FRAP) experiments to measure the spreading of FLIM-AKAR. **(A)** Image of FLIM-AKAR donor fluorescence of a dendritic region of a hippocampal CA1 neuron cultured in organotypic slices. The arrowhead shows the spine that was photobleached. The dashed line shows the region being imaged by line scan. **(B)** Fluorescence measured in line scans for the region indicated by dashed line in **(A)**. **(C)** Quantification of FRAP for the spine shown in **(A,B)**. The trace was an average of 4 acquisitions. The red trace shows curve fitting with a single exponential decay. **(D,E)** Cumulative distribution of τ from FRAP experiments to examine FLIM-AKAR spreading from spines **(D)** and for aspiny regions of dendrites **(E)** in hippocampal CA1 neurons.

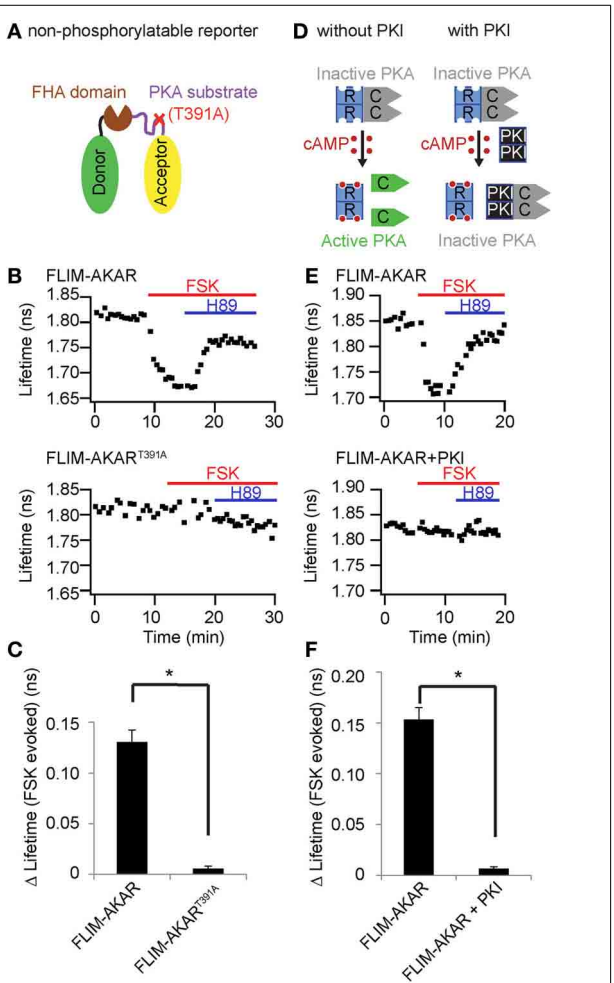

FIGURE 5 | FLIM-AKAR is specific for PKA following adenylate cyclase activation by forskolin. All experiments were performed in HEK cells. Red line indicates bath application of 50 μM forsklin (FSK) and blue line indicates bath application of 10 μM H89. **(A)** Schematic illustrating introduction of the point mutation T391A that renders the PKA phosophorylation site of FLIM-AKAR non-functional. **(B)** Example plots showing FLIM-AKAR lifetime response to adenylate cyclase activation by forskolin (red) and subsequent PKA inhibition by H89 (blue) (top), and the lack of a response from the non-phosphorylatable point mutant FLIM-AKAR^{T391A} (bottom). **(C)** Summary bar graph showing Δlifetime from baseline to forskolin treatment for FLIM-AKAR ($n = 9$ cells) and the non-phosphorylatable mutant of FLIM-AKAR ($n = 11$ cells). $^*p < 10^{-5}$. **(D)** Schematic illustrating how PKI inhibits PKA activity. Without PKI, binding of cAMP to the regulatory subunits of PKA (R) frees the catalytic subunits (C), resulting in activation of PKA. With PKI, even though cAMP binding to the regulatory subunits dissociates them from the catalytic subunits, PKI can bind to the catalytic subunits of PKA and inhibit their activity. **(E)** Example plot showing FLIM-AKAR response to adenylate cyclase activation and subsequent PKA inhibition (top), and the lack of a lifetime response by FLIM-AKAR when it was co-transfected with PKIα (bottom). **(F)** Summary bar graph showing Δlifetimes from baseline to forskolin treatment for FLIM-AKAR, in the absence ($n = 18$ cells) and presence ($n = 10$ cells) of PKI. $^*p < 10^{-9}$.

receptor $A_{2A}R$, whereas direct pathway SPNs (dSPNs) preferentially express the Gαs-coupled dopamine D1 receptors (D1R) (Kreitzer and Malenka, 2008; Lerner and Kreitzer, 2011). Thus, we would expect that activation of the preferentially expressed Gαs-coupled receptors would result in net PKA activity in the corresponding type of SPNs. Here, we first injected AAV encoding Cre-dependent FLIM-AKAR into the striatum of *Adora2a* BAC-Cre mice (Heintz, 2004; Durieux et al., 2009) to target iSPNs. FLIM-AKAR showed robust expression in the striatum (**Figures 7B,C**). Application of the $A_{2A}R$ agonist CGS21680 decreased lifetime in both the cytoplasm and the nucleus (**Figure 7D**). We also used *Drd1* BAC-Cre mice (Heintz, 2004; Gong et al., 2007), and injected virus carrying the Cre-dependent FLIM-AKAR into the striatum in order to target dSPNs. The D1R agonist SKF81297 produced a rapid lifetime decrease in the cytoplasm and a slower decrease in the nucleus (**Figure 7E**). Therefore, FLIM-AKAR can be targeted to genetically defined cell types, was able to report regulation of PKA activity by distinct endogenous GPCR in these cell types, and revealed differential kinetics in different subcellular compartments.

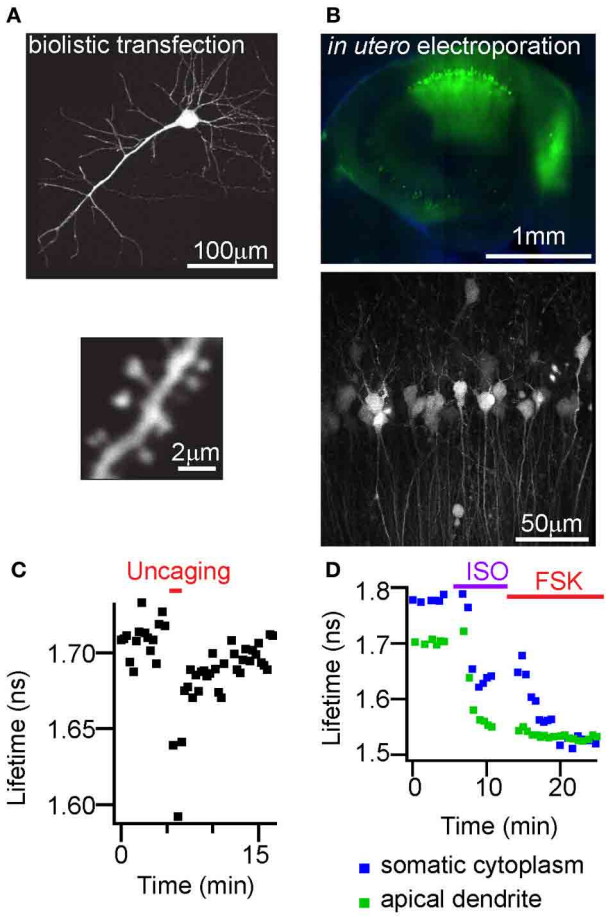

FIGURE 6 | FLIM-AKAR shows high expression with different transfection methods and lifetime responses to glutamate or GPCR activation in the hippocampus. (A) Images showing a hippocampal CA1 neuron (top), dendrite and spines (bottom) from an organotypic slice transfected with FLIM-AKAR with biolistic method. **(B)** Image showing a 300 μm acute hippocampal slice expressing FLIM-AKAR in CA1 region after *in utero* electroporation. **(C)** Example plot of lifetime change of FLIM-AKAR in a stimulated spine in response to photolysis of caged glutamate adjacent to the spine. A CA1 pyramidal neuron in an organotypic hippocampal slice was biolistically transfected with FLIM-AKAR, and the stimulated spine shows enlargement following 2-photon photolysis of caged glutamate (MNI-glutamate). The temporal window of uncaging is indicated in the red bar above. **(D)** Lifetime response of FLIM-AKAR upon isoproterenol (1 μM, ISO) treatment to activate β-adrenergic receptors followed by forskolin (50 μM, FSK) treatment to activate adenylate cyclases. The experiment was done in acute hippocampal slice expressing FLIM-AKAR in CA1 region after *in utero* electroporation.

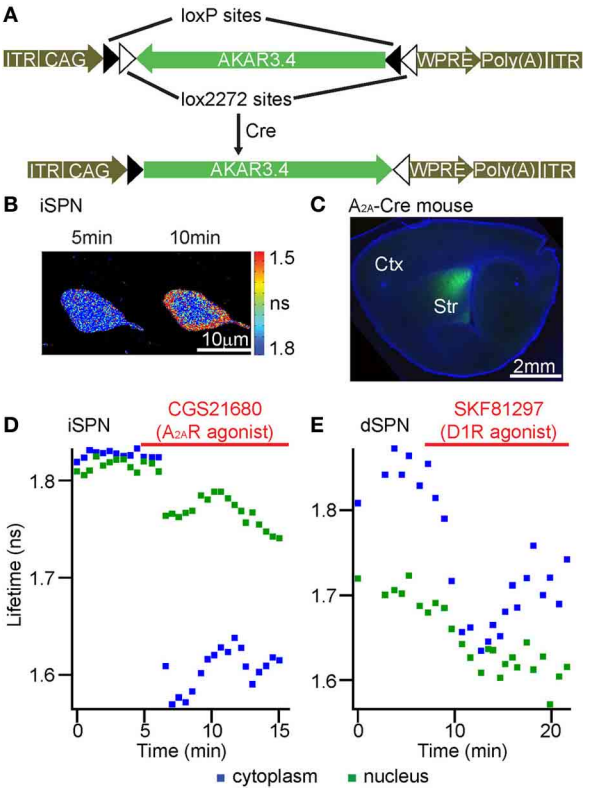

FIGURE 7 | FLIM-AKAR can be targeted to genetically defined cell types and reports modulation of PKA by endogenous GPCRs in the striatum. (A) AAV plasmid map showing how Cre recombinase leads to AKAR3.4 expression. **(B–D)** A Cre-dependent AAV virus carrying FLIM-AKAR was delivered to the striatum of an *Adora2a* BAC-Cre mouse. **(B)** Lifetime heat map of an indirect pathway striatal spiny projection neuron (iSPN) in an acute striatal slice. Following $A_{2A}R$ activation by its agonist CGS21680, cytoplasmic FLIM-AKAR became rapidly phosphorylated, while nuclear FLIM-AKAR showed a slower response. **(C)** A parasagittal brain section showing FLIM-AKAR expression in the striatum. **(D)** Example plots showing modulation of PKA by $A_{2A}R$ in an iSPN. 1 μM CGS21680 was used to activate $A_{2A}R$. **(E)** A Cre-dependent AAV virus carrying FLIM-AKAR was delivered to the striatum of a *Drd1* BAC-Cre mouse to target direct pathway SPNs (dSPNs). Example plots showing modulation of PKA by SKF81297 in a dSPN. 1 μM SKF81297 was used to activate D1R.

DISCUSSION

In order to image the spatiotemporal dynamics of net PKA activity in brain tissue, we developed and characterized a 2pFLIM-compatible PKA reporter FLIM-AKAR for quantitative imaging. FLIM-AKAR shows a large dynamic range of FLIM signals and little pH sensitivity. In addition, it is a diffusible cytoplasmic protein and shows specificity to PKA following forskolin stimulation. Finally, it can be targeted to genetically defined neurons, and can report net PKA activity in response to both glutamate and endogenous neuromodulator GPCR activation. Thus, FLIM-AKAR enables the study of PKA activity in response to neurotransmitter and neuromodulator inputs in genetically identified cell types, and promises to shed light on the intracellular dynamics of endogenous GPCR signaling.

A PKA REPORTER FOR 2pFLIM

2p microscopy facilitates fluorescence imaging within living tissue such as the brain due to its diminished sensitivity to light scattering and the restriction of fluorophore excitation to the focus. However, it poses considerable challenges for ratiometric FRET imaging. This is mainly because the 2p excitation spectra of many fluorophores are broad, resulting in overlapping donor and acceptor excitation and emission spectra, which leads to noisy intensity measurements since the noise is amplified during correction for spectral bleedthrough during ratiometric imaging (Yasuda et al., 2006).

2pFLIM is advantageous over ratiometric imaging for FRET measurement in thick brain tissue (Bastiaens and Squire, 1999; Yasuda, 2006). Since 2pFLIM directly measures the lifetime of the donor fluorophore, it is insensitive to many of the technical challenges that accompany ratiometric FRET imaging in brain tissue such as fluorophore concentration and wavelength-dependent light scattering. 2pFLIM is also not sensitive to artifacts introduced by low intensity measurements in ratiometric FRET, notably focus change, tissue movement, and differential photobleaching. Furthermore, with the use of dark acceptor fluorophores (Ganesan et al., 2006; Murakoshi et al., 2008), 2pFLIM avoids the propagation of noise due to corrections for spectral bleedthrough and ectopic excitation of fluorophores involved in ratiometric FRET. Finally, 2pFLIM is inherently quantitative: donor lifetime analysis directly gives the fractions of fluorophores that are free and that have undergone FRET. Thus, although the PKA reporter AKAR3 and two of its derivatives have proven valuable for ratiometric FRET imaging (Allen and Zhang, 2006; Depry et al., 2011; Lam et al., 2012), a 2pFLIM reporter of PKA activity offers advantages for the study of endogenous GPCR signaling in intact brain tissue.

The 2pFLIM compatible reporter FLIM-AKAR shows robust responses to neuromodulator and neurotransmitter receptor activation in brain tissue. We found net PKA activity increase with activation of β-adrenergic receptors in hippocampal CA1 neurons, of $A_{2A}R$ in iSPNs, and of D1R in dSPNs, all of which are consistent with the Gαs-coupling of these receptors (Lefkowitz, 2007; Lerner and Kreitzer, 2011). Beside neuromodulator receptor activation, FLIM-AKAR also revealed an increase in net PKA activity in response to glutamate, potentially due to metabotropic glutamate receptor activation (Wang and Zhuo, 2012). Thus, based on the characterization of the reporter and its demonstrated utility in brain tissue, FLIM-AKAR is suitable for studying how PKA activity responds to endogenous GPCR signaling in the brain.

For future use of FLIM-AKAR, a couple of points should be noted. First, we confirmed the specificity of FLIM-AKAR for PKA with multiple methods (**Figure 5**). However, true specificity of any reporter has to be demonstrated for each biological application and experimental context, since each stimulus may elicit a different range of intracellular signals. Therefore, similar specificity tests should be performed for every new stimulus used in the future. Second, the expression level of a FLIM sensor is important. Since autofluorescence with a non-uniform lifetime distribution can contaminate actual signals, sufficient expression level is required for an accurate measurement of the lifetime of a sensor. The amount of expression required can be estimated by simulation of data combined with autofluorescence measurements in the biological system. In the biological experiments described here, the expression level is sufficient with all three methods of transfection.

DIFFUSION OF THE 2pFLIM REPORTER AND COMPARTMENTALIZED NET PKA ACTIVITY

Compartmentalization of intracellular signals is a key feature of neuronal processing that gives rise to synaptic specificity, and allows spatially segregated responses to different types of signals (Chen and Sabatini, 2012). Differential PKA kinetics has been demonstrated between cellular membrane, cytoplasm and nucleus (Dipilato et al., 2004; Allen and Zhang, 2006; Gervasi et al., 2007). Since FLIM-AKAR is a PKA substrate that is not specifically tagged, the diffusibility of the 2pFLIM reporter is important for interpreting the localization of PKA activity. Using FRAP we determined that FLIM-AKAR spreads like a diffusible cytoplasmic protein (**Figure 4**), with a time constant of hundreds of milliseconds.

Comparing time constants between FLIM-AKAR diffusion and biological experiments can reveal true temporal persistence and spatial compartmentalization of net PKA activity. Following structural plasticity induction, the lifetime decrease must be attributable to long-lasting net PKA activity within the spine, since the time of recovery to baseline lifetime (minutes) is far longer than the time constant of FLIM-AKAR diffusion from the spine (hundreds of milliseconds). In addition to the temporal persistence, we also demonstrate spatial heterogeneity in net PKA activity, both during basal states, and in the kinetics and amplitudes of response to GPCR activation (**Figures 6**, **7**). These spatial differences are due to differential net PKA activity between dendrites, nucleus and somatic cytoplasm, since the differences persist on the order of minutes. This raises the interesting possibility that different physiological signals (different types, duration, and amplitude of stimulus) can change net PKA activity in different subcellular compartments, resulting in distinct functional consequences. FLIM-AKAR allows future investigations addressing the types of stimuli that result in net PKA activity changes in specific compartments, and the active mechanisms that maintain net PKA activity differences across subcellular compartments.

QUANTIFICATION OF FLIM CHANGES AND RELATIONSHIP TO GPCR SIGNALING

An important advantage of FLIM is that it is inherently quantitative, such that the fitting of the fluorescence decay curve with a double-exponential decay function gives the fractions of donor fluorophores that are free and that have undergone FRET (for simplicity of notation, the convolution term with instrument response curve is not included here) (Yasuda, 2006):

$$F(t) = F_0(P_{free}e^{-\frac{t}{\tau_{free}}} + P_{FRET}e^{-\frac{t}{\tau_{FRET}}})$$

where $F(t)$ is the fluorescence over time, F_0 is the peak fluorescence, τ_{free} and τ_{FRET} are fluorescence lifetimes of donors that are free and that have undergone FRET respectively, and P_{free} and P_{FRET} are the corresponding fractions of these two species. In the case of the PKA reporter AKAR,

$$[AKAR] \underset{k_{phosphatase}}{\overset{k_{PKA}}{\rightleftharpoons}} [pAKAR]$$

where pAKAR stands for phosphorylated AKAR. If the expression level of FLIM-AKAR is low, steady state for the reporter can be achieved at all times. If the expression level is high, steady state may only be reached when the lifetime reaches a constant value, and the time course of the reporter lifetime between these constant values may lag the actual kinase or phosphatase activity. At

steady state,

$$k_{PKA}\,[AKAR] = k_{phosphatase}\,[pAKAR]$$

Rearrangement of the equation gives the following:

$$\frac{[AKAR]}{[pAKAR]} = \frac{k_{phosphatase}}{k_{PKA}}$$

Therefore, knowing the fraction of pAKAR ($f_{pAKAR} = \frac{[pAKAR]}{[AKAR]+[pAKAR]}$) would allow for the calculation of $\frac{k_{phosphatase}}{k_{PKA}}$. f_{pAKAR} can be interpolated from the fraction of free donors in a measurement. If $P_{free}\,(experiment)$ is the fraction of free donors in a given experiment, $P_{free}\,(AKAR)$ is the fraction of free donors measured when the reporters are not phosphorylated (for example, with the PKA inhibitor H89 or with the non-phosphorylatable mutant of FLIM-AKAR), $P_{free}\,(pAKAR)$ is the fraction of donors measured when the reporters are completely phosphorylated (for example, with forskolin, phosphodiesterase inhibitor and phosphatase inhibitor), then

$$f_{pAKAR} = \frac{P_{free}\,(AKAR) - P_{free}\,(experiment)}{P_{free}\,(AKAR) - P_{free}\,(pAKAR)}$$

Thus, the fraction of free donors from a given FLIM measurement can give the fraction of pAKAR, which gives the ratio of $\frac{k_{phosphatase}}{k_{PKA}}$ at steady state. Therefore, 2pFLIM measurement with FLIM-AKAR gives valuable quantitative information about the kinetic balance between PKA and phosphatase activity.

Taken together, our 2pFLIM compatible reporter FLIM-AKAR allows analysis of endogenous GPCR signaling in brain tissue, and can reveal previously unavailable quantitative information on the kinetic balance between phosphorylation and dephosphorylation of PKA substrates. Biologically, the reporter has revealed compartmentalization of net PKA activity in the dendrite, somatic cytoplasm and nucleus. With the multiple advantages of 2pFLIM imaging, FLIM-AKAR promises to reveal net PKA activity in response to neuromodulator inputs with high spatial temporal specificity.

MATERIALS AND METHODS

DNA CONSTRUCTS

The original pcDNA3-AKAR3 construct was a gift from Jin Zhang (Johns Hopkins University) (Allen and Zhang, 2006). AAV-AKAR3 was constructed by subcloning the coding region of pcDNA3-AKAR3 into the AAV vector AAV-ChR2-mCherry via EcoRI and BamHI sites. AAV-AKAR3 was used in imaging experiments in this study and referred to as AKAR3. AKAR3.2 was constructed by gene synthesis of codon-optimized truncated mTurquoise (Goedhart et al., 2010) (amino acid 1-227) together with part of the linker region between the donor and acceptor fluorophores, and subcloning of the synthesized fragment into AKAR3 via BamHI and PpumI (Genscript). AKAR3.3 was constructed by gene synthesis of part of the linker region between the donor and acceptor fluorophores together with circularly permuted sReaCh (Murakoshi et al., 2008) (^{175}sReaCh^{173}), and subcloning of the synthesized fragment into AKAR3.2 via SgrAI and EcoRI (Genscript). AKAR3.4 (also called FLIM-AKAR) was constructed by PCR amplification of truncated meGFP (amino acid 1-227) from the template GFP-sReaCh (Murakoshi et al., 2008) (Addgene) followed by recombination-based cloning with CloneEZ into AKAR3.3 to replace truncated mTurquoise (Genscript).

For the construction of the Cre-dependent reporter AAV-FLEX-FLIM-AKAR, the coding region of FLIM-AKAR was amplified by PCR and subcloned into AAV-FLEX-Arch-GFP (Atasoy et al., 2008; Chow et al., 2010) (Addgene Plasmid 22222) to replace Arch-GFP by recombination-based cloning with CloneEZ (Genscript).

The non-phosphorylatable point mutant AAV-FLEX-FLIM-AKAR^{T391A} was made by site-directed mutagenesis of threonine to alanine at amino acid 391 of the construct AAV-FLEX-FLIM-AKAR.

For the construction of AAV-FLEX-PKIalpha-IRES-mRuby2, the coding region of mouse PKIalpha (GenBank ID: NM_008862) was made by gene synthesis, followed by subcloning of the synthesized fragment into AAV-FLEX-tmeGFP-IRES-nls-mRuby2 via AvrII and BglII (Genscript). AAV-FLEX-tmeGFP-IRES-nls-mRuby2 was constructed by gene synthesis of IRES-nls-mRuby2 (Lam et al., 2012) and subsequent cloning into AAV-FLEX-FLIM-AKAR via XbaI and XhoI (Genscript).

pBS-β-actin Cre was a gift from Susan Dymecki (Harvard Medical School).

CELL CULTURE AND TRANSFECTION

HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) at 37°C in 5% CO_2. They were plated on coverslips in 24-well plates and transfected with plasmids using Lipofectamine 2000 (Invitrogen). Approximately 15–48 h after transfection, the cells were imaged in solutions containing either HEPES-based buffer (containing in mM: 130 KCl, 1 EGTA, 1 $MgCl_2$, 25 HEPES, 10 glucose, 20 sucrose, pH with KOH to 7.5 or as specified in the manuscript), or ACSF (containing in mM: 127 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 25 glucose) with carbogen (95% O_2, 5% CO_2).

For data in **Figure 5**, pBS-β-actin Cre was cotransfected with AAV-FLEX-FLIM-AKAR, AAV-FLEX-FLIM-AKAR^{T391A} or AAV-FLEX-PKIalpha-IRES-mRuby2.

ANIMAL HUSBANDRY

All procedures for animal husbandry and surgery were performed following protocols approved by the Harvard Standing Committee on Animal Care and in accordance with National Institutes of Health guidelines.

BRAIN SLICE PREPARATIONS

Organotypic hippocampal slices were cultured from 6 to 8 day old Spraque Dawley rats (Stoppini et al., 1991). The brain was dissected and immediately placed in cold dissection media. Transverse hippocampal slices were cut with 400 μm thickness and placed above a sterile culture insert (Millicell-CM, Millipore) in 6-well plates containing prewarmed culture media. DNA

plasmids were biolistically transfected with a Helios Gene Gun (Biorad) 2 days after culturing. Bullets were made with 60 μg of DNA.

For acute slices, mice were anesthetized with isoflurane. For hippocampal slices, the brain of C57BL/6 mice was rapidly dissected out. Horizontal sections were cut at 300 μm thickness using a Leica VT1000S vibratome (Leica Instruments, Nussloch, Germany) in cold sucrose cutting solution containing (in mM) 87 NaCl, 25 NaHCO3, 1.25 NaH2PO4 2.5 KCl, 75 sucrose, 25 glucose, 7.5 MgCl2. For striatal slices, mice first underwent intracardiac perfusion with cold ACSF. Coronal or parasagittal sections were then cut at 300 μm thickness in cold choline cutting solution containing (in mM) 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 7 $MgCl_2$, 25 glucose, 1 CaCl2, 110 choline chloride, 11.6 ascorbic acid, and 3.1 pyruvic acid. Slices were transferred to ACSF after sectioning. The slices were incubated at 34°C for 10–15 min and then kept in ACSF at room temperature. Slices were then transferred to a microscope chamber and imaging was performed in perfusing ACSF with a flow rate of 2–4 ml/min. Both cutting and ACSF solutions were constantly bubbled with carbogen.

In utero ELECTROPORATION

To target hippocampal pyramidal neurons by *in utero* electroporation, glass injection micropipettes were pulled, and the tip broken to be approximately 60 μm in diameter, and beveled at 18° (NARISHIGE, Japan). E15 timed-pregnant female C57BL/6 mice (Charles River, MA, United States) were anesthetized with 2% isoflurane. 1–2 μl of DNA (1 μg/μl) with the dye FastGreen (0.005%) were injected into the left lateral ventricle. The embryo head was then held with a tweezer with round plate electrodes (0.5 mm diameter) and electric pulses were delivered five times per second (50 V, 50 ms) with the cathode placed at the right cortex and the anode at the left cortex (CUY21 electroporator, NEPA GENE, Japan). Warm PBS was dripped onto embryos periodically. The uterus was placed back into the pregnant mother, and the muscle and the skin were sutured separately. Pups were housed with the mother until they were used.

VIRUS PRODUCTION AND STEREOTAXIC VIRAL INJECTIONS

AAV-FLEX-FLIM-AKAR was packaged as serotype 8 at University of North Carolina Gene Therapy Center Virus Core Facility. For stereotaxic viral injections, P2–4 pups were anesthetized with isofluorane and placed on a small stereotaxic frame (David Kopf Instruments). For striatal injections, 1 μl of virus ($2 \times 10\hat{}12$ genome copies/ml of AAV-FLEX-FLIM-AKAR) were injected into the right hemisphere of either an *Adora2a* BAC-Cre pup (GENSAT, founder line KG139) to target iSPNs (Heintz, 2004; Durieux et al., 2009), or a *Drd1* BAC-Cre pup (GENSAT, founder line EY262) to target dSPNs (Heintz, 2004; Gong et al., 2007). Injection coordinates were approximately lateral 1.5 mm from Bregma, and 2.3 mm beneath the skull. The injection was at a rate of 200 nl/min through a UMP3 microsyringe pump (World Precision Instruments). After injection, pups were returned to their home cage with their mother, and kept for 16–22 days before being used for experimentation.

TWO-PHOTON IMAGING AND FLUORESCENCE LIFETIME IMAGING MICROSCOPY

Two-photon imaging was achieved by a custom-built microscope (Carter and Sabatini, 2004) together with a mode-locked Ti:sappire laser (Chameleon Vision II, 80MHz, Coherent, Santa Clara, CA). Photons were collected with fast photomultiplier tubes (PMTs) (H7422-40MOD Hamamatsu). Excitation wavelengths of 820, 860, and 920 nm were used to excite donor fluorophores of CFP, Turquoise, and meGFP respectively. On the emission path, a 700SP filter (Semrock) was used to filter off excitation light. For meGFP imaging, a 565LP dichroic mirror (Chroma) and 525/50 emission filter (Semrock) were used. For CFP and Turquoise imaging, a 520LP dichroic mirror (Semrock) and 480/40 emission filter (Chroma) were used.

The custom-written software ScanImage (Pologruto et al., 2003) was run in Matlab to acquire imaging data. The FLIM data acquisition and analysis modules were modified from the software from Ryohei Yasuda (Max Planck Florida Institute). Scan mirror control and fluorescence signal acquisition were achieved with the data acquisition (DAQ) board PCI 6110E (National Instruments). An additional DAQ board PCI 6713 (National Instruments) is used to generate frame and line clocks to synchronize ScanImage and the FLIM board SPC-150 (Becker and Hickl GmbH).

The epifluorescence PMTs were used for 2pFLIM, and transfluorescence PMTs for regular imaging. Time-domain single photon counting was used for FLIM and the data were collected in either 64 or 256 time channels. Lifetime decay curve was constructed by comparing times of laser pulses detected by photodiode and photon pulses from the fast PMT.

FLUORESCENCE LIFETIME CURVE FITTING AND IMAGE ANALYSIS

Instrument response curve (IRF) for photon spreading was measured with double harmonic generation of urea crystals. It was then used to deconvolve the fluorescence decay curve.

FLIM data were processed by first determining the offset arrival time from a full field-of-view to increase the accuracy of fitting, followed by calculation of FRET fractions of photons for individual regions of interests (ROIs). The processing procedure was as described in Yasuda et al. (2006), Harvey et al. (2008), except that a measured IRF rather than a Gaussian IRF was used to fit the fluorescence decay curve.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

For FRAP experiments with FLIM-AKAR, 920 nm laser was used to photobleach meGFP. Fluorescence data were collected 1 s before bleaching and then 10 s after a step perturbation of bleaching. Laser pulse width (4, 50, or 100 ms) and laser power were adjusted to give 30–50% bleaching. Three to ten acquisitions were collected for each spine or dendritic region. For FRAP in spines, mushroom spines were selected; for FRAP in dendrites, relatively aspiny regions of dendrites (aspiny in a 5.5 ×5.5 μm square) were selected. Less than 3 spines or dendrites were imaged from each cell. Linescan was used to achieve good temporal resolution of FRAP data.

For processing of FRAP data, fluorescence data from each acquisition was normalized against baseline before photobleaching. The data were then averaged and fitted with a

single exponential decay curve (not specifying return to baseline) to calculate τ.

STATISTICS

Student *t*-tests (unpaired, assuming unequal variance) were used to compare different conditions. In cases where various reporters were compared with AKAR3.4, Bonferroni correction was used to counteract the problem of multiple comparisons.

2-PHOTON PHOTOLYSIS OF CAGED GLUTAMATE

A second laser was tuned to 720 nm for 2-photon photolysis of caged glutamate. The bath solution consists of 9 ml of circulating ACSF, with no magnesium, 4 mM CaCl2, 1 μM TTX, 10 μM D-serine, 200 μM Trolox (Sigma), 2 mM pyruvate, 5 U/ml glutamic-pyruvate transaminase (Sigma), and 3.3 mM MNI-glutamate (Tocris). Light power of 75 mW at the back aperture of the 60X (NA1.1) objective was used. Light pulses were delivered at 0.5 ms duration for each pulse, and 40 pulses were delivered in 1 min. 2-photon uncaging was performed adjacent to a spine, and both the spine morphology and FLIM-AKAR response were monitored.

PHARMACOLOGY

Drugs were applied *via* bath perfusion, with the final concentrations in the brackets: forskolin (50 μM), H89 (10 μM), isoproterenol (1 μM), CGS21680 (1 μM), and SKF81297 (1 μM) were from Tocris Bioscience; nigericin (5 μM) was from Sigma. The specified concentration of chemicals were either spiked into the circulating buffer, or premade buffers with the correct drug concentrations were switched from one to another via a custom-made solution exchanger.

AUTHOR NOTE

During the preparation of this manuscript, a single-fluorophore PKA biosensor has been developed that is also 2pFLIM compatible (Bonnot et al., 2014), and future experiments will be needed to compare the detailed characteristics of our sensor and the single-fluorophore PKA biosensor.

AUTHOR CONTRIBUTIONS

Yao Chen and Bernardo L. Sabatini designed the experiments. Yao Chen, Gary Yellen, and Bernardo L. Sabatini implemented the hardware and software of the FLIM setup. Jessica L. Saulnier generated organotypic slices and performed some *in utero* electroporation surgeries. Yao Chen performed the rest of the experiments and data analysis. Yao Chen, Gary Yellen, and Bernardo L. Sabatini wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphar.2014.00056/abstract

Supplementary Figure 1 | Diagram illustrating how FRET changes fluorescence lifetime. Free donor fluorophores show a single exponential decay of fluorescence lifetime distribution (green). When donor and acceptor fluorophores interact via FRET, an additional decay process occurs, resulting in bi-exponential decay and shorter lifetime (red). P_{free} and P_{FRET} represent the fractions of donors that are free and that have undergone FRET respectively. $P_{free} + P_{FRET} = 1$.

Supplementary Table 1 | Optical characteristics of the three donors and two acceptors used in the 4 PKA reporters. The brightness is relative to eGFP. The optical characteristics are based on literature (Piston et al.; Ganesan et al., 2006; Murakoshi et al., 2008) and our own data.

REFERENCES

Albin, R. L., Young, A. B., and Penney, J. B. (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci.* 12, 366–375. doi: 10.1016/0166-2236(89)90074-X

Allen, M. D., and Zhang, J. (2006). Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem. Biophys. Res. Commun.* 348, 716–721. doi: 10.1016/j.bbrc.2006.07.136

Ashby, C. D., and Walsh, D. A. (1972). Characterization of the interaction of a protein inhibitor with adenosine 3′,5′-monophosphate-dependent protein kinases. I. Interaction with the catalytic subunit of the protein kinase. *J. Biol. Chem.* 247, 6637–6642.

Ashby, C. D., and Walsh, D. A. (1973). Characterization of the interaction of a protein inhibitor with adenosine 3′,5′-monophosphate-dependent protein kinases. II. Mechanism of action with the holoenzyme. *J. Biol. Chem.* 248, 1255–1261.

Atasoy, D., Aponte, Y., Su, H. H., and Sternson, S. M. (2008). A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J. Neurosci.* 28, 7025–7030. doi: 10.1523/JNEUROSCI.1954-08.2008

Bastiaens, P. I., and Squire, A. (1999). Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. *Trends Cell Biol.* 9, 48–52. doi: 10.1016/S0962-8924(98)01410-X

Baxter, M. G. (2003). Age-related memory impairment. Is the cure worse than the disease? *Neuron* 40, 669–670. doi: 10.1016/S0896-6273(03)00715-3

Berg, J., Hung, Y. P., and Yellen, G. (2009). A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nat. Methods* 6, 161–166. doi: 10.1038/nmeth.1288

Berke, J. D., and Hyman, S. E. (2000). Addiction, dopamine, and the molecular mechanisms of memory. *Neuron* 25, 515–532. doi: 10.1016/S0896-6273(00)81056-9

Bloodgood, B. L., and Sabatini, B. L. (2005). Neuronal activity regulates diffusion across the neck of dendritic spines. *Science* 310, 866–869. doi: 10.1126/science.1114816

Bonnot, A., Guiot, E., Hepp, R., Cavellini, L., Tricoire, L., and Lambolez, B. (2014). Single-fluorophore biosensors based on conformation-sensitive GFP variants. *FASEB J.* 28, 1375–1385. doi: 10.1096/fj.13-240507

Brandon, E. P., Idzerda, R. L., and McKnight, G. S. (1997). PKA isoforms, neural pathways, and behaviour: making the connection. *Curr. Opin. Neurobiol.* 7, 397–403. doi: 10.1016/S0959-4388(97)80069-4

Brunelli, M., Castellucci, V., and Kandel, E. R. (1976). Synaptic facilitation and behavioral sensitization in Aplysia: possible role of serotonin and cyclic AMP. *Science* 194, 1178–1181. doi: 10.1126/science.186870

Carter, A. G., and Sabatini, B. L. (2004). State-dependent calcium signaling in dendritic spines of striatal medium spiny neurons. *Neuron* 44, 483–493. doi: 10.1016/j.neuron.2004.10.013

Chen, Y., and Sabatini, B. L. (2012). Signaling in dendritic spines and spine microdomains. *Curr. Opin. Neurobiol.* 22, 389–396. doi: 10.1016/j.conb.2012.03.003

Chow, B. Y., Han, X., Dobry, A. S., Qian, X., Chuong, A. S., Li, M., et al. (2010). High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature* 463, 98–102. doi: 10.1038/nature08652

Dalton, G. D., and Dewey, W. L. (2006). Protein kinase inhibitor peptide (PKI): a family of endogenous neuropeptides that modulate neuronal cAMP-dependent protein kinase function. *Neuropeptides* 40, 23–34. doi: 10.1016/j.npep.2005.10.002

Davis, R. L. (1996). Physiology and biochemistry of Drosophila learning mutants. *Physiol. Rev.* 76, 299–317.

Depry, C., Allen, M. D., and Zhang, J. (2011). Visualization of PKA activity in plasma membrane microdomains. *Mol. Biosyst.* 7, 52–58. doi: 10.1039/c0mb00079e

Dipilato, L. M., Cheng, X., and Zhang, J. (2004). Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16513–16518. doi: 10.1073/pnas.0405973101

Durieux, P. F., Bearzatto, B., Guiducci, S., Buch, T., Waisman, A., Zoli, M., et al. (2009). D2R striatopallidal neurons inhibit both locomotor and drug reward processes. *Nat. Neurosci.* 12, 393–395. doi: 10.1038/nn.2286

Ganesan, S., Ameer-Beg, S. M., Ng, T. T., Vojnovic, B., and Wouters, F. S. (2006). A dark yellow fluorescent protein (YFP)-based Resonance Energy-Accepting Chromoprotein (REACh) for Forster resonance energy transfer with GFP. *Proc. Natl. Acad. Sci. U.S.A.* 103, 4089–4094. doi: 10.1073/pnas.0509922103

Gervasi, N., Hepp, R., Tricoire, L., Zhang, J., Lambolez, B., Paupardin-Tritsch, D., et al. (2007). Dynamics of protein kinase A signaling at the membrane, in the cytosol, and in the nucleus of neurons in mouse brain slices. *J. Neurosci.* 27, 2744–2750. doi: 10.1523/JNEUROSCI.5352-06.2007

Goedhart, J., Van Weeren, L., Hink, M. A., Vischer, N. O., Jalink, K., and Gadella, T. W. Jr. (2010). Bright cyan fluorescent protein variants identified by fluorescence lifetime screening. *Nat. Methods* 7, 137–139. doi: 10.1038/nmeth.1415

Gong, S., Doughty, M., Harbaugh, C. R., Cummins, A., Hatten, M. E., Heintz, N., et al. (2007). Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs. *J. Neurosci.* 27, 9817–9823. doi: 10.1523/JNEUROSCI.2707-07.2007

Greengard, P. (2001). The neurobiology of slow synaptic transmission. *Science* 294, 1024–1030. doi: 10.1126/science.294.5544.1024

Harvey, C. D., Yasuda, R., Zhong, H., and Svoboda, K. (2008). The spread of Ras activity triggered by activation of a single dendritic spine. *Science* 321, 136–140. doi: 10.1126/science.1159675

Heintz, N. (2004). Gene expression nervous system atlas (GENSAT). *Nat. Neurosci.* 7:483. doi: 10.1038/nn0504-483

Higley, M. J., and Sabatini, B. L. (2010). Competitive regulation of synaptic Ca(2+) influx by D2 dopamine and A2A adenosine receptors. *Nat. Neurosci.* 13, 958–966. doi: 10.1038/nn.2592

Hikosaka, O., Bromberg-Martin, E., Hong, S., and Matsumoto, M. (2008). New insights on the subcortical representation of reward. *Curr. Opin. Neurobiol.* 18, 203–208. doi: 10.1016/j.conb.2008.07.002

Kandel, E., and Abel, T. (1995). Neuropeptides, adenylyl cyclase, and memory storage. *Science* 268, 825–826. doi: 10.1126/science.7754367

Kreitzer, A. C., and Malenka, R. C. (2008). Striatal plasticity and basal ganglia circuit function. *Neuron* 60, 543–554. doi: 10.1016/j.neuron.2008.11.005

Lam, A. J., St-Pierre, F., Gong, Y., Marshall, J. D., Cranfill, P. J., Baird, M. A., et al. (2012). Improving FRET dynamic range with bright green and red fluorescent proteins. *Nat. Methods* 9, 1005–1012. doi: 10.1038/nmeth.2171

Lee, S. J., Escobedo-Lozoya, Y., Szatmari, E. M., and Yasuda, R. (2009). Activation of CaMKII in single dendritic spines during long-term potentiation. *Nature* 458, 299–304. doi: 10.1038/nature07842

Lefkowitz, R. J. (2007). Seven transmembrane receptors: something old, something new. *Acta Physiol.* 190, 9–19. doi: 10.1111/j.1365-201X.2007.01693.x

Le Merrer, J., Becker, J. A., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009

Lerner, T. N., and Kreitzer, A. C. (2011). Neuromodulatory control of striatal plasticity and behavior. *Curr. Opin. Neurobiol.* 21, 322–327. doi: 10.1016/j.conb.2011.01.005

Matsuzaki, M., Honkura, N., Ellis-Davies, G. C., and Kasai, H. (2004). Structural basis of long-term potentiation in single dendritic spines. *Nature* 429, 761–766. doi: 10.1038/nature02617

Murakoshi, H., Lee, S. J., and Yasuda, R. (2008). Highly sensitive and quantitative FRET-FLIM imaging in single dendritic spines using improved non-radiative YFP. *Brain Cell Biol.* 36, 31–42. doi: 10.1007/s11068-008-9024-9

Murakoshi, H., Wang, H., and Yasuda, R. (2011). Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. *Nature* 472, 100–104. doi: 10.1038/nature09823

Nemeroff, C. B., and Owens, M. J. (2002). Treatment of mood disorders. *Nat. Neurosci.* 5(Suppl.), 1068–1070. doi: 10.1038/nn943

Piston, D. W., Patterson, G. H., Lippincott-Schwartz, J., Claxton, N. S., and Davidson, M. W. *Introduction to Fluorescent Proteins*. Nikon. Available online at: http://www.microscopyu.com/articles/livecellimaging/fpintro.html

Pologruto, T. A., Sabatini, B. L., and Svoboda, K. (2003). ScanImage: flexible software for operating laser scanning microscopes. *Biomed. Eng. Online* 2:13. doi: 10.1186/1475-925X-2-13

Pologruto, T. A., Yasuda, R., and Svoboda, K. (2004). Monitoring neural activity and [Ca2+] with genetically encoded Ca2+ indicators. *J. Neurosci.* 24, 9572–9579. doi: 10.1523/JNEUROSCI.2854-04.2004

Raimondo, J. V., Irkle, A., Wefelmeyer, W., Newey, S. E., and Akerman, C. J. (2012). Genetically encoded proton sensors reveal activity-dependent pH changes in neurons. *Front. Mol. Neurosci.* 5:68. doi: 10.3389/fnmol.2012.00068

Rathje, M., Fang, H., Bachman, J. L., Anggono, V., Gether, U., Huganir, R. L., et al. (2013). AMPA receptor pHluorin-GluA2 reports NMDA receptor-induced intracellular acidification in hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14426–14431. doi: 10.1073/pnas.1312982110

Schultz, W. (1998). Predictive reward signal of dopamine neurons. *J. Neurophysiol.* 80, 1–27.

Shaner, N. C., Patterson, G. H., and Davidson, M. W. (2007). Advances in fluorescent protein technology. *J. Cell Sci.* 120, 4247–4260. doi: 10.1242/jcs.005801

Shaywitz, A. J., and Greenberg, M. E. (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* 68, 821–861. doi: 10.1146/annurev.biochem.68.1.821

Shen, W., Flajolet, M., Greengard, P., and Surmeier, D. J. (2008). Dichotomous dopaminergic control of striatal synaptic plasticity. *Science* 321, 848–851. doi: 10.1126/science.1160575

Skeberdis, V. A., Chevaleyre, V., Lau, C. G., Goldberg, J. H., Pettit, D. L., Suadicani, S. O., et al. (2006). Protein kinase A regulates calcium permeability of NMDA receptors. *Nat. Neurosci.* 9, 501–510. doi: 10.1038/nn1664

Steiner, P., Higley, M. J., Xu, W., Czervionke, B. L., Malenka, R. C., and Sabatini, B. L. (2008). Destabilization of the postsynaptic density by PSD-95 serine 73 phosphorylation inhibits spine growth and synaptic plasticity. *Neuron* 60, 788–802. doi: 10.1016/j.neuron.2008.10.014

Stoppini, L., Buchs, P. A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* 37, 173–182. doi: 10.1016/0165-0270(91)90128-M

Tantama, M., Hung, Y. P., and Yellen, G. (2011). Imaging intracellular pH in live cells with a genetically encoded red fluorescent protein sensor. *J. Am. Chem. Soc.* 133, 10034–10037. doi: 10.1021/ja202902d

Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979). Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* 18, 2210–2218. doi: 10.1021/bi00578a012

Tronson, N. C., Wiseman, S. L., Olausson, P., and Taylor, J. R. (2006). Bidirectional behavioral plasticity of memory reconsolidation depends on amygdalar protein kinase A. *Nat. Neurosci.* 9, 167–169. doi: 10.1038/nn1628

Tzounopoulos, T., Janz, R., Sudhof, T. C., Nicoll, R. A., and Malenka, R. C. (1998). A role for cAMP in long-term depression at hippocampal mossy fiber synapses. *Neuron* 21, 837–845. doi: 10.1016/S0896-6273(00)80599-1

Vincent, P., Gervasi, N., and Zhang, J. (2008). Real-time monitoring of cyclic nucleotide signaling in neurons using genetically encoded FRET probes. *Brain Cell Biol.* 36, 3–17. doi: 10.1007/s11068-008-9035-6

Wang, H., and Zhuo, M. (2012). Group I metabotropic glutamate receptor-mediated gene transcription and implications for synaptic plasticity and diseases. *Front. Pharmacol.* 3:189. doi: 10.3389/fphar.2012.00189

Yasuda, R. (2006). Imaging spatiotemporal dynamics of neuronal signaling using fluorescence resonance energy transfer and fluorescence lifetime imaging microscopy. *Curr. Opin. Neurobiol.* 16, 551–561. doi: 10.1016/j.conb.2006.08.012

Yasuda, R., Harvey, C. D., Zhong, H., Sobczyk, A., Van Aelst, L., and Svoboda, K. (2006). Supersensitive Ras activation in dendrites and spines revealed by two-photon fluorescence lifetime imaging. *Nat. Neurosci.* 9, 283–291. doi: 10.1038/nn1635

Yasuda, R., and Murakoshi, H. (2011). The mechanisms underlying the spatial spreading of signaling activity. *Curr. Opin. Neurobiol.* 21, 313–321. doi: 10.1016/j.conb.2011.02.008

Zhang, J., Hupfeld, C. J., Taylor, S. S., Olefsky, J. M., and Tsien, R. Y. (2005). Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes. *Nature* 437, 569–573. doi: 10.1038/nature04140

Zhang, J., Ma, Y., Taylor, S. S., and Tsien, R. Y. (2001). Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14997–15002. doi: 10.1073/pnas.211566798

Zhong, H., Sia, G. M., Sato, T. R., Gray, N. W., Mao, T., Khuchua, Z., et al. (2009). Subcellular dynamics of type II PKA in neurons. *Neuron* 62, 363–374. doi: 10.1016/j.neuron.2009.03.013

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Does the kappa opioid receptor system contribute to pain aversion?

Catherine M. Cahill[1,2,3] *, Anna M. W. Taylor[1,4], Christopher Cook[1,2], Edmund Ong[1,3], Jose A. Morón[5] and Christopher J. Evans[4]

[1] Department of Anesthesiology and Perioperative Care, University of California Irvine, Irvine, CA, USA
[2] Department of Pharmacology, University of California Irvine, Irvine, CA, USA
[3] Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada
[4] Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA
[5] Department of Anesthesiology, Columbia University Medical Center, New York, NY, USA

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Lynn G. Kirby, University of Pennsylvania, USA
Clifford John Woolf, Boston Children's Hospital, USA

***Correspondence:**
Catherine M. Cahill, Department of Anesthesiology and Perioperative Care, University of California Irvine, 837 Health Sciences Road, 2117 Gillespie Neuroscience, Zot Code 4265, Irvine, CA 92697, USA
e-mail: cmcahill@uci.edu

The kappa opioid receptor (KOR) and the endogenous peptide-ligand dynorphin have received significant attention due the involvement in mediating a variety of behavioral and neurophysiological responses, including opposing the rewarding properties of drugs of abuse including opioids. Accumulating evidence indicates this system is involved in regulating states of motivation and emotion. Acute activation of the KOR produces an increase in motivational behavior to escape a threat, however, KOR activation associated with chronic stress leads to the expression of symptoms indicative of mood disorders. It is well accepted that KOR can produce analgesia and is engaged in chronic pain states including neuropathic pain. Spinal studies have revealed KOR-induced analgesia in reversing pain hypersensitivities associated with peripheral nerve injury. While systemic administration of KOR agonists attenuates nociceptive sensory transmission, this effect appears to be a stress-induced effect as anxiolytic agents, including delta opioid receptor agonists, mitigate KOR agonist-induced analgesia. Additionally, while the role of KOR and dynorphin in driving the dysphoric and aversive components of stress and drug withdrawal has been well characterized, how this system mediates the negative emotional states associated with chronic pain is relatively unexplored. This review provides evidence that dynorphin and the KOR system contribute to the negative affective component of pain and that this receptor system likely contributes to the high comorbidity of mood disorders associated with chronic neuropathic pain.

Keywords: kappa opioid receptor, pain, aversion, reward system ventral tegmental area, dopamine, negative reinforcement

INTRODUCTION

Chronic pain may be considered an epidemic in many westernized countries affecting 25% of the population, and where quality of life of chronic pain patients is reported to be lower than other disorders such as heart failure, renal failure and even depression (O'Connor, 2009). Pain is a multidimensional experience comprised of sensory, cognitive, and emotional (subjective) components, which are processed within discreet but interacting brain structures. Many chronic pain states are accompanied by dramatic sensory disturbances that result in pain hypersensitivity (allodynia and hyperalgesia) and tonic (unprovoked) ongoing pain. However, the negative affect, or how much the pain is 'bothersome' significantly impacts the quality of life of the sufferer. Notably, the emotional component of pain has been argued to be a greater metric of quality of life than its sensory component, and thus understanding the processes that influence this pain characteristic is essential to developing novel treatment strategies.

Neuroplasticity in brain regions important for the expression of affect may underlie the comorbidity between chronic pain and Axis I disorders of the DSM-V, including depression, anxiety disorders, bipolar disorder, and ADHD. Comorbidities with each of these disorders in chronic pain patients have has been well documented, where depression is the most common comorbidity, with some studies finding a prevalence rate approaching 100% among clinical chronic pain samples (reviewed by Nicholson and Verma, 2004). In fact, chronic pain is second only to bipolar disorder as the major cause of suicide among all medical illnesses, further highlighting the importance of negative affect in this condition (Juurlink et al., 2004; Asmundson and Katz, 2009; Elman et al., 2013). Nevertheless, it remains debated whether mood disorders are a consequence of, or a pre-existing susceptibility for the genesis of chronic pain (Von Korff et al., 1993; Fishbain et al., 1997; Blackburn-Munro and Blackburn-Munro, 2001; Miller and Cano, 2009). Clinical studies specifically aimed at identifying risk factors that may predict the incidence of or transition to chronic pain are now being pursued (Attal et al., 2014; Mundal et al., 2014).

Dysfunction of reward mesolimbic circuitry underlies the etiology of many psychiatric disorders, including depression. Because it is common for chronic pain to be comorbid with diseases known to have deficits in the dopamine mesolimbic system, it is posited

that this dysfunction also contributes to the genesis of chronic pain (Taylor, 2013; Cahill et al., 2014). For example, a high prevalence of chronic pain is common in disorders linked with deficits in the dopamine system, including disorders of mood and affect, substance abuse, and Parkinson's disease (Jarcho et al., 2012). The statistic that substance abusers are six times more likely to develop chronic pain than its prevalence in society (Gureje et al., 1998; Verhaak et al., 1998; Jamison et al., 2000; Rosenblum et al., 2003) is not surprising, if dysfunction of mesolimbic reward system contributes to chronic pain states. In contrast, clinical conditions associated with elevated mesolimbic dopamine (e.g., schizophrenia) have higher pain thresholds (Dworkin, 1994; Boettger et al., 2013). It should be noted that an alternative explanation for the increased prevalence of chronic pain in substance abusers is the occurrence of opioid-induced hyperalgesia. Opioid-induced hyperalgesia is a paradoxical increase in pain sensitivity following opioid administration via either chronic exposure [e.g., morphine, hydrocodone, oxycodone and methadone, or single exposure (e.g., Remifentanil) Chu et al., 2008; Lee et al., 2011; Fletcher and Martinez, 2014]. Studies have identified various mechanisms that may account for the occurrence of opioid-induced hyperalgesia including sensitization of pro-nociceptive pathways caused by long term potentiation of synapses between nociceptive C fibers and spinal dorsal horn neurons (Drdla et al., 2009) and neuroimmune responses reducing GABAergic inhibition (Ferrini et al., 2013).

Pain and reward are considered opponent processes but are processed within overlapping or interacting brain structures (e.g., anterior cingulate cortex, dorsal and ventral striatum, and amygdala). It has been demonstrated that rewarding stimuli such as food and pleasurable music decrease pain sensitivity (Leknes and Tracey, 2008), whereas pain can impair reward processing, which can lead to an anhedonic state (Marbach and Lund, 1981; Nicholson and Verma, 2004; Elman et al., 2013). Canonical neurotransmitters involved in affect and reward are dopamine, serotonin, norepinephrine, and endogenous opioids. Modulating the function of these neurotransmitters is associated with altered mood states. The mesolimbic system, which includes the ventral tegmental area (VTA) and the nucleus accumbens (NAc, part of the ventral striatum), is responsible for the expression of positively motivated behaviors and reinforcement learning produced by natural and drug rewarding stimuli (Fields et al., 2007; Sun, 2011). Few studies have examined dysfunction of this circuitry in chronic pain, and whether the mesolimbic dopaminergic system contributes to the aversive component of ongoing persistent pain. Some clues have emerged from functional imaging studies on healthy volunteers and chronic pain patients. Functional magnetic resonance imaging studies of clinical pain cohorts demonstrate altered connectivity between the mesolimbic system and various cortical structures (Apkarian et al., 2005; Jensen et al., 2013; Ichesco et al., 2014). For example, greater functional connectivity of the NAc with the prefrontal cortex predicted pain persistence, implying that corticostriatal circuitry is causally involved in the transition from acute to chronic pain (Baliki et al., 2012). Functional connectivity analysis in neuropathic pain animals also revealed that changes in connectivity were primarily (97%) localized within the limbic system (NAc, septum and ventral pallidum, amygdala and hippocampus), as well as between the limbic and nociceptive systems (thalamus, primary sensory cortices, insula cortex, and periaqueductal gray; Baliki et al., 2012, yet no connectivity changes were observed within the nociceptive network). A corollary study in patients reported that chronic back pain patients exhibited brain activity in regions responsible for emotion-related circuitry, whereas acute back pain patients demonstrated activity in nociceptive circuitry (Hashmi et al., 2013). These studies suggest that the limbic system is engaged in clinical and experimental models of chronic pain. It is unknown how or why greater functional connectivity with limbic structures contributes to chronic pain, although this system is likely engaged to modulate the affective component of pain and gives salience to the pain experience via release of dopamine. The fact that dopamine release in the ventral striatum is associated with placebo-induced analgesia and anticipation of analgesia (Scott et al., 2008; Tracey, 2010; Abhishek and Doherty, 2013) also suggests that dopamine release in the mesolimbic system may be important in modulating the negative affect component of pain. The interplay between reward pathways and pain validate the importance of this circuitry, not only in the chronicity of pain, but also the lack of opioid effectiveness in treating chronic pain (including that of neuropathic origin).

Opioids and their receptors play a central role in various physiological effects throughout the peripheral and central nervous systems. In addition to their ability to modulate the sensory component of pain (the intensity), opioids also modulate the emotional, aversive component of pain (affective, unpleasant component). For example, a patient being treated with opioids for post-operative pain may still feel the sensory component of pain, but it no longer bothers them. There is strong evidence that release of dopamine within the ventral striatum is responsible for the mood altering properties of opioids. However, opioid-evoked release of dopamine also contributes to their abuse potential, where an allostatic shift in reward signaling leads to the pathological state of addiction. Mu opioid receptor (MOR) agonists positively modulate mood and are the predominant opioid drugs used for clinical and recreational purposes. However, both delta (DOR) and kappa opioid receptors (KORs) also modulate mood and emotion, but in opposite directions (Lutz and Kieffer, 2013). Activation of the KOR causes dysphoria (defined as unpleasant or profound feeling of unwell/unease) in humans and an aversive response in animals, evidenced by its ability to produce a conditioned place aversion in animals (Land et al., 2009; Tejeda et al., 2013). One of the underlying mechanisms thought to account for the dysphoric effects of KOR drugs is their ability to suppress mesolimbic dopamine release within reward circuitry. This review will posit that disruption in mesolimbic cortical circuitry plays an important role in chronic pain and that activity at the KOR is an important regulator of this circuitry. It will also highlight inferences that this opioid receptor contributes to the high incidence of mood disorder comorbidity in various chronic pain states.

THE VENTRAL TEGMENTAL AREA IS A CENTRAL LOCUS FOR PAIN AND PLEASURE

A decrease or suppression of mesolimbic dopaminergic transmission that originates in the VTA is one mechanistic commonality

between a stress response, the precipitation of an aversive state, and chronic pain. Salience is one of the key functions of the mesolimbic dopaminergic circuitry that is encoded via interactions between tonic and phasic spikes in dopamine neurons (McClure et al., 2003). The 'pain neuromatrix' has been described as a salience network where the neurocircuitry related to emotion rather than the sensory aspects of pain are considered to have salient value (Legrain et al., 2011; Mouraux et al., 2011). It was recently hypothesized that aberrant functioning of the brain circuits which assign salience values to stimuli may contribute to chronic pain (Borsook et al., 2013). We will focus the discussion on the circuitry of inputs and outputs of midbrain dopaminergic neurons, as this neurocircuitry is engaged by salience attributed to a range of stimuli, including pain (Berridge, 2007; Leknes and Tracey, 2008; Elman et al., 2013). Moreover, this system is engaged by punishment and contributes to negative reinforcement learning (i.e., removal of a negative stimulus, including pain, is rewarding). Alterations in dopamine signaling are associated with motivational deficits, and animals in chronic pain show impaired motivated responses to natural and drug reward (Navratilova and Porreca, 2014; Schwartz et al., 2014). The motivational effect for place preference of analgesic drugs hypothesized to reflect the rewarding component of pain relief is currently being used to assess the affective or tonic-aversive component of pain. Magnussen et al. (2009) were the first to report that non-rewarding analgesics produce a place preference in chronic pain, but not in pain-naïve animals. Subsequently, King et al. (2009) reported that intrathecal lidocaine produced a place preference in an animal model of neuropathic pain, but not in pain-naïve animals. Many studies have now used this paradigm to understand the mechanisms underlying the tonic-aversive component of pain (De Felice et al., 2013; Cahill et al., 2014; Roughan et al., 2014; Xie et al., 2014), which is predicted to have construct validity for screening novel analgesic drugs for clinical development. Analgesic place preference was blocked by intra-NAc injections of dopamine receptor antagonists (Navratilova et al., 2012), suggesting that dopamine release is important for the expression of negative reinforcement associated with pain relief. A clinical correlate to these studies has been described whereby reciprocal negative/positive signals in the NAc correlated with pain onset/offset, respectively (Becerra and Borsook, 2008). Additionally, negative correlations between pain and mesolimbic dopamine activity in humans has been described (Borsook et al., 2007; Wood et al., 2007; Jarcho et al., 2012). It is worth noting that there is no evidence that non-rewarding drugs that produce negative reinforcement in models of chronic pain become rewarding after prolonged use, (i.e., produce psychological dependence). Evidence against this argument is the lack of reported dependence for non-rewarding analgesics including local anesthetic patches, clonidine or tricyclic antidepressants used to manage pain in various clinical pain populations.

The VTA is the origin of dopaminergic neurons within the mesocorticolimbic system that mediates reward, motivation, and arousal. There are various inputs to the VTA that result in the inhibition of VTA dopaminergic neurons and are attributed to the expression of an aversive state (**Figure 1**). These brain structures include the habenula, rostromedial tegmental nucleus (RMTg),

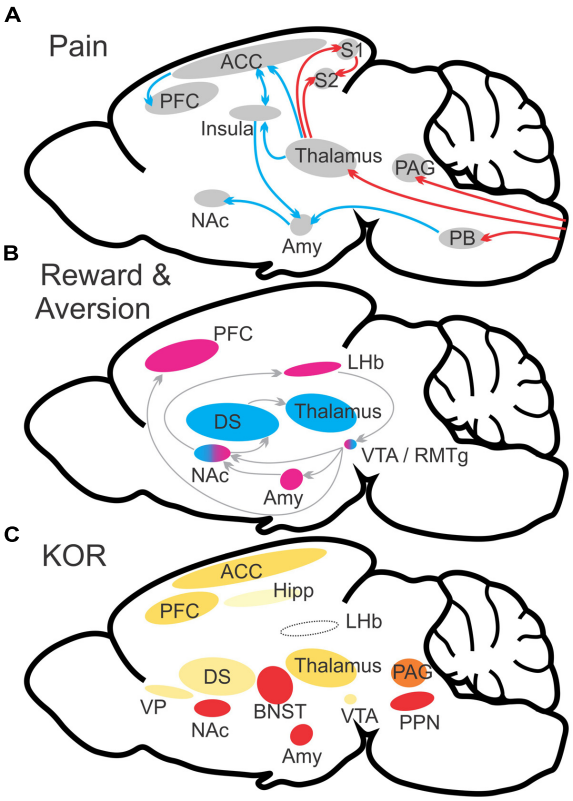

FIGURE 1 | Schematic illustration of major brain networks involved in pain and reward processes (A,B). (A) Ascending projections convey pain signaling to multiple brain structures including the periaqueductal gray, thalamus, and parabrachial nucleus. Continued and distinct processing occurs for both sensory (red arrows) and affective (blue arrows) dimensions of pain perception. **(B)** Reward/aversion processes involve multiple overlapping and interacting networks. Shown are the major structures involved in reward (blue) and aversion (magenta). **(C)** KOR abundance in relevant brain structures. Heat map color-coded (Red = most abundant) by reported radioligand binding (Mansour et al., 1987; Le Merrer et al., 2009). ACC, anterior cingulate cortex; Amy, amygdala; BST, bed nucleus of the stria terminalis; DS, dorsal striatum; LHb, lateral habenula; NAc, nucleus accumbens; OFC, orbitofrontal cortex; PAG, periaqueductal gray; PB, parabrachial nucleus; PFC, prefrontal cortex; PPN, pedunculopontine nucleus/pedunculopontine tegmental nucleus; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; VP, ventral pallidum; VTA, ventral tegmental area. **(A,B)** adapted from Cahill et al. (2014).

and ventral pallidum. The habenula is a small brain structure located near the pineal gland and the third ventricle, sometimes called the tail of the VTA. Recent reviews highlight the critical role this brain structure has in influencing the brain's response to pain, stress, anxiety, sleep, and reward (Shelton et al., 2012; Velasquez et al., 2014). The habenula evaluates external stimuli and directs the motivation of appropriate behavioral response, thereby contributing to reward-related learning to reinforce or avoid actions based on previous outcomes. It primarily contains GABAergic neurons that control activity of the VTA, substantia nigra, locus coeruleus, and raphe nucleus. The RMTg is a midbrain structure located at the caudal tail of the VTA. Its function is to convey salient positive and negative signals to dopamine neurons and participate in appetitive behavioral responses (Bourdy

and Barrot, 2012). The ventral pallidum is a brain structure within the basal ganglia located along the external segment of the globus pallidus. It projects to the VTA (Haber et al., 1985), subthalamic nucleus, thalamus, and lateral hypothalamus, and has reciprocal projections to the ventral striatum (including the NAc). It is part of the striatopallidal indirect cortico-basal ganglia pathway that regulates emotion, motivation, and movement. The periaqueductal gray (PAG) also projects directly to the VTA, providing the third heaviest subcortical source of glutamate input to the VTA (Geisler et al., 2007) synapsing onto both gamma-aminobutyric acid (GABA) and dopaminergic neurons (Omelchenko and Sesack, 2010). Based on its functions, the PAG is likely to supply VTA neurons with information important for processing nociceptive signals, defensive and stress behaviors, and rewarding responses to opiates.

Gamma-aminobutyric acid is the primary neurotransmitter in RMTg neurons that project to the VTA (Jhou et al., 2009). Activation of these neurons release GABA directly on VTA dopamine neurons leading to suppression of dopaminergic transmission. Functionally, when the RMTg is surgically lesioned, the response to aversive stimuli is attenuated, which suggests a convergence of aversive inputs within the RMTg (Jhou et al., 2009). The habenula is another input to the VTA that suppresses VTA dopaminergic transmission, and it does so via a direct and indirect pathway (Omelchenko and Sesack, 2010). The habenula is divided into medial and lateral (LHb) components that have different afferent and efferent connections (Velasquez et al., 2014). The LHb is topographically organized with the medial division sending excitatory glutamatergic projections to the VTA that synapse on GABAergic interneurons (Ji and Shepard, 2007; Gonçalves et al., 2012). Activation of this pathway leads to an increase in inhibitory postsynaptic currents in dopamine neurons. The lateral division of the LHb sends excitatory projections to the GABAergic neurons in the RMTg (Gonçalves et al., 2012). Hence, LHb glutamatergic terminals in the RMTg excite GABAergic neurons that in turn synapse with VTA dopaminergic neurons, resulting in an inhibition of dopaminergic neuronal firing (**Figure 2**).

One prominent feature of the habenula is that it is involved in the processing of aversive information, including pain. In addition, repeated or continuous stress can lead to expression of depression-like behavior and exacerbate chronic pain. Importantly, sensitization of the LHb-dopamine circuitry occurs in depressive states (Hikosaka, 2010). Indeed, humans with depression or animal models of depression exhibit hyperactivity within the LHb (Caldecott-Hazard et al., 1988; Morris et al., 1999). Whether the LHb exhibits hyperactivity in chronic pain and contributes to the high comorbidity of mood disorders with chronic pain states remains unexplored. Pain transmission directly and indirectly activates the habenula. Reports show that an aversive stimulus increases the LHb excitatory drive onto GABAergic RMTg neurons (Jhou et al., 2009; Hong et al., 2011; Stamatakis and Stuber, 2012), leading to a decrease in dopamine output (Ji and Shepard, 2007; Matsui and Williams, 2011). The spinal cord projects to the Lhb directly (Craig, 2003) or indirectly via the lateral hypothalamus (Dafny et al., 1996), another brain region well established to modulate pain. Studies using anterograde tracing identified that while spinal cord lamina I nociceptive neurons project primarily to thalamic nuclei, some terminals were found in the dorsomedial hypothalamus (Craig, 2003). The deep dorsal spinal cord projects to the thalamus, globus pallidus, substantia innominata, amygdala, and hypothalamus (Gauriau and Bernard, 2004), and many of these structures influence mesolimbic dopamine circuitry.

Because the habenula is such as small brain structure, imaging studies to examine changes in activity within this region are challenging. Nevertheless, the habenula circuitry is proposed to undergo neuroplastic changes in chronic pain (Shelton et al., 2012), where the hedonic deficit due to dysfunction of reward systems generates a facilitation of pain. Several lines of evidence suggest that LHb neurons are hyperactive in individuals with depression. Such studies led to the successful use of deep brain stimulation (DBS) to manipulate the activity of the habenula as a treatment of major depression (Sartorius and Henn, 2007; Hauptman et al., 2008; Sartorius et al., 2010). The positive outcomes are thought to result from the ability of DBS to suppress the abnormally elevated activity of the habenula. Interestingly the habenula has one of the richest MOR expression patterns in the brain (Zastawny et al., 1994; Bunzow et al., 1995; Kitchen et al., 1997). Morphine injection into the habenula produces analgesia (Cohen and Melzack, 1986; Darcq et al., 2012), and intra-habenular injection of the opioid antagonist naloxone blocks the analgesic effects that result from an injection of morphine into the PAG (Ma et al., 1992). Taken together, pain modulatory systems likely engage this structure for the expression of pain affect.

It cannot be assumed that the inputs to the VTA discussed above result in modulation of the dopaminergic projections to the NAc implicated in reward. The VTA-NAc projection is also implicated in the pathogenesis of stress-related behaviors. Dopaminergic neurons within the VTA project to various brain structures, including the medial prefrontal cortex (mPFC), amygdala, and hippocampus, as well as the NAc. Importantly, there is evidence that discrete subpopulations of VTA dopaminergic neurons exclusively project to only one of these regions and that they are engaged by different stimuli and inputs (Volman et al., 2013). It is well accepted that activation of the VTA dopaminergic neurons projecting to the NAc produces reward-like behavior. Thus, it is not unexpected that aversive stimuli strongly inhibit VTA dopamine neurons (Ungless et al., 2004; Hong et al., 2011; Tan et al., 2012), and optogenetic activation of VTA GABAergic neurons or inhibition of VTA dopaminergic neurons produces a conditioned place aversion (Tan et al., 2012). Similarly, dopamine neurons in the caudal VTA increase firing to aversive stimuli such as a foot shock (Brischoux et al., 2009). However, various studies have demonstrated that salient but aversive stimuli, restraint stress, or even social defeat stress will increase VTA dopaminergic transmission (Anstrom and Woodward, 2005; Anstrom et al., 2009). Studies using fast scan cyclic voltammetry and microdialysis have shown elevated dopamine output in the NAc and mPFC in response to aversive stimuli (Bassareo et al., 2002; Budygin et al., 2012). Although, a recent study in non-human primates argues that aversion does not cause dopamine release (Fiorillo, 2013). Ventral tegmental area dopaminergic projections to other areas such as the mPFC and amygdala are also involved in stress-related behaviors.

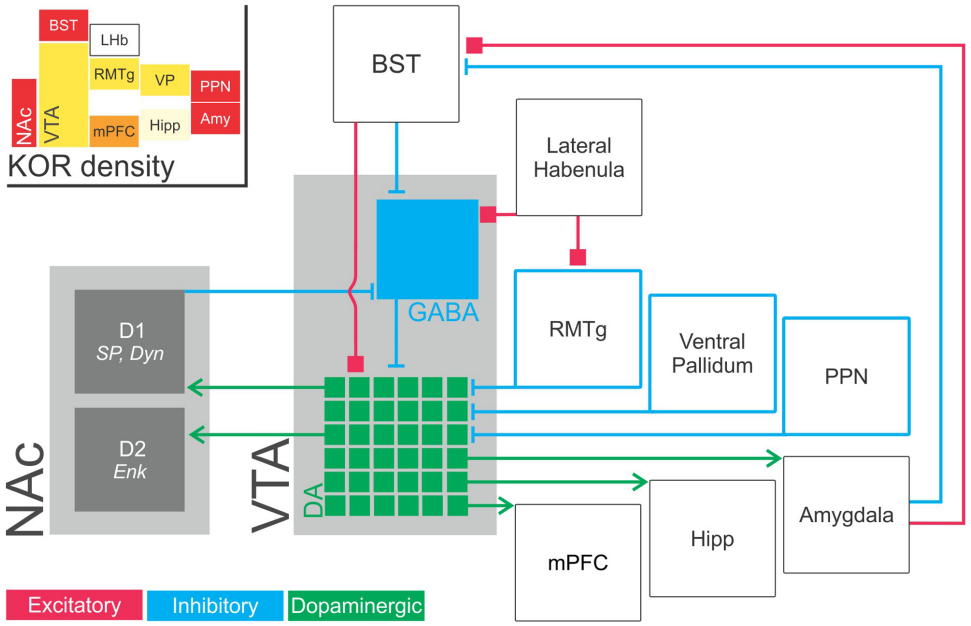

FIGURE 2 | Schematic illustration of inputs to and outputs from VTA dopaminergic neurons. VTA dopaminergic neurons project to the NAc (required for responding to reward prediction cues), medial prefrontal cortex (implicated in working memory and attentional processes), basolateral amygdala (implicated in emotion, reward, fear conditioning, and avoidance) and hippocampus (memory; green, dopaminergic components). Notably, VTA dopaminergic outputs are extensively differentiated with distinct projections from specific populations of dopaminergic neurons to specific downstream structures. In this way, mesolimbic dopaminergic activity functions in seemingly contradictory processes such as reward and aversion (McCullough et al., 1993; Stevenson et al., 2003; Baliki et al., 2010; McCutcheon et al., 2012). That is, the functions of ventral tegmental area dopaminergic projections is determined by network topology rather than simply by the choice of neurotransmitter. VTA dopaminergic neurons receive dense GABAergic modulatory input from numerous extrinsic structures and from GABAergic interneurons within the ventral tegmental area itself (blue). GABAergic projections to the VTA has been identified from the NAc (Nauta et al., 1978); ventral pallidum (Haber et al., 1985); RMTg (Jhou et al., 2009) and the pedunculopontine tegmental nucleus/lateral dorsal tegmentun (Omelchenko and Sesack, 2005; Good and Lupica, 2009). These neurons are also modulated by excitatory inputs (red) from the bed nucleus of the stria terminalis (Georges and Aston-Jones, 2001, 2002; Watabe-Uchida et al., 2012). (Inset) KOR abundance in these structures. Heat map color-coded (Red = most abundant) by reported radioligand binding (Mansour et al., 1987; Le Merrer et al., 2009). Amy, amygdala; BST, bed nucleus of the stria terminalis, D1, dopamine receptor D1; D2, dopamine receptor D2; Enk, enkephalin; Hipp, hippocampus; LHb, lateral habenula; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; PPN, pedunculopontine nucleus/pedunculopontine tegmental nucleus; RMTg, rostromedial tegmental nucleus; VP, ventral pallidum; SP, substance P; VTA, ventral tegmental area.

The mPFC both receives dopaminergic projections from the VTA and sends projections back to the VTA and the NAc, thus forming a regulatory feedback system (Nestler and Carlezon, 2006). An elegant study recently reported that activation of laterodorsal tegmentum terminals synapsing on VTA dopaminergic neurons that project to the NAc produces reward-related behavior, whereas activation of the LHb or RMTg terminals within the VTA that modulate dopamine neurons projecting to the mPFC produces aversion (Lammel et al., 2012). This study highlights the topographical input to the VTA and may explain the conflicting reports of whether aversive stimuli excite or inhibit VTA dopaminergic activity.

Other brain structures that either directly or indirectly modulate VTA dopaminergic circuitry are the NAc, amygdala, and the bed nucleus of the stria terminalis (BST). Medium spiny neurons within the NAc are GABAergic neurons that comprise the striatonigral (direct) and striatopallidal (indirect) cortico-basal ganglia pathways. There are two subtypes of medium spiny neurons within the NAc that respond to different patterns of dopaminergic firing patterns (Grace et al., 2007; Schultz, 2007). A burst of phasic firing is responsible for activation of medium spiny neurons containing low-affinity D1 dopamine receptors, substance P, and dynorphin. Activation of these neurons encodes reward-like behavior (Mirenowicz and Schultz, 1994; Grace et al., 2007; Carlezon and Thomas, 2009; Hikida et al., 2010, 2013). They project back to the VTA, synapsing primarily on GABAergic interneurons (Xia et al., 2011) producing a disinhibition that results in excitation of dopaminergic transmission. However, a recent study challenges the exclusive feedback onto only VTA GABAergic interneurons. Using a transgenic mouse that expresses MORs only in D1 medium spiny neurons, Cui et al. (2014) demonstrated that morphine evokes dopamine release in the NAc suggesting that these neurons may also synapse directly on VTA dopaminergic neurons. Medium spiny neurons of the indirect pathway contain D2 dopamine receptors. Slow single spike or tonic firing activates D2 dopamine receptors on medium spiny neurons that co-express enkephalin and produce aversion by modulating VTA circuitry via the ventral pallidum (Mirenowicz and Schultz, 1996; Ungless et al., 2004; Grace et al., 2007; Hikida et al., 2010). As in the VTA, there is some evidence that medium spiny neurons within the NAc may be topographically organized, in that hedonic 'hot spots'

have been described (Peciña et al., 2006; Richard et al., 2013; reviewed by McCutcheon et al., 2012; Berridge and Kringelbach, 2013). Interestingly, interruption of NAc activity (via lidocaine infusion) reversibly alleviates neuropathic pain (Chang et al., 2014).

The amygdala is involved in a wide array of functions including decision-making, memory, attention and fear. The amygdala is another limbic structure that is thought to attribute affective significance to environmental stimuli by forming a link between brain regions that process sensory information and areas involved in the production of emotional responses. A number of clinical and animal studies have indicated that the amygdala, along with the anterior cingulate cortex, plays a critical role in the processing of affective components of pain (Bie et al., 2011). Hence, excitotoxic lesions of the central amygdaloid nucleus or basolateral amygdaloid nucleus suppress intraplantar formalin-induced aversive responses (Tanimoto et al., 2003; Gao et al., 2004). Glutamatergic transmission within the basolateral amygdala via *N*-methyl-D-aspartate (NMDA) receptors has been shown to play a critical role in these aversive responses. The amygdala sends projections to, among other areas, the hypothalamus, VTA, and the cortex, making it a neuroanatomical structure well positioned to mediate the negative affect (aversiveness) associated with chronic pain (Murray, 2007; Jennings et al., 2013). The extended amygdala includes the BST and the central nucleus of the amygdala. The amygdala modulates the mesolimbic circuitry by sending projections to the NAc and to the BST. There is also evidence for a prominent direct projection from the ventral BST to the VTA (Georges and Aston-Jones, 2001) and local glutamate microinfusion into the ventral BST increased the firing and bursting activity of VTA dopamine neurons (Georges and Aston-Jones, 2002).

OPIOID RECEPTORS MODULATE PAIN AND REWARD

The opioid system is involved in modulating pain and reward. Opioid receptors are a group of G-protein coupled receptors divided into three families: the MOR, DOR, and KORs. These receptors are activated by three classes of endogenous opioid peptides, beta-endorphin, dynorphin, and enkephalin, that are derived from three precursor peptides (proopiomelanocortin, proenkephalin, and prodynorphin, respectively). The selectivity and distribution of the opioid peptide and receptor systems suggests enkephalin and beta-endorphin act through the MOR and DOR, and dynorphin via the KOR. A fourth opioid receptor family, nociceptin, is distinct from the classical opioid receptor family, in that the endogenous opioid peptides do not bind to it with high affinity (Mollereau et al., 1994). Rather, peptides derived from the pro-orphanin FQ/nociceptin peptide are considered the primary endogenous ligand (Meunier et al., 1995; Reinscheid et al., 1995). Activation of nociceptin receptors opposes the analgesic and rewarding actions of the classical opioid receptors (Mogil et al., 1996; Murphy et al., 1999; Vazquez-DeRose et al., 2013).

The opioid receptors and their peptides are distributed throughout the central and peripheral nervous system in a distinct but overlapping manner (Mansour et al., 1988). The MOR is widely distributed throughout the brainstem, midbrain, and forebrain structures, and mediates most of the analgesia and reinforcing effects of opioid agonists, such as morphine (Kieffer and Gavériaux-Ruff, 2002). DORs, on the other hand, are highly expressed in forebrain regions, including the olfactory bulb, striatum, and cortex (Mansour et al., 1993). Activation of the DOR produces minimal analgesia in acute pain models but develops an analgesic effect in rodent models of chronic pain, where the DOR responses are up-regulated (Cahill et al., 2007; Pradhan et al., 2011). Like the MOR, the DOR positively modulates hedonic state, but to a lesser extent. DOR agonists are anxiolytic (Saitoh and Yamada, 2012), but they are not self-administered and have lower abuse liability than MOR agonists (Negus et al., 1998; Brandt et al., 2001; Stevenson et al., 2005). KOR and MOR expression widely overlaps throughout the brain. However, in contrast to the MOR, activation of the KOR negatively modulates mood and is aversive (Wadenberg, 2003). Systemic KOR agonists also produce robust analgesia (Kolesnikov et al., 1996). KORs are located in the spinal cord and brain stem, and part of their analgesic effect is due to the direct inhibition of pain pathways (Simonin et al., 1995). Recently, we have shown another element of KOR analgesia is a result of their engagement of stress pathways (Taylor et al., 2014). The dynorphin-KOR system plays a central role in the dysphoric elements of stress. Stress induces the release of the opioid peptide dynorphin, an agonist at the KOR, and the aversive effects of stress are mimicked by activation of KORs in various limbic structures in the brain (Knoll and Carlezon, 2010). Dynorphin is released in response to stress via corticotrophin releasing factor (CRF), where it activates KORs in several brain regions involved in affect, including the dorsal raphe nucleus, basal lateral amygdala, hippocampus, and VTA (Nabeshima et al., 1992; Land et al., 2008). Blocking KOR signaling or dynorphin through antibodies or gene disruption blocks stress-induced immobility and produces antidepressant-like effects (Newton et al., 2002; Mague et al., 2003; Mclaughlin et al., 2003; Shirayama et al., 2004). Further, interfering with KOR signaling blocks the development of avoidance behavior associated with a stressful cue (Land et al., 2008). This suggests the dynorphin/KOR system plays a central role in the aversive stress experience.

While some studies implicate a positive role for the dynorphin/KOR system in anxiety-like behavior (Knoll et al., 2007; Wittmann et al., 2009), other studies have reported that the dynorphin/KOR system decreases anxiety-like behavior (Kudryavtseva et al., 2004; Bilkei-Gorzo et al., 2008). Additionally, transgenic mice with deletion of the KOR show no difference in behavior using a common test of anxiety (elevated plus maze) that is accepted to have predictive validity for pharmacological screening of anxiolytic drugs that reduce anxiety in humans, suggesting a minimal role for KOR in such behaviors (Simonin et al., 1998). Although is not inconceivable that some of these studies are confounded by the side effect profile of KOR agonists, which includes being hallucinogenic (Roth et al., 2002), producing dysphoria (Pfeiffer et al., 1986; Land et al., 2008), and inducing hypo-locomotor activity (Simonin et al., 1998). Nevertheless, salvinorin A, an illicitly used agonist at KORs, is a psychotropic that produces hallucinations, suggesting that activation of KORs may not cause dysphoria in all individuals.

Chronic pain produces anxiety and dysphoria that suggests the engagement of the dynorphin/kappa opioid system (Narita

et al., 2006a). In the spinal cord, chronic pain leads to the sustained release of dynorphin, which is hypothesized to be an analgesic response to a sustained pain state (Iadarola et al., 1988; Wagner et al., 1993; Spetea et al., 2002). Inhibiting KOR activation, either through KOR antagonists or in KOR knockout mice, enhanced tactile allodynia after a peripheral nerve lesion (Obara et al., 2003; Xu et al., 2004; Aita et al., 2010). This is in contrast to the results observed in dynorphin knockout mice, in which loss of dynorphin facilitated the return to normal nociceptive baselines after a peripheral nerve lesion (Wang et al., 2001). This is suggestive of a pronociceptive role for dynorphin in chronic pain, and is in contrast to the antinociceptive effects of KOR agonists described above. While the mechanism behind the pronociceptive effects of dynorphin is unknown, intrathecal injection of dynorphin has been reported to have neurotoxic effects and may exacerbate neuronal damage (Walker et al., 1982; Caudle and Isaac, 1988; Long et al., 1988; Sherwood and Askwith, 2009). Xu et al. (2004) hypothesized that sustained release of dynorphin in chronic pain desensitizes KORs. This would reveal the non-opioid mediated pronociceptive effects of dynorphin, and provide a possible explanation for the discrepancy between the results from dynorphin knockout and KOR knockout mice in chronic pain models. Additionally, pain-induced KOR desensitization is supported by the evidence that KOR agonists have a lowered analgesic potency in chronic pain animals (Xu et al., 2004).

In addition to direct effects on neurons, KORs have also been localized to astrocytes, and KOR agonists induce glial activation *in vivo* (Stiene-Martin and Hauser, 1991; Ruzicka et al., 1995; Stiene-Martin et al., 1998; Aita et al., 2010). Chronic pain leads to astrocyte activation in the spinal cord, and glial activation has been identified as a critical mechanism contributing to the sensitization of peripheral afferents leading to chronic pain (Raghavendra et al., 2003). Dynorphin KO animals do not show astrocyte activation after peripheral nerve injury, suggesting the kappa opioid system may act as a critical neuron-glia signal in chronic pain states (Xu et al., 2007). In primary astrocytes, U-69,593, a KOR agonist, produced the same effects as seen in immortalized astrocytes. Another KOR agonist, 2-methoxymethyl-salvinorin B, elicited sustained ERK1/2 activation, which was correlated with increased primary astrocyte proliferation. Proliferative actions of KOR agonists were abolished by either inhibition of ERK1/2, G-protein subunits or β-arrestin 2, suggesting that both G-protein dependent and independent ERK pathways are required for this outcome (McLennan et al., 2008).

While the bulk of studies investigating the contribution of the dynorphin/KOR system in chronic pain have focused on the spinal cord, there is evidence that this system is affected in supraspinal sites as well. Dynorphin is increased in the parietal cortex after spinal cord injury (Abraham et al., 2000). Increased GTPgS binding of KOR-specific ligands in the amygdala of chronic pain animals has also been described (Narita et al., 2006b).

KOR REGULATION OF MESOLIMBIC CIRCUITRY

The effect of chronic pain on the supraspinal actions of the dynorphin/KOR system, including anxiety and dysphoria, is an area that remains to be studied. Opioid receptors are widely expressed throughout the brain. This expression is highly regulated and varies by cell type, structure, and activity. Each of the three opioid receptor types is differentially expressed uniquely from each other type. As such, the mix of opioid receptor complements of any given structure varies substantially. KORs are widely expressed throughout the brain, spinal cord, and peripheral tissues. KORs are present in many of the major structures involved in pain and addiction processing. High expression levels of KOR have been detected in the VTA, NAc, prefrontal cortex, hippocampus, striatum, amygdala, BST, locus coeruleus, substantia nigra, dorsal raphe nucleus, pedunculopontine nucleus, and hypothalamus of both the rat and human brains (Peckys and Landwehrmeyer, 1999). These brain areas are implicated in the modulation of reward, mood state, and cognitive function. KORs are also expressed at several levels of pain circuitry, including areas such as the dorsal root ganglia, dorsal spinal cord, rostral ventromedial medulla, PAG, sensory thalamus, and the limbic regions. Activation of KORs *in vivo* produces many effects including analgesia, dysphoria, anxiety, depression, water diuresis, corticosteroid elevations, immunomodulation, relapse to cocaine seeking, and decreases in pilocarpine-induced seizure (Bruijnzeel, 2009; Van't Veer and Carlezon, 2013). KOR agonists have attracted considerable attention for their ability to exert potent analgesic effects without high abuse potential and to antagonize various MOR-mediated actions in the brain, including analgesia, tolerance, reward, and memory processes (Pan, 1998).

Mounting evidence indicates that KORs play a defining role in modulating dopamine transmission. An early PET study identified that glucose metabolism was increased in the NAc and lateral habenular nucleus following peripheral injection of the KOR agonist U-50488 (Ableitner and Herz, 1989). KOR signaling is also able to modulate synaptic transmission of monoamines in a variety of brain structures involved in reward including the VTA and NAc (Margolis et al., 2003, 2005, 2006; Ford et al., 2007). Two microdialysis studies in rats demonstrated that systemic administration of U-50488 and the KOR antagonist nor-BNI decreased and increased dopamine concentrations in the NAc, respectively (Di Chiara and Imperato, 1988; Maisonneuve et al., 1994). Additionally, KOR receptors are present both on dopaminergic neuron cell bodies in the VTA and the presynaptic terminals in the NAc. It has been reported that dopaminergic cell bodies in the VTA expressing KORs selectively project to the prefrontal cortex (Margolis et al., 2006). Here, the authors demonstrated that local injection of a KOR agonist in the VTA of rats selectively inhibited neurons projecting to the prefrontal cortex, and not the NAc. A contradictory study demonstrated, however, that administration of the KOR antagonist U-69539 was able to inhibit NAc projecting neurons from the VTA, whereas met-enkephalin (via MOR or DOR action) inhibited projections to the basolateral amygdala (Ford et al., 2006). It is unclear why there are discrepancies between these two studies, however the topographic organization of VTA neurons involved in reward and aversion may contribute to such differences. Although, biased agonism observed between different KOR agonists may also be an important factor that would explain such discrepancies (Bruchas et al., 2006; Chavkin, 2011; Rives et al., 2012; Negri et al., 2013; Zhou et al., 2013).

Kappa opioid receptors also modulate dopaminergic tone within the NAc. Significant evidence demonstrates that KORs are highly expressed in the both the ventral and dorsal striatum, with the highest concentration in the medial shell of the NAc (Mansour et al., 1996). Further, electron microscopy data has localized the receptors predominantly in synaptic vesicles in axons terminals within the NAc (Meshul and McGinty, 2000). Thus, they are poised to negatively modulate dopamine transmission in this brain region and may serve to affect mood and reward function. Donzanti et al. (1992) demonstrated that application of multiple KOR agonists directly into the NAc inhibited dopamine as measured by microdialysis. In another study, U-50488 was able to inhibit release of dopamine from rat accumbal slices (Heijna et al., 1990).

Both the KOR and its endogenous opioid peptide dynorphin are expressed in the BST and central nucleus of the amygdala (Poulin et al., 2009). The extended amygdala projects to the BST, which plays a critical role in the regulation of anxiety behavior (Walker and Davis, 2008) via release of corticotropin releasing factor (CRF) to enhance glutamate release. GABA is also a transmitter in this projection and it is hypothesized that GABA counteracts the effects of CRF. A recent study demonstrated that the GABAergic transmission is depressed by activation of KORs via a pre-synaptic mechanism within the BST (Li et al., 2012). Thus, CRF and dynorphin release in the extended amygdala act to increase anxiety-like behavior. Indeed, an interaction between CRF and dynorphin is evidenced by the report that anxiogenic effects of stress are encoded by dynorphin in the basolateral amygdala where CRF triggered activation of the dynorphin/KOR system (Bruchas et al., 2009).

Kappa opioid receptors are coupled to heterotrimer Gi/o proteins. Activation of KORs leads to an inhibition of adenylyl cyclase through the Gα subunit and induces increased potassium channel conductance and decreased calcium conductance via the Gβγ subunit. KORs can signal not only through activation of G proteins but also through recruitment of β-arrestins. While β-arrestins are regulatory scaffolding proteins involved in receptor desensitization, they are also signal transducers able to recruit and activate mitogen activated protein kinases (MAPKs). In fact, development of biased agonists for these pharmacological effects has the potential to mitigate some of the side effects associated with KOR activation (Chavkin, 2011). It has been proposed that activation of the MAPK p38 pathway mediates the dysphoric effects produced by selective KOR agonists (Bruchas et al., 2006, 2007). The development of novel KOR agonists that have the potential to be effective analgesics lacking the aversive and dysphoric side effects led to the synthesis of novel small molecule KOR agonists (6′-GNTI, MCKK1-22, triazole and isoquinolinone analogs). These agonists activate the G protein with minimal activity at β-arrestin-MAPK signaling pathway (Rives et al., 2012; Negri et al., 2013; Zhou et al., 2013).

DO KORs CONTRIBUTE TO PAIN AVERSIVENESS?

What remains unclear, and difficult to ascertain, is whether KORs modulate mesolimbic circuitry and drive the emotional, aversive nature of pain. KOR agonists have dysphoric and psychotomimetic properties in humans and will mediate place aversion in rodents (Shippenberg et al., 1993; Knoll and Carlezon, 2010). These effects can be elicited by direct injection of receptor selective ligands into the VTA (Bals-Kubik et al., 1993). A positive correlation has been demonstrated between dynorphin expression and dysphoria/anhedonia in depressive disorders and withdrawal associated with chronic drug use (Carlezon and Thomas, 2009; Wise and Koob, 2014). Administration of dynorphin and synthetic KOR agonists produces identical anhedonic and dysphoric symptoms characteristic of these disorders (Pfeiffer et al., 1986; Shippenberg and Herz, 1987; Lindholm et al., 2000; Frankel et al., 2008; Isola et al., 2009; Solecki et al., 2009; Knoll and Carlezon, 2010). Non-noxious stressors also activate dynorphin/KORs to produce depressive-like effects that can be blocked by KOR antagonists (Mclaughlin et al., 2003; Chartoff et al., 2009; Bruchas et al., 2010). There is convincing evidence that the aversive properties of KOR agonists are mediated by a negative modulation of the mesolimbic dopamine system (Shippenberg et al., 1993; Chefer et al., 2013), although serotonergic neurons within the dorsal raphe nucleus projecting to the rostral NAc are also proposed to underlie KOR mediated aversion (Land et al., 2009). Further evidence that modulation of serotonergic circuitry contributes to KOR mediated aversion is demonstrated by the observation that serotonin transporter knockout mice do not exhibit KOR-mediated aversion, but restoring this transporter via lentiviral injection in the ventral striatum recovered the pro-depressive effects (Schindler et al., 2012). In contrast, others have reported that KOR agonists continue to produce a place aversion in serotonin transporter knockout mice (Thompson et al., 2013) and that U50,488 produced a hypodopaminergic and hyposerotonergic state in the absence of the serotonin transporter. The observation that selective serotonin re-uptake inhibitor (SSRI) drugs show little efficacy in alleviating chronic pain of various etiologies suggests that serotonin may not be an important monoamine in the aversive component of pain (Moja et al., 2005; Gilron et al., 2006; Sumpton and Moulin, 2014). Nevertheless, activity of medium spiny neurons expressing dopamine receptors within the NAc appears necessary for KOR mediated aversion. Concomitant with altered dopamine transmission, interaction with KORs has been demonstrated to modulate brain reward function, both to natural reward and to drugs of abuse. KOR agonists have been shown to increase food intake in mice and rats, including a direct administration of dynorphin A into the VTA (Hamilton and Bozarth, 1988; Badiani et al., 2001). Though the exact mechanism behind KOR mediated food intake is unclear, it may be a process by which the animal attempts to offset decreased dopamine levels resulting from administration of KOR agonists. Intracranial self-stimulation (ICSS) can be used experimentally to measure alterations in reward thresholds. In one study, the KOR agonist U-69,593 was shown to increase brain reward thresholds for ICSS, indicating a depressive-like state, which was reversed with administration of a KOR antagonist (Todtenkopf et al., 2004). Altered reward states resulting from KOR activation are likely intimately linked with changes in dopamine transmission. For instance, both intra-VTA and intra-NAc administration of U-50488 results in conditioned place aversion in rats (Bals-Kubik et al., 1993). As evidence for a role in altered KOR-driven dopamine transmission in mediating these

aversive behaviors, genetic deletion of KORs from dopamine neurons was requisite for systemic KOR agonist place aversion (Chefer et al., 2013). Interestingly, the authors were able to rescue U-69593 mediated place aversion by intra-VTA injection of AAV to re-express KORs on dopamine neurons. Anhedonia and negative affect are also observed in the generation of comorbid mood disorders in neuropathic pain (Yalcin and Barrot, 2014). Thus, KOR modulation of dopamine circuitry and reward may serve as a putative mechanism for mediating the onset of negative emotional states and affect in chronic pain.

Evidence for a role of dynorphin in linking the depression of both behavior and dopaminergic transmission in chronic pain states remains sparse. It is recognized that acute pain (like euphorogenic drugs) activates dopaminergic transmission in brain reward circuitry including the NAc (Boutelle et al., 1990; Scott et al., 2006), whereas chronic or prolonged on-going pain produces the opposite effect (Wood et al., 2007; Geha et al., 2008; Pais-Vieira et al., 2009). Thus, it would be predicted that KOR involvement in modulating pain aversion would occur in chronic pain states where dopamine dysfunction has been described. A recent study demonstrated that CRF is a salient stressor in animal models of chronic pain where either CRF antagonists or CRF-saporin alleviated pain hypersensitivities (Hummel et al., 2010). Stress has been shown to activate the transcription factor CREB (cAMP response element-binding protein) in the NAc, and CREB-mediated increases in dynorphin function in this region contribute to depressive-like behavioral signs including anhedonia in the ICSS test (Pliakas et al., 2001; Chartoff et al., 2009; Muschamp et al., 2011). Additionally, KOR activation in the mPFC causes local reductions in dopamine levels and establishes conditioned place aversions (Tejeda et al., 2013), suggesting that elevated dynorphin function in this region can produce dysphoria. CRF is increased in the limbic system of chronic pain conditions (Rouwette et al., 2012), and injection of CRF into the VTA suppresses dopamine output to the NAc (Wanat et al., 2013). Since KOR antagonists block CRF induced stress responses (Bruchas et al., 2009), it has been hypothesized that KOR may modulate the dysphoric/aversive component of pain via regulation of CRF. However, a recent studies by Leitl et al. (2014a,b) recently reported that KORs are not involved in pain-induced changes in dopamine transmission. Both acute visceral pain (via intraperitoneal injection of lactic acid) and tonic pain (intraplantar injection of formalin) caused reduction in NAc dopamine release and a depression of ICSS, which was not recovered by pretreatment with a KOR antagonist. These studies highlight the influence of pain on dopamine transmission but argue that KOR is not involved in regulation of dopaminergic transmission by an acute or tonic pain stimulus within relatively short time periods. Previous studies demonstrated that KOR activation depressed both ICSS and NAc dopamine release (Todtenkopf et al., 2004; Zhang et al., 2005; Carlezon et al., 2006; Negus et al., 2010). It remains unclear if KORs are not involved in pain modulation of dopaminergic circuitry or if the negative outcome of the Leitl studies (Leitl et al., 2014a,b) was due to study design. The occurrence of anxiety and depressive behaviors that accompany chronic pain states in rodents do not typically begin to manifest until weeks 4 and 6–8 respectively (Yalcin et al., 2011). Thus, the KOR system may only be engaged at later time points following tissue or nerve damage which induces a chronic pain state. Alternatively, KORs may not be critical for the expression of chronic pain but contributes to the modest effects of analgesics in treating some forms of chronic pain including neuropathic pain. Opioid-induced dopamine release in the NAc is attenuated in rodents with neuropathic pain (Ozaki et al., 2002). This result was proposed to explain the lack of opioid addiction in chronic pain. However, an alternative interpretation is that the lack of opioid-induced dopamine release may account for the blunted analgesic properties of opioids in treating this type of pain or in the precipitation of comorbidities such as depression. There is evidence that KORs are responsible for the blunted rewarding effects of opioids induced by a tonic inflammatory pain stimulus. Hence, the effects of morphine induced place preference and morphine induced dopamine release in rats were attenuated by formalin treatment, which was prevented by KOR antagonist pre-treatment (Narita et al., 2005). In line with these results, morphine evoked dopamine release was blunted in the NAc of formalin injected animals, an effect that was reversed with microinjection of an anti-dynorphin antibody in this brain region.

CONCLUSION

The perception of pain and processes of reward and aversion are complex, multifaceted phenomena manifested through extensive processing in and between multiple brain structures. Of note, these networks exhibit extensive anatomical overlap with several major brain structures are important nodes in pain, pleasure and aversion processing. The mesolimbic system is one point of convergence that lends credence and consilience to the extensive evidence for interactions between pain, reward, and aversion.

The aforementioned studies provide evidence for the role of KOR in modulating dopaminergic neurotransmission in reward circuitry and the influence of dopamine in the transduction and generation of pain processing. Pharmacological manipulation of KOR can be used to modify dopamine transmission and negative affect. An engaging hypothesis holds the upregulation of dynorphin/KOR in chronic pain states to be causal in the generation of concomitant depression and mood disorders. This remains to be fully tested, however supporting evidence includes upregulation of dynorphin following chronic drug use and in post-mortem suicide patients where stress, depression, and anxiety disorders have developed.

There is a clear a role for the dynorphin/KOR system in modulating the interplay of pain and reward processing. Through modulation of limbic neurotransmission, this system produces aversion, stress affect, and depression. The manifestation of these processes as corresponding psychiatric disorders is highly comorbid with chronic pain and suicide is exceedingly prevalent in chronic pain patients. These linked conditions have profound and severely deleterious effects on patients' quality of life. Despite the implication of the KOR system in this progression, accepted treatments targeting it are lacking, thus manipulation of the KOR system may prove valuable in ameliorating chronic pain-induced negative affect.

REFERENCES

Abhishek, A., and Doherty, M. (2013). Mechanisms of the placebo response in pain in osteoarthritis. *Osteoarthr. Cartil.* 21, 1229–1235. doi: 10.1016/j.joca.2013.04.018

Ableitner, A., and Herz, A. (1989). Limbic brain structures are important sites of κ-opioid receptor-mediated actions in the rat: a [^{14}C]-2-deoxyglucose study. *Brain Res.* 478, 326–336. doi: 10.1016/0006-8993(89)91513-8

Abraham, K. E., Brewer, K. L., and McGinty, J. F. (2000). Opioid peptide messenger RNA expression is increased at spinal and supraspinal levels following excitotoxic spinal cord injury. *Neuroscience* 99, 189–197. doi: 10.1016/S0306-4522(00)00150-0

Aita, M., Byers, M. R., Chavkin, C., and Xu, M. (2010). Trigeminal injury causes kappa opioid-dependent allodynic, glial and immune cell responses in mice. *Mol. Pain* 6, 8. doi: 10.1186/1744-8069-6-8

Anstrom, K. K., Miczek, K. A., and Budygin, E. A. (2009). Increased phasic dopamine signaling in the mesolimbic pathway during social defeat in rats. *Neuroscience* 161, 3–12. doi: 10.1016/j.neuroscience.2009.03.023

Anstrom, K. K., and Woodward, D. J. (2005). Restraint increases dopaminergic burst firing in awake rats. *Neuropsychopharmacology* 30, 1832–1840. doi: 10.1038/sj.npp.1300730

Apkarian, A. V., Bushnell, M. C., Treede, R.-D., and Zubieta, J.-K. (2005). Human brain mechanisms of pain perception and regulation in health and disease. *Eur. J. Pain* 9, 463–484. doi: 10.1016/j.ejpain.2004.11.001

Asmundson, G. J. G., and Katz, J. (2009). Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. *Depress. Anxiety* 26, 888–901. doi: 10.1002/da.20600

Attal, N., Masselin-Dubois, A., Martinez, V., Jayr, C., Albi, A., Fermanian, J., et al. (2014). Does cognitive functioning predict chronic pain? Results from a prospective surgical cohort. *Brain* 137, 904–917. doi: 10.1093/brain/awt354

Badiani, A., Rajabi, H., Nencini, P., and Stewart, J. (2001). Modulation of food intake by the kappa opioid U-50,488H: evidence for an effect on satiation. *Behav. Brain Res.* 118, 179–186. doi: 10.1016/S0166-4328(00)00325-9

Baliki, M. N., Geha, P. Y., Fields, H. L., and Apkarian, A. V. (2010). Predicting value of pain and analgesia: nucleus accumbens response to noxious stimuli changes in the presence of chronic pain. *Neuron* 66, 149–160. doi: 10.1016/j.neuron.2010.03.002

Baliki, M. N., Petre, B., Torbey, S., Herrmann, K. M., Huang, L., Schnitzer, T. J., et al. (2012). Corticostriatal functional connectivity predicts transition to chronic back pain. *Nat. Neurosci.* 15, 1117–1119. doi: 10.1038/nn.3153

Bals-Kubik, R., Ableitner, A., Herz, A., and Shippenberg, T. S. (1993). Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats. *J. Pharmacol. Exp. Ther.* 264, 489–495.

Bassareo, V., De Luca, M. A., and Di Chiara, G. (2002). Differential expression of motivational stimulus properties by dopamine in nucleus accumbens shell versus core and prefrontal cortex. *J. Neurosci.* 22, 4709–4719.

Becerra, L., and Borsook, D. (2008). Signal valence in the nucleus accumbens to pain onset and offset. *Eur. J. Pain* 12, 866–869. doi: 10.1016/j.ejpain.2007.12.007

Berridge, K. C. (2007). The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology (Berl.)* 191, 391–431. doi: 10.1007/s00213-006-0578-x

Berridge, K. C., and Kringelbach, M. L. (2013). Neuroscience of affect: brain mechanisms of pleasure and displeasure. *Curr. Opin. Neurobiol.* 23, 294–303. doi: 10.1016/j.conb.2013.01.017

Bie, B., Brown, D. L., and Naguib, M. (2011). Synaptic plasticity and pain aversion. *Eur. J. Pharmacol.* 667, 26–31. doi: 10.1016/j.ejphar.2011.05.080

Bilkei-Gorzo, A., Racz, I., Michel, K., Mauer, D., Zimmer, A., Klingmüller, D., et al. (2008). Control of hormonal stress reactivity by the endogenous opioid system. *Psychoneuroendocrinology* 33, 425–436. doi: 10.1016/j.psyneuen.2007.12.010

Blackburn-Munro, G., and Blackburn-Munro, R. E. (2001). Chronic pain, chronic stress and depression: coincidence or consequence? *J. Neuroendocrinol.* 13, 1009–1023. doi: 10.1046/j.0007-1331.2001.00727.x

Boettger, M. K., Grossmann, D., and Bär, K.-J. (2013). Increased cold and heat pain thresholds influence the thermal grill illusion in schizophrenia. *Eur. J. Pain* 17, 200–209. doi: 10.1002/j.1532-2149.2012.00188.x

Borsook, D., Becerra, L., Carlezon, W. A., Shaw, M., Renshaw, P., Elman, I., et al. (2007). Reward-aversion circuitry in analgesia and pain: implications for psychiatric disorders. *Eur. J. Pain* 11, 7–20. doi: 10.1016/j.ejpain.2005.12.005

Borsook, D., Edwards, R., Elman, I., Becerra, L., and Levine, J. (2013). Pain and analgesia: the value of salience circuits. *Prog. Neurobiol.* 104, 93–105. doi: 10.1016/j.pneurobio.2013.02.003

Bourdy, R., and Barrot, M. (2012). A new control center for dopaminergic systems: pulling the VTA by the tail. *Trends Neurosci.* 35, 681–690. doi: 10.1016/j.tins.2012.06.007

Boutelle, M. G., Zetterström, T., Pei, Q., Svensson, L., and Fillenz, M. (1990). In vivo neurochemical effects of tail pinch. *J. Neurosci. Methods* 34, 151–157. doi: 10.1016/0165-0270(90)90053-I

Brandt, M. R., Furness, M. S., Rice, K. C., Fischer, B. D., and Negus, S. S. (2001). Studies of tolerance and dependence with the delta-opioid agonist SNC80 in rhesus monkeys responding under a schedule of food presentation. *J. Pharmacol. Exp. Ther.* 299, 629–637.

Brischoux, F., Chakraborty, S., Brierley, D. I., and Ungless, M. A. (2009). Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4894–4899. doi: 10.1073/pnas.0811507106

Bruchas, M. R., Land, B. B., Aita, M., Xu, M., Barot, S. K., Li, S., et al. (2007). Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. *J. Neurosci.* 27, 11614–11623. doi: 10.1523/JNEUROSCI.3769-07.2007

Bruchas, M. R., Land, B. B., and Chavkin, C. (2010). The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res.* 1314, 44–55. doi: 10.1016/j.brainres.2009.08.062

Bruchas, M. R., Land, B. B., Lemos, J. C., and Chavkin, C. (2009). CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. *PLoS ONE* 4:e8528. doi: 10.1371/journal.pone.0008528

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

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

Budygin, E. A., Park, J., Bass, C. E., Grinevich, V. P., Bonin, K. D., and Wightman, R. M. (2012). Aversive stimulus differentially triggers subsecond dopamine release in reward regions. *Neuroscience* 201, 331–337. doi: 10.1016/j.neuroscience.2011.10.056

Bunzow, J. R., Zhang, G., Bouvier, C., Saez, C., Ronnekleiv, O. K., Kelly, M. J., et al. (1995). Characterization and distribution of a cloned rat mu-opioid receptor. *J. Neurochem.* 64, 14–24. doi: 10.1046/j.1471-4159.1995.64010014.x

Cahill, C. M., Cook, C., and Pickens, S. (2014). Migraine and reward system-or is it aversive? *Curr. Pain Headache Rep.* 18, 410. doi: 10.1007/s11916-014-0410-y

Cahill, C. M., Holdridge, S. V., and Morinville, A. (2007). Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. *Trends Pharmacol. Sci.* 28, 23–31. doi: 10.1016/j.tips.2006.11.003

Caldecott-Hazard, S., Mazziotta, J., and Phelps, M. (1988). Cerebral correlates of depressed behavior in rats, visualized using 14C-2-deoxyglucose autoradiography. *J. Neurosci.* 8, 1951–1961.

Carlezon, W. A., Béguin, C., DiNieri, J. A., Baumann, M. H., Richards, M. R., Todtenkopf, M. S., et al. (2006). Depressive-like effects of the kappa-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats. *J. Pharmacol. Exp. Ther.* 316, 440–447. doi: 10.1124/jpet.105.092304

Carlezon, W. A., and Thomas, M. J. (2009). Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. *Neuropharmacology* 56(Suppl. 1), 122–132. doi: 10.1016/j.neuropharm.2008.06.075

Caudle, R. M., and Isaac, L. (1988). A novel interaction between dynorphin(1–13) and an *N*-methyl-D-aspartate site. *Brain Res.* 443, 329–332. doi: 10.1016/0006-8993(88)91628-9

Chang, P.-C., Pollema-Mays, S. L., Centeno, M. V., Procissi, D., Contini, M., Baria, A. T., et al. (2014). Role of nucleus accumbens in neuropathic pain: linked multi-scale evidence in the rat transitioning to neuropathic pain. *Pain* 155, 1128–1139. doi: 10.1016/j.pain.2014.02.019

Chartoff, E. H., Papadopoulou, M., MacDonald, M. L., Parsegian, A., Potter, D., Konradi, C., et al. (2009). Desipramine reduces stress-activated dynorphin expression and CREB phosphorylation in NAc tissue. *Mol. Pharmacol.* 75, 704–712. doi: 10.1124/mol.108.051417

Chavkin, C. (2011). The therapeutic potential of κ-opioids for treatment of pain and addiction. *Neuropsychopharmacology* 36, 369–370. doi: 10.1038/npp.2010.137

Chefer, V. I., Bäckman, C. M., Gigante, E. D., and Shippenberg, T. S. (2013). Kappa opioid receptors on dopaminergic neurons are necessary for kappa-mediated place aversion. *Neuropsychopharmacology* 38, 2623–2631. doi: 10.1038/npp.2013.171

Chu, N., Xia, W., Yu, P., Hu, L., Zhang, R., and Cui, C. (2008). Chronic morphine-induced neuronal morphological changes in the ventral tegmental area in rats are reversed by electroacupuncture treatment. *Addict. Biol.* 13, 47–51. doi: 10.1111/j.1369-1600.2007.00092.x

Cohen, S. R., and Melzack, R. (1986). Habenular stimulation produces analgesia in the formalin test. *Neurosci. Lett.* 70, 165–169. doi: 10.1016/0304-3940(86)90457-X

Craig, A. D. (2003). Distribution of trigeminothalamic and spinothalamic lamina I terminations in the cat. *Somatosens. Mot. Res.* 20, 209–222. doi: 10.1080/08990220310001623013

Cui, Y., Ostlund, S. B., James, A. S., Park, C. S., Ge, W., Roberts, K. W., et al. (2014). Targeted expression of μ-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nat. Neurosci.* 17, 254–261. doi: 10.1038/nn.3622

Dafny, N., Dong, W. Q., Prieto-Gomez, C., Reyes-Vazquez, C., Stanford, J., and Qiao, J. T. (1996). Lateral hypothalamus: site involved in pain modulation. *Neuroscience* 70, 449–460. doi: 10.1016/0306-4522(95)00358-4

Darcq, E., Befort, K., Koebel, P., Pannetier, S., Mahoney, M. K., Gaveriaux-Ruff, C., et al. (2012). RSK2 signaling in medial habenula contributes to acute morphine analgesia. *Neuropsychopharmacology* 37, 1288–1296. doi: 10.1038/npp.2011.316

De Felice, M., Eyde, N., Dodick, D., Dussor, G. O., Ossipov, M. H., Fields, H. L., et al. (2013). Capturing the aversive state of cephalic pain preclinically. *Ann. Neurol.* 74, 257–265. doi: 10.1002/ana.23922

Di Chiara, G., and Imperato, A. (1988). Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *J. Pharmacol. Exp. Ther.* 244, 1067–1080.

Donzanti, B. A., Althaus, J. S., Payson, M. M., and Von Voigtlander, P. F. (1992). Kappa agonist-induced reduction in dopamine release: site of action and tolerance. *Res. Commun. Chem. Pathol. Pharmacol.* 78, 193–210.

Drdla, R., Gassner, M., Gingl, E., and Sandkühler, J. (2009). Induction of synaptic long-term potentiation after opioid withdrawal. *Science* 325, 207–210. doi: 10.1126/science.1171759

Dworkin, R. H. (1994). Pain insensitivity in schizophrenia: a neglected phenomenon and some implications. *Schizophr. Bull.* 20, 235–248. doi: 10.1093/schbul/20.2.235

Elman, I., Borsook, D., and Volkow, N. D. (2013). Pain and suicidality: insights from reward and addiction neuroscience. *Prog. Neurobiol.* 109, 1–27. doi: 10.1016/j.pneurobio.2013.06.003

Ferrini, F., Trang, T., Mattioli, T.-A. M., Laffray, S., Del'Guidice, T., Lorenzo, L.-E., et al. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl-homeostasis. *Nat. Neurosci.* 16, 183–192. doi: 10.1038/nn.3295

Fields, H. L., Hjelmstad, G. O., Margolis, E. B., and Nicola, S. M. (2007). Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu. Rev. Neurosci.* 30, 289–316. doi: 10.1146/annurev.neuro.30.051606.094341

Fiorillo, C. D. (2013). Two dimensions of value: dopamine neurons represent reward but not aversiveness. *Science* 341, 546–549. doi: 10.1126/science.1238699

Fishbain, D. A., Cutler, R., Rosomoff, H. L., and Rosomoff, R. S. (1997). Chronic pain-associated depression: antecedent or consequence of chronic pain? A review. *Clin. J. Pain* 13, 116–137. doi: 10.1097/00002508-199706000-00006

Fletcher, D., and Martinez, V. (2014). Opioid-induced hyperalgesia in patients after surgery: a systematic review and a meta-analysis. *Br. J. Anaesth.* 112, 991–1004. doi: 10.1093/bja/aeu137

Ford, C. P., Beckstead, M. J., and Williams, J. T. (2007). Kappa opioid inhibition of somatodendritic dopamine inhibitory postsynaptic currents. *J. Neurophysiol.* 97, 883–891. doi: 10.1152/jn.00963.2006

Ford, C. P., Mark, G. P., and Williams, J. T. (2006). Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. *J. Neurosci.* 26, 2788–2797. doi: 10.1523/JNEUROSCI.4331-05.2006

Frankel, P. S., Alburges, M. E., Bush, L., Hanson, G. R., and Kish, S. J. (2008). Striatal and ventral pallidum dynorphin concentrations are markedly increased in human chronic cocaine users. *Neuropharmacology* 55, 41–46. doi: 10.1016/j.neuropharm.2008.04.019

Gao, Y.-J., Ren, W.-H., Zhang, Y.-Q., and Zhao, Z.-Q. (2004). Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. *Pain* 110, 343–353. doi: 10.1016/j.pain.2004.04.030

Gauriau, C., and Bernard, J.-F. (2004). A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: the forebrain. *J. Comp. Neurol.* 468, 24–56. doi: 10.1002/cne.10873

Geha, P. Y., Baliki, M. N., Wang, X., Harden, R. N., Paice, J. A., and Apkarian, A. V. (2008). Brain dynamics for perception of tactile allodynia (touch-induced pain) in postherpetic neuralgia. *Pain* 138, 641–656. doi: 10.1016/j.pain.2008.02.021

Geisler, S., Derst, C., Veh, R. W., and Zahm, D. S. (2007). Glutamatergic afferents of the ventral tegmental area in the rat. *J. Neurosci.* 27, 5730–5743. doi: 10.1523/JNEUROSCI.0012-07.2007

Georges, F., and Aston-Jones, G. (2001). Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. *J. Neurosci.* 21, RC160.

Georges, F., and Aston-Jones, G. (2002). Activation of ventral tegmental area cells by the bed nucleus of the stria terminalis: a novel excitatory amino acid input to midbrain dopamine neurons. *J. Neurosci.* 22, 5173–5187.

Gilron, I., Watson, C. P. N., Cahill, C. M., and Moulin, D. E. (2006). Neuropathic pain: a practical guide for the clinician. *CMAJ* 175, 265–275. doi: 10.1503/cmaj.060146

Gonçalves, L., Sego, C., and Metzger, M. (2012). Differential projections from the lateral habenula to the rostromedial tegmental nucleus and ventral tegmental area in the rat. *J. Comp. Neurol.* 520, 1278–1300. doi: 10.1002/cne.22787

Good, C. H., and Lupica, C. R. (2009). Properties of distinct ventral tegmental area synapses activated via pedunculopontine or ventral tegmental area stimulation *in vitro*. *J. Physiol.* 587, 1233–1247. doi: 10.1113/jphysiol.2008.164194

Grace, A. A., Floresco, S. B., Goto, Y., and Lodge, D. J. (2007). Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci.* 30, 220–227. doi: 10.1016/j.tins.2007.03.003

Gureje, O., Von Korff, M., Simon, G. E., and Gater, R. (1998). Persistent pain and well-being: a World Health Organization Study in Primary Care. *JAMA* 280, 147–151. doi: 10.1001/jama.280.2.147

Haber, S. N., Groenewegen, H. J., Grove, E. A., and Nauta, W. J. (1985). Efferent connections of the ventral pallidum: evidence of a dual striato pallidofugal pathway. *J. Comp. Neurol.* 235, 322–335. doi: 10.1002/cne.902350304

Hamilton, M. E., and Bozarth, M. A. (1988). Feeding elicited by dynorphin (1–13) microinjections into the ventral tegmental area in rats. *Life Sci.* 43, 941–946. doi: 10.1016/0024-3205(88)90271-8

Hashmi, J. A., Baliki, M. N., Huang, L., Baria, A. T., Torbey, S., Hermann, K. M., et al. (2013). Shape shifting pain: chronification of back pain shifts brain representation from nociceptive to emotional circuits. *Brain* 136, 2751–2768. doi: 10.1093/brain/awt211

Hauptman, J. S., DeSalles, A. A. F., Espinoza, R., Sedrak, M., and Ishida, W. (2008). Potential surgical targets for deep brain stimulation in treatment-resistant depression. *Neurosurg. Focus* 25, E3. doi: 10.3171/FOC/2008/25/7/E3

Heijna, M. H., Padt, M., Hogenboom, F., Portoghese, P. S., Mulder, A. H., and Schoffelmeer, A. N. (1990). Opioid receptor-mediated inhibition of dopamine and acetylcholine release from slices of rat nucleus accumbens, olfactory tubercle and frontal cortex. *Eur. J. Pharmacol.* 181, 267–278. doi: 10.1016/0014-2999(90)90088-N

Hikida, T., Kimura, K., Wada, N., Funabiki, K., and Nakanishi, S. (2010). Distinct roles of synaptic transmission in direct and indirect striatal pathways to reward and aversive behavior. *Neuron* 66, 896–907. doi: 10.1016/j.neuron.2010.05.011

Hikida, T., Yawata, S., Yamaguchi, T., Danjo, T., Sasaoka, T., Wang, Y., et al. (2013). Pathway-specific modulation of nucleus accumbens in reward and aversive behavior via selective transmitter receptors. *Proc. Natl. Acad. Sci. U.S.A.* 110, 342–347. doi: 10.1073/pnas.1220358110

Hikosaka, O. (2010). The habenula: from stress evasion to value-based decision-making. *Nat. Rev. Neurosci.* 11, 503–513. doi: 10.1038/nrn2866

Hong, S., Jhou, T. C., Smith, M., Saleem, K. S., and Hikosaka, O. (2011). Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. *J. Neurosci.* 31, 11457–11471. doi: 10.1523/JNEUROSCI.1384-11.2011

Hummel, M., Cummons, T., Lu, P., Mark, L., Harrison, J. E., Kennedy, J. D., et al. (2010). Pain is a salient "stressor" that is mediated by corticotropin-releasing factor-1 receptors. *Neuropharmacology* 59, 160–166. doi: 10.1016/j.neuropharm.2010.05.001

Iadarola, M. J., Brady, L. S., Draisci, G., and Dubner, R. (1988). Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding. *Pain* 35, 313–326. doi: 10.1016/0304-3959(88)90141-8

Ichesco, E., Schmidt-Wilcke, T., Bhavsar, R., Clauw, D. J., Peltier, S. J., Kim, J., et al. (2014). Altered resting state connectivity of the insular cortex in individuals with fibromyalgia. *J. Pain* 15, 815.e1–826.e1. doi: 10.1016/j.jpain.2014.04.007

Isola, R., Zhang, H., Tejwani, G. A., Neff, N. H., and Hadjiconstantinou, M. (2009). Acute nicotine changes dynorphin and prodynorphin mRNA in the striatum. *Psychopharmacology (Berl.)* 201, 507–516. doi: 10.1007/s00213-008-1315-4

Jamison, R. N., Kauffman, J., and Katz, N. P. (2000). Characteristics of methadone maintenance patients with chronic pain. *J. Pain Symptom Manage.* 19, 53–62. doi: 10.1016/S0885-3924(99)00144-X

Jarcho, J. M., Mayer, E. A., Jiang, Z. K., Feier, N. A., and London, E. D. (2012). Pain, affective symptoms, and cognitive deficits in patients with cerebral dopamine dysfunction. *Pain* 153, 744–754. doi: 10.1016/j.pain.2012.01.002

Jennings, J. H., Sparta, D. R., Stamatakis, A. M., Ung, R. L., Pleil, K. E., Kash, T. L., et al. (2013). Distinct extended amygdala circuits for divergent motivational states. *Nature* 496, 224–228. doi: 10.1038/nature12041

Jensen, K. B., Srinivasan, P., Spaeth, R., Tan, Y., Kosek, E., Petzke, F., et al. (2013). Overlapping structural and functional brain changes in patients with long-term exposure to fibromyalgia pain. *Arthritis Rheum.* 65, 3293–3303. doi: 10.1002/art.38170

Jhou, T. C., Fields, H. L., Baxter, M. G., Saper, C. B., and Holland, P. C. (2009). The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron* 61, 786–800. doi: 10.1016/j.neuron.2009.02.001

Ji, H., and Shepard, P. D. (2007). Lateral habenula stimulation inhibits rat midbrain dopamine neurons through a $GABA_A$ receptor-mediated mechanism. *J. Neurosci.* 27, 6923–6930. doi: 10.1523/JNEUROSCI.0958-07.2007

Juurlink, D. N., Herrmann, N., Szalai, J. P., Kopp, A., and Redelmeier, D. A. (2004). Medical illness and the risk of suicide in the elderly. *Arch. Intern. Med.* 164, 1179–1184. doi: 10.1001/archinte.164.11.1179

Kieffer, B. L., and Gavériaux-Ruff, C. (2002). Exploring the opioid system by gene knockout. *Prog. Neurobiol.* 66, 285–306. doi: 10.1016/S0301-0082(02)00008-4

King, T., Vera-Portocarrero, L., Gutierrez, T., Vanderah, T. W., Dussor, G., Lai, J., et al. (2009). Unmasking the tonic-aversive state in neuropathic pain. *Nat. Neurosci.* 12, 1364–1366. doi: 10.1038/nn.2407

Kitchen, I., Slowe, S. J., Matthes, H. W., and Kieffer, B. (1997). Quantitative autoradiographic mapping of μ-, δ- and κ-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res.* 778, 73–88. doi: 10.1016/S0006-8993(97)00988-8

Knoll, A. T., and Carlezon, W. A. (2010). Dynorphin, stress, and depression. *Brain Res.* 1314, 56–73. doi: 10.1016/j.brainres.2009.09.074

Knoll, A. T., Meloni, E. G., Thomas, J. B., Carroll, F. I., and Carlezon, W. A. (2007). Anxiolytic-like effects of kappa-opioid receptor antagonists in models of unlearned and learned fear in rats. *J. Pharmacol. Exp. Ther.* 323, 838–845. doi: 10.1124/jpet.107.127415

Kolesnikov, Y., Jain, S., Wilson, R., and Pasternak, G. W. (1996). Peripheral kappa 1-opioid receptor-mediated analgesia in mice. *Eur. J. Pharmacol.* 310, 141–143. doi: 10.1016/0014-2999(96)00520-1

Kudryavtseva, N. N., Gerrits, M. A. F. M., Avgustinovich, D. F., Tenditnik, M. V., and Van Ree, J. M. (2004). Modulation of anxiety-related behaviors by mu- and kappa-opioid receptor agonists depends on the social status of mice. *Peptides* 25, 1355–1363. doi: 10.1016/j.peptides.2004.05.005

Lammel, S., Lim, B. K., Ran, C., Huang, K. W., Betley, M. J., Tye, K. M., et al. (2012). Input-specific control of reward and aversion in the ventral tegmental area. *Nature* 491, 212–217. doi: 10.1038/nature11527

Land, B. B., Bruchas, M. R., Lemos, J. C., Xu, M., Melief, E. J., and Chavkin, C. (2008). The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *J. Neurosci.* 28, 407–414. doi: 10.1523/JNEUROSCI.4458-07.2008

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

Lee, M., Silverman, S. M., Hansen, H., Patel, V. B., and Manchikanti, L. (2011). A comprehensive review of opioid-induced hyperalgesia. *Pain Physician* 14, 145–161.

Legrain, V., Iannetti, G. D., Plaghki, L., and Mouraux, A. (2011). The pain matrix reloaded: a salience detection system for the body. *Prog. Neurobiol.* 93, 111–124. doi: 10.1016/j.pneurobio.2010.10.005

Leitl, M. D., Onvani, S., Bowers, M. S., Cheng, K., Rice, K. C., Carlezon, W. A., et al. (2014a). Pain-related depression of the mesolimbic dopamine system in rats: expression, blockade by analgesics, and role of endogenous κ-opioids. *Neuropsychopharmacology* 39, 614–624. doi: 10.1038/npp.2013.236

Leitl, M. D., Potter, D. N., Cheng, K., Rice, K. C., Carlezon, W. A., and Negus, S. S. (2014b). Sustained pain-related depression of behavior: effects of intraplantar formalin and complete freund's adjuvant on intracranial self-stimulation (ICSS) and endogenous kappa opioid biomarkers in rats. *Mol. Pain* 10, 62. doi: 10.1186/1744-8069-10-62

Leknes, S., and Tracey, I. (2008). A common neurobiology for pain and pleasure. *Nat. Rev. Neurosci.* 9, 314–320. doi: 10.1038/nrn2333

Le Merrer, J., Becker, J. A. J., Befort, K., and Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412. doi: 10.1152/physrev.00005.2009

Li, C., Pleil, K. E., Stamatakis, A. M., Busan, S., Vong, L., Lowell, B. B., et al. (2012). Presynaptic inhibition of gamma-aminobutyric acid release in the bed nucleus of the stria terminalis by kappa opioid receptor signaling. *Biol. Psychiatry* 71, 725–732. doi: 10.1016/j.biopsych.2011.11.015

Lindholm, S., Ploj, K., Franck, J., and Nylander, I. (2000). Repeated ethanol administration induces short- and long-term changes in enkephalin and dynorphin tissue concentrations in rat brain. *Alcohol* 22, 165–171. doi: 10.1016/S0741-8329(00)00118-X

Long, J. B., Petras, J. M., Mobley, W. C., and Holaday, J. W. (1988). Neurological dysfunction after intrathecal injection of dynorphin A (1–13) in the rat. II. Nonopioid mechanisms mediate loss of motor, sensory and autonomic function. *J. Pharmacol. Exp. Ther.* 246, 1167–1174.

Lutz, P.-E., and Kieffer, B. L. (2013). Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* 36, 195–206. doi: 10.1016/j.tins.2012.11.002

Ma, Q. P., Shi, Y. S., and Han, J. S. (1992). Further studies on interactions between periaqueductal gray, nucleus accumbens and habenula in antinociception. *Brain Res.* 583, 292–295. doi: 10.1016/S0006-8993(10)80036-8

Magnussen, C., Sutherland, K., Olmstead, M., and Cahill, C. (2009). "Conditioned place preference predicts drug effectiveness in alleviating neuropathic pain," *Proceedings of the International Narcotics Research Conference*, Abstr. 57, 71.

Mague, S. D., Pliakas, A. M., Todtenkopf, M. S., Tomasiewicz, H. C., Zhang, Y., Stevens, W. C., et al. (2003). Antidepressant-like effects of kappa-opioid receptor antagonists in the forced swim test in rats. *J. Pharmacol. Exp. Ther.* 305, 323–330. doi: 10.1124/jpet.102.046433

Maisonneuve, I. M., Archer, S., and Glick, S. D. (1994). U50,488, a kappa opioid receptor agonist, attenuates cocaine-induced increases in extracellular dopamine in the nucleus accumbens of rats. *Neurosci. Lett.* 181, 57–60. doi: 10.1016/0304-3940(94)90559-2

Mansour, A., Burke, S., Pavlic, R. J., Akil, H., and Watson, S. J. (1996). Immunohistochemical localization of the cloned kappa 1 receptor in the rat CNS and pituitary. *Neuroscience* 71, 671–690. doi: 10.1016/0306-4522(95)00464-5

Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J. (1987). Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *J. Neurosci.* 7, 2445–2464.

Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J. (1988). Anatomy of CNS opioid receptors. *Trends Neurosci.* 11, 308–314. doi: 10.1016/0166-2236(88)90093-8

Mansour, A., Thompson, R. C., Akil, H., and Watson, S. J. (1993). Delta opioid receptor mRNA distribution in the brain: comparison to delta receptor binding and proenkephalin mRNA. *J. Chem. Neuroanat.* 6, 351–362. doi: 10.1016/0891-0618(93)90010-2

Marbach, J. J., and Lund, P. (1981). Depression, anhedonia and anxiety in temporomandibular joint and other facial pain syndromes. *Pain* 11, 73–84. doi: 10.1016/0304-3959(81)90140-8

Margolis, E. B., Hjelmstad, G. O., Bonci, A., and Fields, H. L. (2003). Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons. *J. Neurosci.* 23, 9981–9986.

Margolis, E. B., Hjelmstad, G. O., Bonci, A., and Fields, H. L. (2005). Both kappa and mu opioid agonists inhibit glutamatergic input to ventral tegmental area neurons. *J. Neurophysiol.* 93, 3086–3093. doi: 10.1152/jn.00855.2004

Margolis, E. B., Lock, H., Chefer, V. I., Shippenberg, T. S., Hjelmstad, G. O., and Fields, H. L. (2006). Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2938–2942. doi: 10.1073/pnas.0511159103

Matsui, A., and Williams, J. T. (2011). Opioid-sensitive GABA inputs from rostromedial tegmental nucleus synapse onto midbrain dopamine neurons. *J. Neurosci.* 31, 17729–17735. doi: 10.1523/JNEUROSCI.4570-11.2011

McClure, S. M., Daw, N. D., and Montague, P. R. (2003). A computational substrate for incentive salience. *Trends Neurosci.* 26, 423–428. doi: 10.1016/S0166-2236(03)00177-2

McCullough, L. D., Sokolowski, J. D., and Salamone, J. D. (1993). A neurochemical and behavioral investigation of the involvement of nucleus accumbens dopamine in instrumental avoidance. *Neuroscience* 52, 919–925. doi: 10.1016/0306-4522(93)90538-Q

McCutcheon, J. E., Ebner, S. R., Loriaux, A. L., and Roitman, M. F. (2012). Encoding of aversion by dopamine and the nucleus accumbens. *Front. Neurosci.* 6:137. doi: 10.3389/fnins.2012.00137

Mclaughlin, J. P., Marton-popovici, M., and Chavkin, C. (2003). κ opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J. Neurosci.* 23, 5674–5683.

McLennan, G. P., Kiss, A., Miyatake, M., Belcheva, M. M., Chambers, K. T., Pozek, J. J., et al. (2008). Kappa opioids promote the proliferation of astrocytes via Gβγ and β-arrestin 2-dependent MAPK-mediated pathways. *J. Neurochem.* 107, 1753–1765. doi: 10.1111/j.1471-4159.2008.05745.x

Meshul, C. K., and McGinty, J. F. (2000). Kappa opioid receptor immunoreactivity in the nucleus accumbens and caudate-putamen is primarily associated with synaptic vesicles in axons. *Neuroscience* 96, 91–99. doi: 10.1016/S0306-4522(99)90481-5

Meunier, J. C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377, 532–535. doi: 10.1038/377532a0

Miller, L. R., and Cano, A. (2009). Comorbid chronic pain and depression: who is at risk? *J. Pain* 10, 619–627. doi: 10.1016/j.jpain.2008.12.007

Mirenowicz, J., and Schultz, W. (1994). Importance of unpredictability for reward responses in primate dopamine neurons. 72, 1024–1027.

Mirenowicz, J., and Schultz, W. (1996). Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. *Nature* 379, 449–451. doi: 10.1038/379449a0

Mogil, J. S., Grisel, J. E., Zhangs, G., Belknap, J. K., and Grandy, D. K. (1996). Functional antagonism of μ-, δ- and κ-opioid antinociception by orphanin FQ. *Neurosci. Lett.* 214, 131–134. doi: 10.1016/0304-3940(96)12917-7

Moja, P. L., Cusi, C., Sterzi, R. R., and Canepari, C. (2005). Selective serotonin re-uptake inhibitors (SSRIs) for preventing migraine and tension-type headaches. *Cochrane Database Syst. Rev.* 1:CD002919. doi: 10.1002/14651858.CD002919.pub2

Mollereau, C., Parmentier, M., Mailleux, P., Butour, J. L., Moisand, C., Chalon, P., et al. (1994). ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* 341, 33–38. doi: 10.1016/0014-5793(94)80235-1

Morris, J. S., Smith, K. A., Cowen, P. J., Friston, K. J., and Dolan, R. J. (1999). Covariation of activity in habenula and dorsal raphé nuclei following tryptophan depletion. *Neuroimage* 10, 163–172. doi: 10.1006/nimg.1999.0455

Mouraux, A., Diukova, A., Lee, M. C., Wise, R. G., and Iannetti, G. D. (2011). A multisensory investigation of the functional significance of the "pain matrix." *Neuroimage* 54, 2237–2249. doi: 10.1016/j.neuroimage.2010.09.084

Mundal, I., Gråwe, R. W., Bjørngaard, J. H., Linaker, O. M., and Fors, E. A. (2014). Psychosocial factors and risk of chronic widespread pain: an 11-year follow-up study—the HUNT study. *Pain* 155, 1555–1561. doi: 10.1016/j.pain.2014.04.033

Murphy, N. P., Lee, Y., and Maidment, N. T. (1999). Orphanin FQ/nociceptin blocks acquisition of morphine place preference. *Brain Res.* 832, 168–170. doi: 10.1016/S0006-8993(99)01425-0

Murray, E. A. (2007). The amygdala, reward and emotion. *Trends Cogn. Sci.* 11, 489–497. doi: 10.1016/j.tics.2007.08.013

Muschamp, J. W., Van't Veer, A., Parsegian, A., Gallo, M. S., Chen, M., Neve, R. L., et al. (2011). Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. *J. Neurosci.* 31, 3095–3103. doi: 10.1523/JNEUROSCI.5973-10.2011

Nabeshima, T., Katoh, A., Wada, M., and Kameyama, T. (1992). Stress-induced changes in brain Met-enkephalin, Leu-enkephalin and dynorphin concentrations. *Life Sci.* 51, 211–217. doi: 10.1016/0024-3205(92)90077-3

Narita, M., Kaneko, C., Miyoshi, K., Nagumo, Y., Kuzumaki, N., Nakajima, M., et al. (2006a). Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. *Neuropsychopharmacology* 31, 739–750. doi: 10.1038/sj.npp.1300858

Narita, M., Miyatake, M., Narita, M., Shibasaki, M., Shindo, K., Nakamura, A., et al. (2006b). Direct evidence of astrocytic modulation in the development of rewarding effects induced by drugs of abuse. *Neuropsychopharmacology* 31, 2476–2488. doi: 10.1038/sj.npp.1301007

Narita, M., Miyatake, M., Shibasaki, M., Tsuda, M., Koizumi, S., Narita, M., et al. (2005). Long-lasting change in brain dynamics induced by methamphetamine: enhancement of protein kinase C-dependent astrocytic response and behavioral sensitization. *J. Neurochem.* 93, 1383–1392. doi: 10.1111/j.1471-4159.2005.03097.x

Nauta, W. J., Smith, G. P., Faull, R. L., and Domesick, V. B. (1978). Efferent connections and nigral afferents of the nucleus accumbens septi in the rat. *Neuroscience* 3, 385–401. doi: 10.1016/0306-4522(78)90041-6

Navratilova, E., and Porreca, F. (2014). Reward and motivation in pain and pain relief. *Nat. Neurosci.* 17, 1304–1312. doi: 10.1038/nn.3811

Navratilova, E., Xie, J. Y., Okun, A., Qu, C., Eyde, N., Ci, S., et al. (2012). Pain relief produces negative reinforcement through activation of mesolimbic reward-valuation circuitry. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20709–20713. doi: 10.1073/pnas.1214605109

Negri, A., Rives, M.-L., Caspers, M. J., Prisinzano, T. E., Javitch, J. A., and Filizola, M. (2013). Discovery of a novel selective kappa-opioid receptor agonist using crystal structure-based virtual screening. *J. Chem. Inf. Model.* 53, 521–526. doi: 10.1021/ci400019t

Negus, S. S., Gatch, M. B., Mello, N. K., Zhang, X., and Rice, K. (1998). Behavioral effects of the delta-selective opioid agonist SNC80 and related compounds in rhesus monkeys. *J. Pharmacol. Exp. Ther.* 286, 362–375.

Negus, S. S., Morrissey, E. M., Rosenberg, M., Cheng, K., and Rice, K. C. (2010). Effects of kappa opioids in an assay of pain-depressed intracranial self-stimulation in rats. *Psychopharmacology (Berl.)* 210, 149–159. doi: 10.1007/s00213-009-1770-6

Nestler, E. J., and Carlezon, W. A. (2006). The mesolimbic dopamine reward circuit in depression. *Biol. Psychiatry* 59, 1151–1159. doi: 10.1016/j.biopsych.2005.09.018

Newton, S. S., Thome, J., Wallace, T. L., Shirayama, Y., Schlesinger, L., Sakai, N., et al. (2002). Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. *J. Neurosci.* 22, 10883–10890.

Nicholson, B., and Verma, S. (2004). Comorbidities in chronic neuropathic pain. *Pain Med.* 5(Suppl. 1), S9–S27. doi: 10.1111/j.1526-4637.2004.04019.x

Obara, I., Mika, J., Schafer, M. K.-H., and Przewlocka, B. (2003). Antagonists of the kappa-opioid receptor enhance allodynia in rats and mice after sciatic nerve ligation. *Br. J. Pharmacol.* 140, 538–546. doi: 10.1038/sj.bjp.0705427

O'Connor, A. B. (2009). Neuropathic pain: quality-of-life impact, costs and cost effectiveness of therapy. 27, 95–112. doi: 10.2165/00019053-200927020-00002

Omelchenko, N., and Sesack, S. R. (2005). Laterodorsal tegmental projections to identified cell populations in the rat ventral tegmental area. *J. Comp. Neurol.* 483, 217–235. doi: 10.1002/cne.20417

Omelchenko, N., and Sesack, S. R. (2010). Periaqueductal gray afferents synapse onto dopamine and GABA neurons in the rat ventral tegmental area. *J. Neurosci. Res.* 88, 981–991. doi: 10.1002/jnr.22265

Ozaki, S., Narita, M., Narita, M., Iino, M., Sugita, J., Matsumura, Y., et al. (2002). Suppression of the morphine-induced rewarding effect in the rat with neuropathic pain: implication of the reduction in mu-opioid receptor functions in the ventral tegmental area. *J. Neurochem.* 82, 1192–1198. doi: 10.1046/j.1471-4159.2002.01071.x

Pais-Vieira, M., Mendes-Pinto, M. M., Lima, D., and Galhardo, V. (2009). Cognitive impairment of prefrontal-dependent decision-making in rats after the onset of chronic pain. *Neuroscience* 161, 671–679. doi: 10.1016/j.neuroscience.2009.04.011

Pan, Z. Z. (1998). mu-Opposing actions of the kappa-opioid receptor. *Trends Pharmacol. Sci.* 19, 94–98. doi: 10.1016/S0165-6147(98)01169-9

Peciña, S., Smith, K. S., and Berridge, K. C. (2006). Hedonic hot spots in the brain. *Neuroscientist* 12, 500–511. doi: 10.1177/1073858406293154

Peckys, D., and Landwehrmeyer, G. B. (1999). Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a ^{33}P *in situ* hybridization study. *Neuroscience* 88, 1093–1135. doi: 10.1016/S0306-4522(98)00251-6

Pfeiffer, A., Brantl, V., Herz, A., and Emrich, H. M. (1986). Psychotomimesis mediated by kappa opiate receptors. *Science* 233, 774–776. doi: 10.1126/science.3016896

Pliakas, A. M., Carlson, R. R., Neve, R. L., Konradi, C., Nestler, E. J., and Carlezon, W. A. (2001). Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J. Neurosci.* 21, 7397–7403.

Poulin, J.-F., Arbour, D., Laforest, S., and Drolet, G. (2009). Neuroanatomical characterization of endogenous opioids in the bed nucleus of the stria terminalis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 1356–1365. doi: 10.1016/j.pnpbp.2009.06.021

Pradhan, A. A., Befort, K., Nozaki, C., Gavériaux-Ruff, C., and Kieffer, B. L. (2011). The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol. Sci.* 32, 581–590. doi: 10.1016/j.tips.2011.06.008

Raghavendra, V., Tanga, F., and DeLeo, J. A. (2003). Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J. Pharmacol. Exp. Ther.* 306, 624–630. doi: 10.1124/jpet.103.052407

Reinscheid, R. K., Nothacker, H. P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., et al. (1995). Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 270, 792–794. doi: 10.1126/science.270.5237.792

Richard, J. M., Castro, D. C., Difeliceantonio, A. G., Robinson, M. J. F., and Berridge, K. C. (2013). Mapping brain circuits of reward and motivation: in the footsteps of Ann Kelley. *Neurosci. Biobehav. Rev.* 37, 1919–1931. doi: 10.1016/j.neubiorev.2012.12.008

Rives, M.-L., Rossillo, M., Liu-Chen, L.-Y., and Javitch, J. A. (2012). 6′-Guanidinonaltrindole (6′-GNTI) is a G protein-biased κ-opioid receptor agonist that inhibits arrestin recruitment. *J. Biol. Chem.* 287, 27050–27054. doi: 10.1074/jbc.C112.387332

Rosenblum, A., Joseph, H., Fong, C., Kipnis, S., Cleland, C., and Portenoy, R. K. (2003). Prevalence and characteristics of chronic pain among chemically dependent patients in methadone maintenance and residential treatment facilities. *JAMA* 289, 2370–2378. doi: 10.1001/jama.289.18.2370

Roth, B. L., Baner, K., Westkaemper, R., Siebert, D., Rice, K. C., Steinberg, S., et al. (2002). Salvinorin A: a potent naturally occurring nonnitrogenous kappa opioid selective agonist. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11934–11939. doi: 10.1073/pnas.182234399

Roughan, J. V., Coulter, C. A., Flecknell, P. A., Thomas, H. D., and Sufka, K. J. (2014). The conditioned place preference test for assessing welfare consequences and potential refinements in a mouse bladder cancer model. *PLoS ONE* 9:e103362. doi: 10.1371/journal.pone.0103362

Rouwette, T., Vanelderen, P., de Reus, M., Loohuis, N. O., Giele, J., van Egmond, J., et al. (2012). Experimental neuropathy increases limbic forebrain CRF. *Eur. J. Pain* 16, 61–71. doi: 10.1016/j.ejpain.2011.05.016

Ruzicka, B. B., Fox, C. A., Thompson, R. C., Meng, F., Watson, S. J., and Akil, H. (1995). Primary astroglial cultures derived from several rat brain regions differentially express mu, delta and kappa opioid receptor mRNA. *Brain Res. Mol. Brain Res.* 34, 209–220. doi: 10.1016/0169-328X(95)00165-O

Saitoh, A., and Yamada, M. (2012). Antidepressant-like effects of δ opioid receptor agonists in animal models. *Curr. Neuropharmacol.* 10, 231–238. doi: 10.2174/157015912803217314

Sartorius, A., and Henn, F. A. (2007). Deep brain stimulation of the lateral habenula in treatment resistant major depression. *Med. Hypotheses* 69, 1305–1308. doi: 10.1016/j.mehy.2007.03.021

Sartorius, A., Kiening, K. L., Kirsch, P., von Gall, C. C., Haberkorn, U., Unterberg, A. W., et al. (2010). Remission of major depression under deep brain stimulation of the lateral habenula in a therapy-refractory patient. *Biol. Psychiatry* 67, e9–e11. doi: 10.1016/j.biopsych.2009.08.027

Schindler, A. G., Messinger, D. I., Smith, J. S., Shankar, H., Gustin, R. M., Schattauer, S. S., et al. (2012). Stress produces aversion and potentiates cocaine reward by releasing endogenous dynorphins in the ventral striatum to locally stimulate serotonin reuptake. *J. Neurosci.* 32, 17582–17596. doi: 10.1523/JNEUROSCI.3220-12.2012

Schultz, W. (2007). Multiple dopamine functions at different time courses. *Annu. Rev. Neurosci.* 30, 259–288. doi: 10.1146/annurev.neuro.28.061604.135722

Schwartz, N., Temkin, P., Jurado, S., Lim, B. K., Heifets, B. D., Polepalli, J. S., et al. (2014). Decreased motivation during chronic pain requires long-term depression in the nucleus accumbens. *Science* 345, 535–542. doi: 10.1126/science.1253994

Scott, D. J., Heitzeg, M. M., Koeppe, R. A., Stohler, C. S., and Zubieta, J.-K. (2006). Variations in the human pain stress experience mediated by ventral and dorsal basal ganglia dopamine activity. *J. Neurosci.* 26, 10789–10795. doi: 10.1523/JNEUROSCI.2577-06.2006

Scott, D. J., Stohler, C. S., Egnatuk, C. M., Wang, H., Koeppe, R. A., and Zubieta, J.-K. (2008). Placebo and nocebo effects are defined by opposite opioid and dopaminergic responses. *Arch. Gen. Psychiatry* 65, 220–231. doi: 10.1001/archgenpsychiatry.2007.34

Shelton, L., Becerra, L., and Borsook, D. (2012). Unmasking the mysteries of the habenula in pain and analgesia. *Prog. Neurobiol.* 96, 208–219. doi: 10.1016/j.pneurobio.2012.01.004

Sherwood, T. W., and Askwith, C. C. (2009). Dynorphin opioid peptides enhance acid-sensing ion channel 1a activity and acidosis-induced neuronal death. *J. Neurosci.* 29, 14371–14380. doi: 10.1523/JNEUROSCI.2186-09.2009

Shippenberg, T. S., Bals-Kubik, R., and Herz, A. (1993). Examination of the neurochemical substrates mediating the motivational effects of opioids: role of the mesolimbic dopamine system and D-1 vs. D-2 dopamine receptors. *J. Pharmacol. Exp. Ther.* 265, 53–59.

Shippenberg, T. S., and Herz, A. (1987). Place preference conditioning reveals the involvement of D1-dopamine receptors in the motivational properties of μ- and κ-opioid agonists. *Brain Res.* 436, 169–172. doi: 10.1016/0006-8993(87)91571-X

Shirayama, Y., Ishida, H., Iwata, M., Hazama, G.-I., Kawahara, R., and Duman, R. S. (2004). Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. *J. Neurochem.* 90, 1258–1268. doi: 10.1111/j.1471-4159.2004.02589.x

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

Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., et al. (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J.* 17, 886–897. doi: 10.1093/emboj/17.4.886

Solecki, W., Ziolkowska, B., Krowka, T., Gieryk, A., Filip, M., and Przewlocki, R. (2009). Alterations of prodynorphin gene expression in the rat mesocorticolimbic system during heroin self-administration. *Brain Res.* 1255, 113–121. doi: 10.1016/j.brainres.2008.12.002

Spetea, M., Rydelius, G., Nylander, I., Ahmed, M., Bileviciute-Ljungar, I., Lundeberg, T., et al. (2002). Alteration in endogenous opioid systems due to chronic inflammatory pain conditions. *Eur. J. Pharmacol.* 435, 245–252. doi: 10.1016/S0014-2999(01)01554-0

Stamatakis, A. M., and Stuber, G. D. (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nat. Neurosci.* 15, 1105–1107. doi: 10.1038/nn.3145

Stevenson, C. W., Sullivan, R. M., and Gratton, A. (2003). Effects of basolateral amygdala dopamine depletion on the nucleus accumbens and medial prefrontal cortical dopamine responses to stress. *Neuroscience* 116, 285–293. doi: 10.1016/S0306-4522(02)00553-5

Stevenson, G. W., Folk, J. E., Rice, K. C., and Negus, S. S. (2005). Interactions between delta and mu opioid agonists in assays of schedule-controlled responding, thermal nociception, drug self-administration, and drug versus food choice in rhesus monkeys: studies with SNC80 [(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N,N*-diethylbenzamide] and heroin. *J. Pharmacol. Exp. Ther.* 314, 221–231. doi: 10.1124/jpet.104.082685

Stiene-Martin, A., and Hauser, K. F. (1991). Glial growth is regulated by agonists selective for multiple opioid receptor types in vitro. *J. Neurosci. Res.* 29, 538–548. doi: 10.1002/jnr.490290415

Stiene-Martin, A., Zhou, R., and Hauser, K. F. (1998). Regional, developmental, and cell cycle-dependent differences in μ, δ, and κ-opioid receptor expression

among cultured mouse astrocytes. *Glia* 22, 249–259. doi: 10.1002/(SICI)1098-1136(199803)22:3<249::AID-GLIA4>3.0.CO;2-0

Sumpton, J. E., and Moulin, D. E. (2014). Fibromyalgia. *Handb. Clin. Neurol.* 119, 513–527. doi: 10.1016/B978-0-7020-4086-3.00033-3

Sun, W. (2011). Dopamine neurons in the ventral tegmental area: drug-induced synaptic plasticity and its role in relapse to drug-seeking behavior. *Curr. Drug Abuse Rev.* 4, 270–285. doi: 10.2174/1874473711104040270

Tan, K. R., Yvon, C., Turiault, M., Mirzabekov, J. J., Doehner, J., Labouèbe, G., et al. (2012). GABA neurons of the VTA drive conditioned place aversion. *Neuron* 73, 1173–1183. doi: 10.1016/j.neuron.2012.02.015

Tanimoto, S., Nakagawa, T., Yamauchi, Y., Minami, M., and Satoh, M. (2003). Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. *Eur. J. Neurosci.* 18, 2343–2350. doi: 10.1046/j.1460-9568.2003.02952.x

Taylor, A. (2013). Pain and reward: how the affective-motivational system is perturbed in chronic pain. *Postdoc J.* 1, 75–88.

Taylor, A. M. W., Roberts, K. W., Pradhan, A. A., Akbari, H. A., Walwyn, W., Lutfy, K., et al. (2014). Anti-nociception mediated by a κ opioid receptor agonist is blocked by a δ receptor agonist. *Br. J. Pharmacol.* 15, 1–13. doi: 10.1111/bph.12810

Tejeda, H. A., Counotte, D. S., Oh, E., Ramamoorthy, S., Schultz-Kuszak, K. N., Bäckman, C. M., et al. (2013). Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion. *Neuropsychopharmacology* 38, 1770–1779. doi: 10.1038/npp.2013.76

Thompson, A. B., Yang, H., and Andrews, A. M. (2013). "Systemic administration of the kappa opioid receptor agonist U50,488 decreases extracellular serotonin by a reuptake-independent mechanism in mice lacking the serotonin transporter," in *Proceedings of the 43rd Annual Meeting of the Society for Neuroscience*, San Diego, CA.

Todtenkopf, M. S., Marcus, J. F., Portoghese, P. S., and Carlezon, W. A. (2004). Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats. *Psychopharmacology (Berl.)* 172, 463–470. doi: 10.1007/s00213-003-1680-y

Tracey, I. (2010). Getting the pain you expect: mechanisms of placebo, nocebo and reappraisal effects in humans. *Nat. Med.* 16, 1277–1283. doi: 10.1038/nm.2229

Ungless, M. A., Magill, P. J., and Bolam, J. P. (2004). Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. *Science* 303, 2040–2042. doi: 10.1126/science.1093360

Van't Veer, A., and Carlezon, W. A. (2013). Role of kappa-opioid receptors in stress and anxiety-related behavior. *Psychopharmacology (Berl.)* 229, 435–452. doi: 10.1007/s00213-013-3195-5

Vazquez-DeRose, J., Stauber, G., Khroyan, T. V., Xie, X. S., Zaveri, N. T., and Toll, L. (2013). Retrodialysis of N/OFQ into the nucleus accumbens shell blocks cocaine-induced increases in extracellular dopamine and locomotor activity. *Eur. J. Pharmacol.* 699, 200–206. doi: 10.1016/j.ejphar.2012.11.050

Velasquez, K. M., Molfese, D. L., and Salas, R. (2014). The role of the habenula in drug addiction. *Front. Hum. Neurosci.* 8:174. doi: 10.3389/fnhum.2014.00174

Verhaak, P. F., Kerssens, J. J., Dekker, J., Sorbi, M. J., and Bensing, J. M. (1998). Prevalence of chronic benign pain disorder among adults: a review of the literature. *Pain* 77, 231–239. doi: 10.1016/S0304-3959(98)00117-1

Volman, S. F., Lammel, S., Margolis, E. B., Kim, Y., Richard, J. M., Roitman, M. F., et al. (2013). New insights into the specificity and plasticity of reward and aversion encoding in the mesolimbic system. *J. Neurosci.* 33, 17569–17576. doi: 10.1523/JNEUROSCI.3250-13.2013

Von Korff, M., Le Resche, L., and Dworkin, S. F. (1993). First onset of common pain symptoms: a prospective study of depression as a risk factor. *Pain* 55, 251–258. doi: 10.1016/0304-3959(93)90154-H

Wadenberg, M.-L. G. (2003). A review of the properties of spiradoline: a potent and selective kappa-opioid receptor agonist. *CNS Drug Rev.* 9, 187–198. doi: 10.1111/j.1527-3458.2003.tb00248.x

Wagner, R., DeLeo, J. A., Coombs, D. W., Willenbring, S., and Fromm, C. (1993). Spinal dynorphin immunoreactivity increases bilaterally in a neuropathic pain model. *Brain Res.* 629, 323–326. doi: 10.1016/0006-8993(93)91339-T

Walker, D. L., and Davis, M. (2008). Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer. *Brain Struct. Funct.* 213, 29–42. doi: 10.1007/s00429-008-0183-3

Walker, J. M., Moises, H. C., Coy, D. H., Baldrighi, G., and Akil, H. (1982). Nonopiate effects of dynorphin and des-Tyr-dynorphin. *Science* 218, 1136–1138. doi: 10.1126/science.6128791

Wanat, M. J., Bonci, A., and Phillips, P. E. M. (2013). CRF acts in the midbrain to attenuate accumbens dopamine release to rewards but not their predictors. *Nat. Neurosci.* 16, 383–385. doi: 10.1038/nn.3335

Wang, Z., Gardell, L. R., Ossipov, M. H., Vanderah, T. W., Brennan, M. B., Hochgeschwender, U., et al. (2001). Pronociceptive actions of dynorphin maintain chronic neuropathic pain. *J. Neurosci.* 21, 1779–1786.

Watabe-Uchida, M., Zhu, L., Ogawa, S. K., Vamanrao, A., and Uchida, N. (2012). Whole-brain mapping of direct inputs to midbrain dopamine neurons. *Neuron* 74, 858–873. doi: 10.1016/j.neuron.2012.03.017

Wise, R. A., and Koob, G. F. (2014). The development and maintenance of drug addiction. *Neuropsychopharmacology* 39, 254–262. doi: 10.1038/npp.2013.261

Wittmann, W., Schunk, E., Rosskothen, I., Gaburro, S., Singewald, N., Herzog, H., et al. (2009). Prodynorphin-derived peptides are critical modulators of anxiety and regulate neurochemistry and corticosterone. *Neuropsychopharmacology* 34, 775–785. doi: 10.1038/npp.2008.142

Wood, P. B., Schweinhardt, P., Jaeger, E., Dagher, A., Hakyemez, H., Rabiner, E. A., et al. (2007). Fibromyalgia patients show an abnormal dopamine response to pain. *Eur. J. Neurosci.* 25, 3576–3582. doi: 10.1111/j.1460-9568.2007.05623.x

Xia, Y., Driscoll, J. R., Wilbrecht, L., Margolis, E. B., Fields, H. L., and Hjelmstad, G. O. (2011). Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. *J. Neurosci.* 31, 7811–7816. doi: 10.1523/JNEUROSCI.1504-11.2011

Xie, J. Y., Qu, C., Patwardhan, A., Ossipov, M. H., Navratilova, E., Becerra, L., et al. (2014). Activation of mesocorticolimbic reward circuits for assessment of relief of ongoing pain: a potential biomarker of efficacy. *Pain* 155, 1659–1666. doi: 10.1016/j.pain.2014.05.018

Xu, M., Bruchas, M. R., Ippolito, D. L., Gendron, L., and Chavkin, C. (2007). Sciatic nerve ligation-induced proliferation of spinal cord astrocytes is mediated by kappa opioid activation of p38 mitogen-activated protein kinase. *J. Neurosci.* 27, 2570–2581. doi: 10.1523/JNEUROSCI.3728-06.2007

Xu, M., Petraschka, M., McLaughlin, J.-P., Westenbroek, R. E., Caron, M. G., Lefkowitz, R. J., et al. (2004). Neuropathic pain activates the endogenous kappa opioid system in mouse spinal cord and induces opioid receptor tolerance. *J. Neurosci.* 24, 4576–4584. doi: 10.1523/JNEUROSCI.5552-03.2004

Yalcin, I., and Barrot, M. (2014). The anxiodepressive comorbidity in chronic pain. *Curr. Opin. Anaesthesiol.* 27, 520–527. doi: 10.1097/ACO.0000000000000116

Yalcin, I., Bohren, Y., Waltisperger, E., Sage-Ciocca, D., Yin, J. C., Freund-Mercier, M.-J., et al. (2011). A time-dependent history of mood disorders in a murine model of neuropathic pain. *Biol. Psychiatry* 70, 946–953. doi: 10.1016/j.biopsych.2011.07.017

Zastawny, R. L., George, S. R., Nguyen, T., Cheng, R., Tsatsos, J., Briones-Urbina, R., et al. (1994). Cloning, characterization, and distribution of a mu-opioid receptor in rat brain. *J. Neurochem.* 62, 2099–2105. doi: 10.1046/j.1471-4159.1994.62062099.x

Zhang, Y., Butelman, E. R., Schlussman, S. D., Ho, A., and Kreek, M. J. (2005). Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at kappa opioid receptors. *Psychopharmacology (Berl.)* 179, 551–558. doi: 10.1007/s00213-004-2087-0

Zhou, L., Lovell, K. M., Frankowski, K. J., Slauson, S. R., Phillips, A. M., Streicher, J. M., et al. (2013). Development of functionally selective, small molecule agonists at kappa opioid receptors. *J. Biol. Chem.* 288, 36703–36716. doi: 10.1074/jbc.M113.504381

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Opioid receptor desensitization: mechanisms and its link to tolerance

***Stéphane Allouche*[1], *Florence Noble*[2,3,4] *and Nicolas Marie*[2,3,4]*

[1] Laboratoire de Signalisation, Électrophysiologie et Imagerie des Lésions D'ischémie-Reperfusion Myocardique, Université de Caen, UPRES EA 4650, IFR 146 ICORE, Caen, France
[2] Centre National de la Recherche Scientifique, ERL 3649, Paris, France
[3] Institut National de la Santé et de la Recherche Médicale, UMR-S 1124, Paris, France
[4] Université Paris Descartes, Neuroplasticité et Thérapies des Addictions, Paris, France

Edited by:
Dominique Massotte, Institut des Neurosciences Cellulaires et Intégratives, France

Reviewed by:
Chris Bailey, University of Bath, UK
Seksiri Arttamangkul, Oregon Health and Science University, USA
Catherine Mollereau, Centre National de la Recherche Scientifique, France

****Correspondence:***
Nicolas Marie, CNRS ERL 3649 ≪ Neuroplasticité et Thérapies des Addictions≫ , INSERM UMR-S 1124, Faculté de Pharmacie, 4, Avenue de l'Observatoire, 75006 Paris, France
e-mail: nicolas.marie@parisdescartes.fr

Opioid receptors (OR) are part of the class A of G-protein coupled receptors and the target of the opiates, the most powerful analgesic molecules used in clinic. During a protracted use, a tolerance to analgesic effect develops resulting in a reduction of the effectiveness. So understanding mechanisms of tolerance is a great challenge and may help to find new strategies to tackle this side effect. This review will summarize receptor-related mechanisms that could underlie tolerance especially receptor desensitization. We will focus on the latest data obtained on molecular mechanisms involved in opioid receptor desensitization: phosphorylation, receptor uncoupling, internalization, and post-endocytic fate of the receptor.

Keywords: opioid receptors, desensitization, tolerance mechanisms, biased signaling, receptor trafficking

INTRODUCTION

Opioids are the most potent drugs used for pain relief. However, their therapeutic potential could be limited as a protracted use will lead to tolerance to analgesic effects requiring escalating doses that is associated with side effects such as respiratory depression. A huge work has been devoted to decipher molecular mechanisms of tolerance. It is now well-established that opioid receptors (OR) desensitization and its molecular mechanisms are intimately connected to this phenomenon. Since the beginning of the 1980's when the parallel between tolerance and desensitization has been evoked, many studies came out on the molecular mechanisms underlying OR desensitization. The number of publications related to OR desensitization increased dramatically with the cloning of the opioid receptor 10 years later. In this review, we made an effort to summarize a large amount of these data and point out conflicting results by discussing about the initial conditions (cell models, agonist treatments…). We also integrated the latest developments obtained on the role of receptor trafficking in desensitization and tolerance and the concept of biased agonism.

STRUCTURE AND FUNCTION OF OPIOID RECEPTORS

DIFFERENT TYPES OF OPIOID RECEPTOR

The idea that opiate narcotic analgesics must bind to specific sites or opiate receptors, in the central nervous system and elsewhere, in order to elicit pharmacological responses dates back for half a century. It was based on the finding that there are important structural and steric constraints on most of the actions of opiates. Thus, Beckett and Casy (1954), and Portoghese (1965) postulated the existence of multiple OR based on the relationship between molecular structure of opiate drugs and their analgesic activity. Opioid-binding sites in the central nervous system were demonstrated in mammalian brain tissue in the 1970s by using radioligand-binding assays on isolated brain tissue (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973), followed by the characterization of endogenous opioid peptides (Hughes et al., 1975; Cox et al., 1976; Guillemin et al., 1977; Goldstein et al., 1981). The endogenous opioid system, whose involvement in different physiological functions has been recently reviewed (Bodnar, 2014), consists of four distinct neuronal systems that are widely distributed throughout the CNS and peripheral organs. To date, four OR have been cloned, the mu, kappa, delta and nociceptin/orphanin FQ receptor (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993a,b; Meng et al., 1993; Thompson et al., 1993; Fukuda et al., 1994; Mollereau et al., 1994). This latter, despite its sequence homology with the first three ones, poorly binds peptide and alkaloid opioid ligands (Mollereau et al., 1994; Reinscheid et al., 1995). So, only data on mu (MOR), delta (DOR), and kappa (KOR) OR will be included in this review. The endogenous opioid peptides are generated from four precursors: proopiomelanocortin, proenkephalin, prodynorphin, and pronociceptin/orphanin FQ (Nakanishi et al., 1979; Kakidani et al., 1982; Noda et al., 1982; Meunier et al., 1995; Reinscheid et al., 1995), each generating biologically active peptides that are

released at the synaptic terminals of opioidergic neurons. These peptides exert their physiological actions by interacting with the various classes of OR present on both pre- and post-synaptic membranes of opioid and opioid target neurons (Besse et al., 1990).

Receptor subtypes of mu, delta and kappa OR have been proposed from the pharmacological *in vitro* and *in vivo* studies, but at present there is no molecular evidence to account for a further subclassification. Only one molecular entity for each receptor has been cloned from a given species (Knapp et al., 1995; Dhawan et al., 1996), although functional splice variants of MOR have been discovered (Abbadie et al., 2004; Pasternak et al., 2004; Pan et al., 2005; Pasternak and Pan, 2013). Recent explanations, not mutually exclusives, regarding the diversity of pharmacological responses following activation of a single target, have emerged with the identification of OR heterodimers that appear to have properties different from the monomeric receptors (Fujita et al., 2014; Massotte, 2014; Ong and Cahill, 2014) and the notion of biased agonism (see this review and Violin et al., 2014).

STRUCTURE

Opioid receptors belong to the class A of G protein-coupled receptors (GPCR) which share some common features. They possess seven transmembrane domains linked by three intracellular and three extracellular loops, an extracellular amino-terminus and an intracytoplasmic C-terminus tail. The amino-terminus region has putative glycosylation sites. Whereas O- and N-glycosylation seems to be important for DOR maturation and export to plasma membrane (Petaja-Repo et al., 2000), N-glycosylation of MOR doesn't affect its function (Befort et al., 2001; Rostami et al., 2010). The transmembrane domains are composed of a strong proportion of hydrophobic amino-acids organized in alpha helix and demonstrate the highest sequence homology between the three OR (around 70%) (Mollereau et al., 1994). These domains contain cysteine residues that might be important for ligand binding for MOR (Gioannini et al., 1999) but not for DOR (Ehrlich et al., 1998). The three extracellular loops (most divergent in sequence), including the first two ones linked by a disulfide bond would participate in ligand binding (Metzger and Ferguson, 1995). The three intracellular loops would be more involved in G protein interaction (Metzger and Ferguson, 1995; Georgoussi et al., 1997; Megaritis et al., 2000). The carboxy-terminus tail has a low sequence homology between the three OR. It contains putative phosphorylation sites (Ser, Thr, and Tyr) involved in regulation events after ligand binding and a conserved cysteine residue. This latter could be involved in receptor palmitoylation, a reversible post-translational modification that could regulate DOR surface expression for instance (Petaja-Repo et al., 2006). However, in MOR, mutation of the two Cys residues does not affect palmitoylation (Chen et al., 1998).

In the last few months, an important breakthrough has been made with the crystal structures of MOR (Manglik et al., 2012), DOR (Granier et al., 2012), and KOR (Wu et al., 2012) at high resolution. The results obtained by these studies confirmed some previously discovered important characteristics of OR. Pharmacology of OR has been described with the message/address model: the ligand is composed of two parts, one carrying the activity (agonist or antagonist) at the different subtypes of OR, the "message" and one part, the "address," conveying selectivity toward a given OR (Portoghese et al., 1990). For the opioid peptides, enkephalins, dynorphins and endorphins, the N-terminal tyrosine residue may be considered as the common message and the C-terminal domain presents the variable address. The deep binding pocket responsible for the "message" recognition is conserved between the different OR subtype, whereas the distal binding site responsible for the "address" recognition is divergent (Metzger and Ferguson, 1995; Granier et al., 2012; Manglik et al., 2012; Filizola and Devi, 2013). For instance, the indole group of naltrindole, carrying the selectivity toward DOR, interacts with the Leu7.35 residue. In the MOR, this amino-acid is replaced by a Trp, preventing naltrindole binding by steric hindrance (Granier et al., 2012; Manglik et al., 2012). Interestingly, MOR crystallized in two-fold symmetrical dimer (Manglik et al., 2012) whereas KOR (Wu et al., 2012) and DOR (Granier et al., 2012) were also shown to adopt anti-parallel arrangements. While those data reinforce the existence of OR dimers (Massotte, 2014), one should keep in mind that the non-physiological conditions (i.e., detergents and modified receptors) used for such crystallographic studies could generate artifactual interactions.

SIGNALING AND BIASED AGONISM

OR are mainly coupled to pertussis toxin-sensitive heterotrimeric $G_{\alpha i/o}$ proteins and to a lesser extent to G_z (Law et al., 2000). G_α and $G_{\beta\gamma}$ dimer activate numerous intracellular effectors. The most studied effector is the adenylyl cyclase (ACase) and investigations on OR coupling demonstrated that stimulation of MOR, DOR, and KOR in cellular models or *ex vivo* inhibited ACase mainly via $G_{i/o}$ proteins (Dhawan et al., 1996; Bian et al., 2012). One of the fastest responses obtained after OR activation is the regulation of certain types of ionic channels such as the inhibition of voltage-dependent Ca^{2+} channels or activation of potassium channels such as GIRK (G protein-coupled inwardly rectifying K^+ channels) (Law et al., 2000). Activation of K^+ channels mediates neuronal membrane hyperpolarization and reduces hyperexcitability. The inhibition of voltage-dependent Ca^{2+} channel blocks neurotransmitters release. These two phenomena participate to reduce nociception mediated by OR. OR also activate phospholipase C and mitogen-activated protein (MAP) kinases pathways (Law et al., 2000).

Recently, a new notion has emerged from pharmacological studies of GPCR, called biased agonism or functional selectivity. The binding of different ligands of a single receptor results in distinct conformational changes of receptor; each conformation preferentially interacts with selective partners producing specific signaling cascades (Kenakin, 2011). One could trace back the first data on biased agonism for OR when some authors demonstrated that different ligands for the same OR activate different subsets of $G\alpha_{i/o}$ proteins (Allouche et al., 1999a). Recently, Morse and colleagues revealed a functional selectivity using a large panel of opioid ligands by the label-free dynamic mass redistribution technology which is based on the detection of refractive index alterations measured by biosensor-coated microplates (Morse et al., 2013); this suggests that opioid ligands are able to promote different conformational changes of OR. Many studies have demonstrated the existence of a biased agonism for OR at

different signaling events including desensitization, phosphorylation, endocytosis, trafficking, and *in vivo* effects (see below) (Raehal et al., 2011; Pradhan et al., 2012; Kelly, 2013).

IN VIVO FUNCTION

The anatomical localization of OR in the brain and peripheral tissues has been clearly established using autoradiographic methods with selective radiolabeled ligands and detection of OR transcripts using *in situ* hybridization (Mansour et al., 1995; Dhawan et al., 1996). The different OR are widely distributed throughout the central nervous system that explains the large pharmacological responses observed following administration of opioid agonists.

The highest density of MOR is found in the caudate and putamen, where they exhibit a typical patchy distribution in the rat. High levels of MOR are observed in the cortex, thalamus, nucleus accumbens, hippocampus, and amygdala. Moderate levels are found in the periaqueductal gray matter and raphe nuclei, and low concentrations are seen in the hypothalamus, preoptic area, and globus pallidus (Quirion et al., 1983). MOR are also present in the superficial layers of the dorsal horn of the spinal cord (Besse et al., 1990). This large distribution in both spinal and supraspinal structures, as well as at periphery, shows that MOR play an important role in the control of nociception, in good agreement with the pharmacological studies demonstrating that mu selective agonists are potent antinociceptive drugs. Numerous other physiological functions appear to be controlled by MOR. These include reward, respiration, cardiovascular functions, bowel transit, feeding, learning and memory, locomotor activity, thermoregulation, hormone secretion, and immune functions (Dhawan et al., 1996; Kieffer, 1999; Bodnar, 2014).

The distribution of KOR demonstrates some of the most striking species differences among the OR types. In the rat, they represent only approximately 10% of the total number of OR, while in most other species (guinea pig, monkey, and human) they represent at least a third of the opioid binding population (Dhawan et al., 1996). KOR have been found to be widely distributed throughout the forebrain, midbrain, and brainstem. They are implicated in the regulation of several functions, including nociception, diuresis, mood, feeding, and neuroendocrine secretions (Tejeda et al., 2012; Bodnar, 2014).

Compared to MOR and KOR, DOR are more restricted in their distribution and are densest in forebrain regions, well-conserved across mammalian species. Dense binding is observed in the caudate, putamen, cerebral cortex, and amygdala, while they are generally sparse to inexistent in thalamus and hypothalamus. They play a role in different functions: nociception, locomotor activity, gastro-intestinal motility, olfaction, cognitive function, and mood driven behavior (Dhawan et al., 1996; Gaveriaux-Ruff and Kieffer, 2002; Bodnar, 2014).

DESENSITIZATION

Chronic opioid use leads to tolerance, defined as a decrease of the drug response. It's possible to reproduce *in vitro* such phenomenon when cellular models expressing OR are exposed to agonists; in that situation, a decrease of signaling is observed and is designated as OR desensitization. Some reports distinguish the OR desensitization from the cellular tolerance. When rats are chronically exposed to morphine, examination of MOR activity on the outward potassium current shows a reduction compared to naive animals which is not reversible even after 6 h in free-morphine medium; this is cellular tolerance (Levitt and Williams, 2012). In contrast, desensitization may be defined as a reduction of signal transduction from OR after acute activation by agonists that recovers when cells or tissues are placed in agonist-free medium. The first works studying the molecular mechanisms underlying OR desensitization were reported more than 30 years ago (Gahwiler, 1981; Law et al., 1982).

Initially, studying desensitization was made possible by using experimental models endogenously expressing OR such as brain membranes, rabbit cerebellum or cell lines (NG 108-15, SH-SY5Y, SK-N-SH, SK-N-BE. . .). Since the cloning of the first OR, those models have been superseded by heterologous expression systems (HEK, CHO, COS-7, *Xenopus laevis* oocyte) in which OR are easily expressed in large amount but whose cellular characteristics are far from neurons in which OR are endogenously expressed.

Desensitization of OR is studied on different signaling pathways including ACase inhibition, activation of MAP kinases, inhibition of voltage-gated calcium channels and activation of GIRK channels. Desensitization is sometimes evaluated by measuring the ability of OR to activate G proteins in [^{35}S]GTPγS binding experiments after opioid agonists exposure. In absence of modification on the downstream signaling pathway, G protein uncoupling is a good marker for desensitization but can't be applied for G protein-independent pathways (i.e., MAP kinases). The comparison between desensitization studies suffers also from the various experimental conditions used. Cellular model, agonist, agonist concentration, time of exposure, level of OR expression or signaling pathway studied are among the different parameters that could influence OR desensitization as previously reviewed (Connor et al., 2004).

DEFINITION

As indicated above, desensitization is defined as a progressive reduction of signal transduction that occurs more or less rapidly after OR activation depending on the agonist and the signaling pathway. The rapid desensitization is mainly observed on the regulation of ion channel conductance from sec to several minutes while a sustained desensitization is rather observed on regulation of enzymes (ACase, MAP kinases) after minutes to several tens of minutes. However, in this latter case, other counter-regulatory mechanisms (internalization, traffic of OR) could participate to desensitization making its description complex. Molecular mechanisms turned out to be complicated for several reasons:

– A single OR can activate simultaneously different signaling pathways such ACase, MAP kinases or ion channels and it is possible to observe different levels of desensitization when considering those cellular responses. For instance, we recently showed that remifentanil, a MOR selective agonist, produces a significant desensitization by 60% on the cAMP pathway after 10 min while at the same time desensitization of the MAP kinases ERK1/2 signaling pathway was not significantly affected (Nowoczyn et al., 2013).

– Two types of desensitization, homologous and heterologous, were described. In homologous desensitization, only agonist-activated receptors are desensitized while in heterologous desensitization, both agonist-activated and non-activated receptors sharing the same signaling pathways are inactivated. Those types of desensitization are related to different mechanisms especially in terms of receptor phosphorylation and kinases (Chu et al., 2010). Cross-desensitization between OR and other GPCRs is not systematically investigated and when it is, the level of desensitization between GPCRs using the same signaling pathway can be different (Namir et al., 1997). Recently, Xu et al. showed a cross-desensitization between the dopamine D1 receptors and DOR. This heterologous desensitization characterized by an uncoupling of G proteins from DOR is neither associated with modifications in receptor number nor in their phosphorylation but involves several kinases [cAMP-dependent protein kinase (PKA), MAP kinases/ERK kinase 1 (MEK1) and phosphoinositide-3 kinase (PI3K)] that could phosphorylate signaling proteins (Xu et al., 2013).
– Desensitization results from several regulatory mechanisms of signal transduction and depends on the number of active receptors at the cell surface, the efficiency of OR/G proteins coupling and the post-endocytic traffic. Recently, desensitization of MOR expressed in the neurons from locus coeruleus was demonstrated to result from a decrease of both number of active receptors and the affinity of residual receptors for the agonist (Williams, 2014).

This part will discuss recent data from literature regarding desensitization of the different OR: the impact of the agonist used through the notion of biased agonism, the role of phosphorylation and consequently the kinases involved, the implication of arrestins and OR internalization and their fate after endocytosis. Regarding MOR, a recent review has been published concerning the molecular mechanisms involved in its regulation (Williams et al., 2013).

EFFECT OF BIASED AGONISM ON OR DESENSITIZATION

The first reports describing a differential desensitization of MOR, DOR, and KOR by various agonists came from Reisine's group (Blake et al., 1997a,b; Bot et al., 1997) suggesting that biased agonism could influence desensitization; but at that time this concept was not established yet. Few studies have been designed to evaluate the impact of biased agonism on OR desensitization. They would require determination of the relationship between agonist concentration and the response from a large panel of ligands. More generally, the comparison of the ability of two ligands to promote OR desensitization is realized using the same concentration regardless their intrinsic efficacy.

Biased agonism at MOR and desensitization

Functional studies revealed that [D-Ala^2-MePhe^4-Gly^5-ol] enkephalin (DAMGO) induced a stronger desensitization of MOR than morphine in different experimental models and signaling pathways (Yu et al., 1997; Whistler and Von Zastrow, 1998; Koch et al., 2001; Blanchet et al., 2003; Bailey et al., 2009). However, such difference was not reported by others (Liu and Prather, 2001; Borgland et al., 2003; Schulz et al., 2004). In contrast, morphine was demonstrated to promote a stronger MOR desensitization than DAMGO on the increase of intracellular [Ca^{2+}] (Chu et al., 2010). In another model, the human neuroblastoma SH-SY5Y, it is possible to observe a huge difference in MOR desensitization produced by morphine and remifentanil on the cAMP pathway but not on the MAP kinases ERK1/2 (Nowoczyn et al., 2013). All those discrepancies could be due to the different level of OR expression, the cellular models and the existence of spare receptors as previously mentioned (Connor et al., 2004).

Biased agonism at DOR and desensitization

Evidence for a different DOR regulation by methadone and morphine was also reported; a pretreatment with methadone but not with morphine produced a cross-desensitization with [D-Ala^2, D-Leu^5]-enkephalin (DADLE) and morphine (Liu et al., 1999a). Similar data were reported by Bot et al. (1997). In our laboratory, we also showed a differential regulation of human DOR (hDOR) on both the inhibition of ACase and the phosphorylation of ERK1/2 in the SK-N-BE cells. Initially, we suggested that peptidic opioid agonists such as [D-Pen^2-D-Pen^5]-enkephalin (DPDPE) and deltorphin I (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) induced a stronger and faster desensitization compared to the alkaloid agonist etorphine (Allouche et al., 1999b). However, using other peptidic ([Leu^5]- and [Met^5]-enkephalins and UFP-512 ([H-Dmt-Tic-NH-CH(CH_2-COOH)-Bid])) and non-peptidic (SNC-80 ((+)-4-[(alpha R)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethyl-benzamide) and ARM-390) ligands we didn't confirm such assumption but our data rather suggest that DOR selective agonists promote profound desensitization compared to non-selective ligands (Marie et al., 2003a; Lecoq et al., 2004; Aguila et al., 2007).

Biased agonism at KOR and desensitization

Very few studies examined the regulation of KOR by different agonists. The group of Pei showed that desensitization of KOR-mediated extracellular acidification response was greater upon dynorphin A (1-13) stimulation than U69,593 ((+)-(5α, 7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide) and etorphine (Ling et al., 1998). On the cAMP pathway, U50,488 (trans-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide) and dynorphin A (1-17) produced a greater KOR desensitization than etorphine or levorphanol (Blake et al., 1997b).

With respect to desensitization, all those data support the idea that agonists are able to promote a different regulation of OR as demonstrated for other GPCR such as the histamine H2 receptors (Alonso et al., 2014). Such differential desensitization demonstrated for each OR by different agonists is probably related to the set of different regulatory molecular mechanisms (see above).

MECHANISMS OF OR DESENSITIZATION

OR phosphorylation

Numerous studies have been carried out to demonstrate the role of OR phosphorylation in desensitization by using chemical

inhibitors of kinases, *in vitro* or *in vivo* knock-out (KO) of kinases using siRNA or transgenic mice, over-expression of dominant negative mutants of kinases, amino acid substitution or truncation on OR. While in some studies the phosphorylation state of OR is clearly determined, in most of them and especially those using kinase inhibitors this major information is lacking. All those data are summarized in **Figures 1A–C**.

MOR phosphorylation. Using metabolic labeling with [^{32}P] and different mutants at the C terminal tail, the group of Law demonstrated that rat MOR (rMOR) displays a basal phosphorylation at S363 and T370 and DAMGO increases phosphorylation at T370 and S375 (El Kouhen et al., 2001). Those results were recently confirmed using specific antibodies directed against the phospho-S363, phospho-T370 and phospho-S375 (Doll et al., 2011). As demonstrated for the DOR (see below), agonist-induced MOR phosphorylation is carried out hierarchically with first of all the S375, considered as the major phosphorylation site, followed by T370 (El Kouhen et al., 2001). Morphine was also shown to increase S375 [or S377 for the human MOR (hMOR)] phosphorylation (Nowoczyn et al., 2013) but failed to phosphorylate T370 (Doll et al., 2011). Recently, Just and collaborators showed that MOR is sequentially phosphorylated at S375, T370, T379, and T376 by DAMGO. Interestingly, low concentrations of this opioid agonist rather promote phosphorylation at S375 and T379 while a strong phosphorylation of T370 and S375 is observed at higher concentrations (Just et al., 2013).

Phosphorylation studies using liquid chromatography-mass spectrometry techniques have led to the characterization of two regions at the C terminal tail of the MOR (Lau et al., 2011): the first region (amino acid 349–365) can be mono- or bi-phosphorylated at S363 and in the cluster 354TSST357. While the basal phosphorylation of S363 is not modified by agonist exposure, morphine or DAMGO can increase phosphorylation at the cluster TSST. The second region 375STANT379 is mono- or bi-phosphorylated upon agonist exposure. Rather than qualitative differences, DAMGO and morphine were shown to induce marked quantitatively different phosphorylation increase in MOR. Using a similar experimental approach, two laboratories showed that rMOR and hMOR were phosphorylated in the absence of agonist at S363 and T370 (Moulédous et al., 2012; Chen et al., 2013). Moulédous et al. showed that DAMGO increases hMOR phosphorylation at S356, T370, S375, and T376 (Moulédous et al., 2012) while Chen et al. compared the phosphorylation mediated by DAMGO and morphine; these latter showed that both agonists increase phosphorylation at S356, T357, T370, and S375 (Chen et al., 2013).

Different kinases are involved in MOR phosphorylation. Using siRNA against various forms of the G protein-coupled receptor kinase (GRK) family, DAMGO was demonstrated to phosphorylate T370 and S375 by GRK2 and 3 while morphine increases S375 phosphorylation by GRK5 (Doll et al., 2012). In SH-SY5Y cells, hMOR phosphorylation at S377 (the equivalent of S375 for the rMOR) upon DAMGO exposure does not rely on GRK2 suggesting the implication of another kinases (Moulédous et al., 2012). *In vivo*, using KO mice for either GRK3 or 5, morphine rather promotes MOR phosphorylation at S375 by both kinases

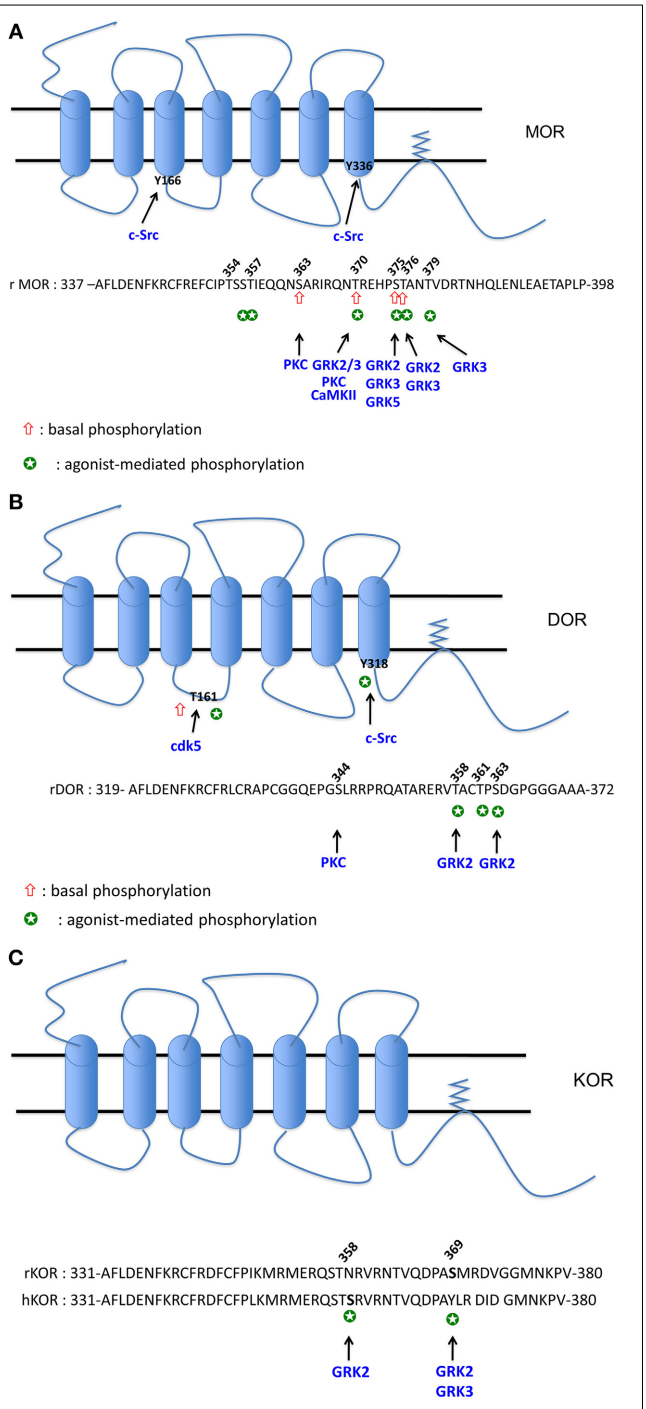

FIGURE 1 | (A) Phosphorylation sites of MOR. The cluster 354TSST357: phosphorylation both by DAMGO and morphine (Lau et al., 2011). The S356 (equivalent to Ser358 in human) is phosphorylated by DAMGO (Moulédous et al., 2012), S356 and T357 are phosphorylated both by DAMGO and morphine (Chen et al., 2013). The S363 (equivalent to S365 in human) is phosphorylated in the absence of agonist (El Kouhen et al., 2001; Lau et al., 2011; Moulédous et al., 2012; Chen et al., 2013). PKC was demonstrated to phosphorylate S363 (Chen et al., 2013; Illing et al., 2014). The T370 (equivalent to T372 in human) is phosphorylated in the absence of agonist

(Continued)

FIGURE 1 | Continued
(Moulédous et al., 2012; Chen et al., 2013). A decrease of phosphorylation level is observed upon DAMGO and 1Dme (a neuropeptide FF analog) exposure (Moulédous et al., 2012). PKC (Illing et al., 2014) and CaMKII (Chen et al., 2013) phophorylate T370. DAMGO, morphine and etonitazene increase phosphorylation at T370 (Doll et al., 2011; Lau et al., 2011). DAMGO-mediated phosphorylation at this residue is ultra-rapid (20 s) (Just et al., 2013) and involves GRK2 and 3 (Doll et al., 2012) but not PKC (Illing et al., 2014). The cluster 375STANT379 displays higher level of phosphorylation upon DAMGO compared to morphine (Lau et al., 2011). S375 or T376 (equivalent to S377 and T378 in human) are phosphorylated upon DAMGO and 1Dme (Moulédous et al., 2012), DAMGO, etonitazene, and morphine (Doll et al., 2011). S375 is considered as the major phosphorylation site as it is rapidly phosphorylated (20 s) upon DAMGO (Just et al., 2013). This agonist-mediated phosphorylation does not implicate PKC (Illing et al., 2014) but rather GRK2 (Chen et al., 2013) or GRK2 and 3 (Doll et al., 2012) upon DAMGO exposure, and GRK5 and to a lesser extent GRK3 upon morphine treatment (Doll et al., 2012). T376 (equivalent to T378 in human) is phosphorylated upon DAMGO and 1Dme (Moulédous et al., 2012), by GRK2 and 3 upon DAMGO exposure but it is considered as a late phosphorylation site (20 min) (Just et al., 2013). T379 is also phosphorylated upon DAMGO exposure after 1 min and required the GRK3 (Just et al., 2013). Y166 (Clayton et al., 2010) and Y336 (Zhang et al., 2009) are phosphorylated by Src. **(B)** Phosphorylation sites of DOR. S344 phosphorylation is mediated by a PKC but is not increased by DPDPE (Xiang et al., 2001). S358 and S363 (Guo et al., 2000; Kouhen et al., 2000) are the two major sites of phosphorylation mediated by GRK2 upon DPDPE exposure. Deltorphin II and morphine are also able to increase phosphorylation at S363 (Navratilova et al., 2005). T361 is phosphorylated by DPDPE but after S358 and S363 phosphorylation (Guo et al., 2000; Kouhen et al., 2000). T161 is phosphorylated by CDK5 in the absence and in the presence of chronic morphine exposure (Xie et al., 2009). Y318 is phosphorylated by Src upon DTLET exposure (Kramer et al., 2000b). **(C)** Phosphorylation sites of KOR. Phosphorylation of S369 (rKOR) is mediated by GRK2 (Mclaughlin et al., 2003) and 3 (Mclaughlin et al., 2004) upon U50488 exposure. In hKOR, S358 is phosphorylated by GRK2 when activated by U50488 (Li et al., 2002).

while only GRK3 was required for fentanyl-induced MOR phosphorylation (Glück et al., 2014). Using the carboxy-terminal region of MOR fused to glutathione S-transferase and purified kinases, PKC, GRK2, and calmodulin-dependent kinase II (CaMKII) were shown to phosphorylate S363, S375 and T370, respectively (Chen et al., 2013). Various PKC isoforms (PKCα, βII, γ, ε) activated by phorbol 12-myristate 13-acetate (PMA) trigger MOR phosphorylation at S363 and T370 but those kinases are not recruited upon DAMGO stimulation (Doll et al., 2011; Feng et al., 2011); those data indicate the role of PKC in the basal and heterologous phosphorylation of MOR (Illing et al., 2014).

The tyrosine kinase Src was also shown to phosphorylate MOR at Y336, located in the NPXXY motif, after sustained morphine treatment followed by naloxone (Zhang et al., 2009). The Y166, located in the DRY motif of the second intracellular loop of MOR, can be phosphorylated by Src but only upon co-activation with DAMGO and epidermal growth factor (EGF) (Clayton et al., 2010).

In summary, those studies revealed that S375 is the main phosphorylation site of MOR but agonists promote a differential and a multi-phosphorylation of this OR as recently reviewed (Mann et al., 2014).

DOR phosphorylation. Pei and colleagues were the first to demonstrate that OR could be phosphorylated upon agonist stimulation (Pei et al., 1995). They showed that DPDPE increases incorporation of [^{32}P] in a GRK-dependent manner. As shown for MOR, the group of Law showed that DOR was sequentially phosphorylated at S363, T358, and T361 upon DPDPE exposure (Kouhen et al., 2000). Those results were confirmed by another group who also demonstrated the critical role of GRK2 in DPDPE-induced phosphorylation of these residues (Guo et al., 2000; Marie et al., 2008). Deltorphin II is also able to increase S363 phosphorylation at hDOR but to a greater extent than morphine (Navratilova et al., 2005). PKC can phosphorylate DOR at S344 but is not required for DPDPE-induced DOR phosphorylation (Xiang et al., 2001). In a similar way as MOR, DOR phosphorylation of the Y318, located in the NPXXY motif, occurred upon DTLET ([D-Thr^2-Leu^5-Thr^6]enkephalin) exposure in a Src dependent manner (Kramer et al., 2000a,b). The cyclin-dependent kinase 5 (Cdk5), a proline-directed S/T kinase, was demonstrated to mediate basal and morphine-activated DOR phosphorylation at the T161 located in the second intracellular loop (Xie et al., 2009).

KOR phosphorylation. Concerning KOR phosphorylation, the data from literature are very scarce. The group of Chavkin showed that rKOR is phosphorylated *in vivo* at S369 by GRK3 upon U50,488 exposure (Mclaughlin et al., 2004) and *in vitro* by GRK2 (Mclaughlin et al., 2003). Upon global evaluation of the hKOR phosphorylation, Li et al. observed that dynorphin A (1-17) and U50,488 promote the highest phosphorylation, etorphine 50% of the maximum and levorphanol failed to induce [^{32}P] incorporation demonstrating that opioid agonists have different potencies to phosphorylate this receptor (Li et al., 2003). It is noteworthy that human and rodent KOR differ substantially in the amino acid composition in the C-terminal region; such difference could explain the absence of rKOR phosphorylation when activated by U50,488 (Li et al., 2002). In hKOR, the S358, substituted by N in the rKOR, is the major phosphorylation site mediated by the GRK2 upon U50,488 exposure.

In summary, the phosphorylation sites for each OR were mapped and showed that activation of a given receptor by different agonists results in a specific pattern involving different kinases (**Figures 1A–C**). Those data are consistent with the model of barcode established for the β-adrenergic receptor, a prototypic GPCR (Nobles et al., 2011), and could determine the selective interactions between the OR and partners such as arrestins.

Uncoupling between G proteins and OR

Any process interfering with the interaction between G proteins and OR can lead to reduction of signal transduction intensity. G protein uncoupling can be evidenced by binding studies on cellular membranes using the radiolabeled non-hydrolyzable GTP analog [^{35}S]GTPγS which binds to a G protein activated by the complex receptor-opioid agonist.

In CHO cells over-expressing hDOR, deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH2) pretreatment induces desensitization after 30 min on the ACase inhibition associated with a G protein uncoupling (Navratilova et al., 2007). In the

neuroblastoma×glioma (NG108-15) hybrid cells, morphine pretreatment failed to promote uncoupling of DOR from G proteins while methadone did (Liu et al., 1999b). Conversely, after 5 days of chronic morphine exposure, it is possible to observe a complete uncoupling between MOR and its cognate G proteins (Bohn et al., 2000). However, upon acute exposure (30 min) morphine failed to promote a reduction of [^{35}S]GTPγS binding compared to DAMGO indicating a great difference between agonists (Whistler and Von Zastrow, 1998). When expressed in the CHO cell line, the hKOR was demonstrated to undergo a time- and concentration-dependent uncoupling from G proteins but with a moderate impact on the inhibition of ACase (a two-fold increase of the EC_{50} value of the KOR agonist U50488) (Zhu et al., 1998).

Relationship between OR phosphorylation and desensitization

In most of these studies, the role of OR phosphorylation in desensitization is indirectly demonstrated by using KO mice or kinases chemical inhibitors; in such situations, we cannot rule out the phosphorylation of other signaling proteins involved in regulatory mechanisms of OR. Mutation of putative phosphorylation sites or truncation of the C terminal tail of OR have been extensively used to delineate the role of phosphorylation in desensitization. All those data are summarized in the **Table 1**.

MOR. Comparison between two truncated MOR in the C terminal tail in HEK cells over-expressing a GRK2 peptide known to block $G_{\beta\gamma}$-mediated recruitment of GRK at the plasma membrane suggest that the amino acids sequence 354TSST357 plays a major role in GRK2-mediated MOR desensitization upon DAMGO exposure (Wang, 2000). In locus coeruleus neurons morphine induced MOR desensitization, measured on K^+ current, in a PKC-dependent manner while GRK2 was required for DAMGO-induced MOR desensitization (Bailey et al., 2009). Such observations were confirmed by others on Ca^{2+} mobilization; PKC-ε was required for morphine-induced MOR desensitization but not upon etorphine, fentanyl and DAMGO (Zheng et al., 2011). Recently, in locus coeruleus neurons and using chemicals activators (phorbol-12,13-dibutyrate and phorbol-12-myristate-13-acetate) or a muscarinic agonist known to activate PKC, acute or sustained desensitization of MOR induced either by morphine or [Met^5]-enkephalin were demonstrated to differentially required PKC activity but such effects were not inhibited by the potent PKC inhibitor staurosporine (Arttamangkul et al., 2014). Those data suggest that the involvement of PKC in MOR desensitization would be cell-type specific. In the presence of DAMGO or [Met^5]-enkephalin, the molecular mechanisms involved in MOR desensitization change during brain development. In the locus coeruleus of young rats, those opioid peptides produce heterologous MOR desensitization with α2 adrenoreceptors in a GRK2-dependent manner but independently of its kinase activity; the high GRK2 expression would sequestrate $G_{\beta\gamma}$ and interfere with K^+ channels activation while in mature rats, homologous MOR desensitization would be due to receptor phosphorylation by this kinase (Llorente et al., 2012). GRK2 was also shown to mediate heterologous desensitization by promoting MOR transphosphorylation upon neuropeptide FF receptor activation (Moulédous et al., 2012). The role of phosphorylation in MOR desensitization has been challenged: using staurosporine as a broad spectrum kinase inhibitor and a GRK2-mutant mice, Arttamangkul et al. showed no modification of [Met^5]-enkephalin-induced receptor desensitization on K^+ channels in locus coeruleus neurons (Arttamangkul et al., 2012).

Table 1 | Role of kinases in OR desensitization/tolerance.

OR	Main results	References
MOR	GRK2-mediated desensitization after DAMGO exposure	Wang, 2000
	DAMGO mediates desensitization in a GRK2-dependent manner while morphine induced-desensitization in a PKC-dependent fashion	Bailey et al., 2009
	Role of PKCε in morphine- but not etorphine-, fentanyl-, and DAMGO-induced desensitization	Zheng et al., 2011
	Role of GRK2 in homologous and heterologous receptor desensitization	Llorente et al., 2012
	Role of GRK2 in heterologous desensitization between MOR and neuropeptide FF receptor	Moulédous et al., 2012
	No evidence for a role of GRK5 in the development of morphine tolerance	Glück et al., 2014
	Staurosporine and GRK inhibitors do not alter desensitization upon [Met^5]-enkephalin exposure	Arttamangkul et al., 2012
	Role of PI3Kγ in desensitization and tolerance after chronic morphine treatment	Konig et al., 2010
	Role of JNK2 in tolerance and uncoupling after chronic morphine but not fentanyl treatment	Melief et al., 2010
	Role of Src in ACase superactivation after chronic morphine treatment and naloxone addition	Zhang et al., 2009
DOR	GRK2, PKC and a tyrosine kinase are involved in desensitization of hDOR when activated by etorphine	Marie et al., 2008
	Role of GRK6 in DPDPE-mediated desensitization	Willets and Kelly, 2001
	Role of PKC in DOR desensitization upon sustained activation by DADLE and [Leu^5]-enkephalin	Yoon et al., 1998; Song and Chueh, 1999
	Role of Src in DPDPE-induced DOR desensitization	Archer-Lahlou et al., 2009; Hong et al., 2009
KOR	Expression of GRK3 or 5 alone is not sufficient to promote desensitization	Appleyard et al., 1999
	Role of GRK3 in development of U50,488 induced tolerance	Mclaughlin et al., 2004

The implication of other kinases than GRK and PKC in MOR desensitization was also investigated. The PI3Kγ was demonstrated to be involved in MOR desensitization on the inhibition of voltage-gated calcium channels induced by chronic morphine treatment (Konig et al., 2010). Using chemical inhibitor and KO mice, c-Jun amino-terminal kinase 2 (JNK2) was demonstrated to play a major role in morphine- but not fentanyl-induced G protein uncoupling (Melief et al., 2010).

Some studies were also conducted to identify the amino acids of MOR involved in desensitization. The T180A substitution abolished MOR desensitization compared to wild type but the phosphorylation state of the receptor was not evaluated (Celver et al., 2004). The S375 was shown to play a major role in MOR desensitization on the cAMP and MAP kinase pathways but only when activated by morphine but not DAMGO (Schulz et al., 2004). Activation of PKC by PMA but not DAMGO pretreatment is able to promote MOR uncoupling from G proteins which is attenuated by the S363A mutation (Feng et al., 2011); this indicates that PKC-mediated phosphorylation of S363 as well as T370 upon substance P receptor activation (Illing et al., 2014) are potentially involved in heterologous desensitization. Using the triple mutant (S363A, T370A, and S375A), Zheng et al. showed that MOR desensitization upon etorphine, fentanyl and DAMGO but not morphine was impaired indicating the different role of amino acids phosphorylation in desensitization (Zheng et al., 2011). As they also demonstrated that PKC mediated morphine-induced MOR desensitization, it can be inferred that PKC would phosphorylate MOR at other sites than S363, T370, and S375. MOR desensitization and phosphorylation at S375 produced by morphine can be modulated by other proteins such as the FK binding protein 12 which would compete with kinase at MOR (Yan et al., 2014).

While all those data indicate that MOR phosphorylation would play a crucial role in desensitization, Qiu and collaborators showed that a truncated mutant of MOR from S363 is able to undergo a similar desensitization to the wild type demonstrating that receptor phosphorylation is not an absolute prerequisite for desensitization (Qiu et al., 2003). However, phosphorylation would rather regulate MOR traffic which could indirectly impact receptor desensitization (see Relationship between OR Internalization and Desensitization).

DOR. In SK-N-BE cells, etorphine-induced hDOR desensitization is totally inhibited by using the dominant negative GRK2 mutant K220R but is only reduced when using PKC and tyrosine kinase inhibitors (Marie et al., 2008). In the NG108-15 cell line, rDOR desensitization promoted by a sustained treatment with DPDPE is mediated by GRK6 but not GRK2 as indicated above for hDOR (Willets and Kelly, 2001). The role of PKC in DADLE- and [Leu^5]-enkephalin-induced DOR desensitization was also demonstrated on the mobilization of Ca^{2+} stores (Yoon et al., 1998; Song and Chueh, 1999). Tyrosine kinases were also suggested to participate in DOR desensitization. Genistein, a broad spectrum tyrosine kinase inhibitor, inhibits hDOR desensitization promoted by DPDPE, deltorphin I, and etorphine (Marie et al., 2008). Hong and collaborators found that DPDPE promotes a tyrosine phosphorylation of DOR which would recruit and activate Src that in turn could phosphorylate and activate GRK2; this latter would then phosphorylates S363 and triggers desensitization (Hong et al., 2009). So, inhibition of Src by PP2 reduces DPDPE-induced DOR phosphorylation of S363 and desensitization on the cAMP pathway but via an indirect mechanism. The role of Src in DOR regulation was also confirmed by the group of Pineyro (Archer-Lahlou et al., 2009).

The major role of DOR phosphorylation at S363 was confirmed using the mutant receptor S363A. While deltorphin II promotes a rapid receptor phosphorylation at this amino acid and desensitization on the cAMP pathway, this latter is totally abolished in the S363A mutant (Navratilova et al., 2007). The T161 of DOR, located in the second intracellular loop and equivalent to the T180 of MOR, also plays a role in DPDPE-induced desensitization; the substitution T161A severely impairs DOR desensitization measured on GIRK channels (Lowe et al., 2002). However, those authors did not evaluate the phosphorylation at this residue. The importance of phosphorylation in DOR desensitization was challenged by the work of Qiu and colleagues who studies those processes using a DOR mutant in which all Ser/Thr residues in the C-terminus region were mutated to Ala (Qiu et al., 2007). They observed that DPDPE-induced desensitization on the inhibition of ACase was significantly delayed but not abolished. This indicates that other mechanisms than phosphorylation could contribute to receptor desensitization.

KOR. In the Xenopus oocyte expression system, examination of rKOR regulation on the activation of potassium channels revealed that over-expression of GRK3 or 5 alone did not promote a significant desensitization which requires both GRK and arrestin 3 (Appleyard et al., 1999). This was confirmed when rKOR and GRK2 were co-expressed in CHO cells; pretreatment with a high concentration of U50,488 failed to promote KOR uncoupling from G proteins (Li et al., 2002). Truncation of the C terminal tail of the receptor or the substitution S369A severely impaired U69,593-induced desensitization. These data were further confirmed when wild type and mutant rKOR were expressed in the pituitary adenoma cell line atT-20 cells (Mclaughlin et al., 2003). As indicated above, S358 is the major phosphorylation site for hKOR and the S358N substitution totally abolished U50,488-induced receptor uncoupling from G proteins (Li et al., 2002).

While most of those studies with either indirect or direct proofs indicate the role of OR phosphorylation in desensitization, some of them clearly ruled out such paradigm. This probably indicates that phosphorylation is not a prerequisite for desensitization but would accelerate such process.

Role of arrestins in OR regulation

From the canonical model of GPCR regulation by Lefkowitz, arrestins (arrestins 2 and 3 also named β-arrestins 1 and 2, respectively) play a pivotal role in receptor regulation by promoting G protein uncoupling and receptor endocytosis (Pierce et al., 2002). As expected, those proteins were also demonstrated to regulate OR functions. Indeed, over-expression of arrestin 2 induces a selective uncoupling of DOR and KOR and reduces inhibition

of ACase (Cheng et al., 1998). However, no significant impact was observed for MOR explaining the lower desensitization rate compared to DOR (Lowe et al., 2002). In recent studies using BRET (Bioluminescence Resonance Energy Transfert) or FRET (Fluorescence Resonance Energy Transfer) techniques, a large panel of opioid ligands were shown to have a different ability to both activate G proteins and recruit arrestins at MOR and DOR (Mcpherson et al., 2010; Molinari et al., 2010; Rivero et al., 2012). For instance, morphine was demonstrated to behave as a partial agonist for DOR and MOR in G protein coupling experiments while almost no interaction with arrestins was detected. This indicates that all opioid ligands do not have the same potency to promote OR desensitization.

Relationship between arrestins and OR desensitization. Genetic ablation of arrestin 3 significantly reduces MOR uncoupling from G proteins upon chronic morphine treatment (Bohn et al., 2000). Using dorsal root ganglion neurons from arrestin 3 KO mice, the role of this protein in mediating inhibitory regulation of MOR by JNK on voltage-dependent calcium channels was evidenced (Mittal et al., 2012). This report suggests that arrestin 3 and not arrestin 2 would promote MOR desensitization by interacting with JNK. However, in dorsal root ganglion neurons obtained from arrestin 3 KO mice, acute MOR desensitization elicited by DAMGO or morphine on the inhibition of voltage-gated calcium channels was not significantly different from wild type mice indicating that arrestin 3 has no major role in those conditions (Walwyn et al., 2007). Similarly, in neurons from locus coeruleus no significant role of arrestin 3 was evidenced in acute MOR desensitization upon [Met^5]-enkephalin exposure on the activation of K^+ currents (Dang et al., 2009). Yet, concomitant inhibition of arrestin 3 expression (arrestin 3 KO mice) and ERK1/2 activity by PD98059 results in reduction of MOR desensitization indicating that this process involves two independent pathways. In the Xenopus oocyte, over-expression of arrestin alone is not sufficient to increase DOR (Kovoor et al., 1999) or KOR (Appleyard et al., 1999) desensitization while in HEK cells, this over-expression enables morphine-induced MOR desensitization probably by increasing both G protein uncoupling and receptor internalization (Whistler and Von Zastrow, 1998). However, such potentiation could be obtained either when arrestin and a GRK are co-expressed or when the constitutive active arrestin mutant R169E is present. This suggests that OR phosphorylation is a pre-requisite for arrestin action. This conclusion is in good agreement with the data obtained by Johnson et al. on MOR desensitization (Johnson et al., 2006). The translocation of arrestin-2-GFP from cytosol to plasma membrane is only observed upon DAMGO exposure which promotes MOR phosphorylation by GRK2. In contrast, no such translocation could be detected in morphine-treated cells which produce a PKC-dependent MOR desensitization. The use of mouse embryonic fibroblast (MEF) from single or double KO mice for arrestins 2 and 3 revealed that DOR desensitization induced by DPDPE relies predominantly on arrestin 3 expression suggesting a preferential interaction between DOR and this arrestin isoform (Qiu et al., 2007). In the SK-N-BE cells, DOR desensitization is reduced when arrestin 2 expression is inhibited by shRNA only upon DPDPE and deltorphin I exposure but not with etorphine (Aguila et al., 2012).

All those data indicate that different mechanisms are responsible for OR desensitization: some are arrestin-dependent and requires GRK while others are arrestin-independent.

OR internalization

The number of active OR at the cell surface is regulated by two processes: endocytosis and export of neosynthesized receptors. Intuitively, when OR internalization is stimulated by agonist exposure, one could expect a reduction in signal transduction. However, the relationship between the number of OR and the cellular response is not linear.

Internalization of OR has been demonstrated in different models with different technical approaches but some discrepancies have been reported. U50,488 and dynorphin A (1-17), but neither etorphine nor levorphanol, promote a time-, and concentration-dependent internalization of hKOR (Li et al., 2003). In several reports, morphine was described as a poor internalizing agonist of MOR in HEK cells (Keith et al., 1998; Whistler and Von Zastrow, 1998; Schulz et al., 2004; Just et al., 2013) but also in enteric neurons (Anselmi et al., 2013) and in brain slice from transgenic mice expressing a FLAG-tagged MOR (Arttamangkul et al., 2008). In few publications, MOR was shown to internalize upon morphine exposure. This was demonstrated for the endogenous MOR in striatal neurons (Haberstock-Debic et al., 2005) and occurred mainly in dendrites (Haberstock-Debic et al., 2003), in the human neuroblastoma cells SH-SY5Y (Nowoczyn et al., 2013) and in double KO MEF for arrestins transfected both with MOR and arrestin 3 (Groer et al., 2011); in those latter publications, morphine-induced receptor internalization was observed for longer time treatment compared to DAMGO. Using a quantitative assay, 30 min morphine exposure promotes half of the MOR internalization induced by DAMGO (Mcpherson et al., 2010). In enteric neurons, morphine promotes a weak internalization of MOR compared to DAMGO as indicated above but chronic morphine exposure results in a significant increase in endocytosis (Patierno et al., 2011).

Role of OR phosphorylation in internalization

The role of OR phosphorylation in endocytosis was mainly investigated using OR mutants defective in phosphorylation. The truncated MOR from S363, which is not phosphorylated by DAMGO treatment, was shown to internalize but with a slower rate than the wild type receptor during the first 30 min (Qiu et al., 2003). The S375A mutation strongly impairs DAMGO-driven MOR endocytosis (Schulz et al., 2004). The T370A substitution has no significant effect on DAMGO-induced MOR internalization while it inhibits endocytosis triggered by PKC activation (Illing et al., 2014). This suggests that PKC is able to phosphorylate MOR at T370 and promotes its internalization. Conversely, the role of PKC in internalization was ruled out using activators of this kinase in the locus coeruleus neurons expressing the FLAG-tagged MOR (Arttamangkul et al., 2014). Herkinorin, a MOR agonist, is unable to promote both phosphorylation and internalization indicating that the two processes could be linked (Groer et al.,

2007). More than a selective phosphorylation on a specific residue of the carboxy-terminal tail of the receptor, the level of MOR internalization would be correlated to the multi-phosphorylation of T370, S375, T376, and T379 (Just et al., 2013).

As demonstrated for MOR, the phosphorylation-deficient DOR mutant (T358A/T361A/S363G) is able to undergo internalization upon DPDPE activation but to a lesser extent than the wild type (Zhang et al., 2005). However, this DOR mutant cannot internalize anymore when arrestin 3 expression is knocked-down suggesting that the non-phosphorylated DOR can internalize but in an arrestin 3-dependent manner. When the major site of phosphorylation of DOR is mutated (S363A), it is possible to observe a deltorphin I-induced endocytosis (Navratilova et al., 2007); however, it is difficult to assume that this mutation has no impact on internalization since no quantitative evaluation was made. This is in contrast with the study of Bradbury et al. who observed a close correlation between the ability of agonists to phosphorylate the S363 and the degree of DOR internalization (Bradbury et al., 2009).

Concerning the rKOR, the phosphorylation-defective mutant S369E is unable to internalize upon U50,488 exposure demonstrating the role of receptor phosphorylation in endocytosis (Mclaughlin et al., 2003).

While those data indicate that MOR and DOR phosphorylation would favor their endocytosis, KOR phosphorylation would be essential to promote its internalization. Other proteins involved in internalization could also be phosphorylated as demonstrated for the MOR. Activation of phospholipase D2 would enhance MOR endocytosis by the activation of p38 kinase which in turn phosphorylates the Rab5 effector early endosome antigen 1 required for this process (Yang et al., 2010).

Role of arrestins in OR internalization

The involvement of arrestins in OR internalization was demonstrated by direct (selective knock-down of arrestin expression) or indirect approaches (visualization of arrestin translocation to plasma membrane) (**Table 2**).

DAMGO-induced MOR internalization in striatal neurons is impaired by over-expression of a dominant negative mutant of arrestin 2 corresponding to the last 100 amino acids (arrestin 2 319–418) (Haberstock-Debic et al., 2005). Etorphine also induces an arrestin-dependent MOR internalization as shown by the reduction of receptor endocytosis when the dominant negative mutant V53D of arrestin is over-expressed (Zhang et al., 1998). While DAMGO triggers MOR internalization by recruiting either arrestin 2 or 3, morphine selectively interacts with arrestin 3 which is recruited at the plasma membrane to promote MOR internalization (Groer et al., 2011). In HEK cells, morphine is a poor inducer of MOR internalization. Whereas over-expression of arrestin 2 alone has not significant impact, over-expression of GRK2 greatly enhances receptor sequestration; such GRK2-mediated MOR internalization is potentiated when both kinase and arrestin 2 are both co- and over-expressed (Zhang et al., 1998). The lack of MOR internalization upon activation with herkinorin would be due to the absence of interaction between receptor and arrestin 3 (Groer et al., 2007). The constitutive MOR internalization is also arrestin 3-dependent (Walwyn et al., 2007). Whereas those reports indicate the crucial role of arrestins in MOR endocytosis, this was recently challenged by Quillinan et al. who still observed a MOR internalization upon [Met^5]-enkephalin exposure in arrestin 3 KO mice (Quillinan et al., 2011). In a recent work, the group of von Zastrow showed that after being recruited by the phosphorylated MOR, arrestin 3 acts as a scaffold, promoting ubiquitination of two lysyl residues in the first intracellular loop by the ubiquitin ligase Smurf2 (Henry et al., 2012). Epsin 1, through its ubiquitin-interacting motifs, recognizes the ubiquitinated MOR contained in the clathrin-coated pits and triggers scission of the vesicle from the cell surface. Those data revealed new inter-relations between MOR phosphorylation and ubiquitination with internalization.

Table 2 | Role of arrestins in OR trafficking.

OR	Main results	References
MOR	Inhibition of DAMGO-induced MOR internalization by a dominant negative mutant of arrestin 2 in striatal neurons	Haberstock-Debic et al., 2005
	Inhibition of etorphine-induced MOR internalization by a dominant negative mutant of arrestin 2	Zhang et al., 1998
	Morphine promotes MOR internalization by arrestin 3 while upon DAMGO exposure both arrestins 2 and 3 are recruited	Groer et al., 2011
	Morphine induces MOR endocytosis only when GRK2 and arrestin 2 are co-expressed	Zhang et al., 1998
	Herkinorin is unable to promote MOR sequestration	Groer et al., 2007
	MOR is still internalized upon [Met^5]-enkephalin exposure in arrestin 3 KO mice	Quillinan et al., 2011
	The arrestin 3 reduces recycling of MOR upon chronic morphine but not methadone exposure	Quillinan et al., 2011
	Role of arrestin 2 in MOR recycling upon sustained activation by DAMGO but not morphine	Groer et al., 2007
DOR	DOR endocytosis promoted by DPDPE involves both arrestins 2 and 3. Only arrestin 3 can mediate sequestration of a non-phosphorylated DOR mutant	Zhang et al., 2005
	Arrestin 2 preferentially interacts with DOR to induce its sequestration	Qiu et al., 2007
	Arrestin 2 is involved in DOR internalization upon etorphine but not DPDPE or deltorphin I exposure	Aguila et al., 2012
	Arrestin 3 targets DOR to lysosome when activated by SNC-80 but not DPDPE	Audet et al., 2012
KOR	Inhibition of U50,488-induced KOR internalization by a dominant negative mutant of arrestin 2	Li et al., 1999

DPDPE also enables arrestin-mediated endocytosis of DOR as shown by the partial reduction of internalization when arrestins 2 or 3 are selectively inhibited (Zhang et al., 2005). The triple DOR mutant T358A/T361A/S363G is still able to internalize but only when arrestin 3 is expressed. This could explain the plasma membrane translocation of arrestin 3-GFP observed in the study of Navratilova and colleagues with the S363A DOR mutant (Navratilova et al., 2007). DOR endocytosis is severely impaired in MEFs obtained from single KO mice for arrestin 2 indicating a preferential interaction between those two proteins (Qiu et al., 2007). It is noteworthy that even when expression of both arrestins 2 and 3 expression is inhibited, a weak proportion of DOR is able to internalize. This is in good agreement with data obtained by Aguila and collaborators who showed that inhibition of arrestin 2 expression reduces etorphine-induced hDOR endocytosis but not upon DPDPE or deltorphin I exposure (Aguila et al., 2012).

As demonstrated for MOR and DOR, KOR also undergoes an arrestin-dependent sequestration when activated by U50,488 as shown by the reduction of internalization when the dominant negative mutant arrestin 2 319–418 is over-expressed (Li et al., 1999).

Together, those data indicate that arrestins are key partners of OR internalization but under specific conditions or agonist exposure, other arrestin-independent mechanisms could occur.

Relationship between OR internalization and desensitization

Arttamangkul and collaborators studied desensitization on potassium currents and internalization in neurons from locus coeruleus of transgenic mice expressing a FLAG-tagged MOR (Arttamangkul et al., 2008). Three kinds of ligands can be identified: those which promote both desensitization and internalization ([Met^5]-enkephalin, etorphine, and methadone), those which induce a desensitization without internalization (morphine and oxymorphone) and oxycodone which promote neither desensitization nor internalization. This reveals the absence of any strong association between internalization and desensitization.

In the Xenopus oocyte expression system, it is possible to observe an acute desensitization of DOR on potassium channels (Kir3) elicited by DPDPE without significant internalization measured by surface biotinylation (Celver et al., 2013). When DOR internalization is significantly inhibited by over-expression of the dominant negative mutant of dynamin (K44E), the desensitization promoted by sustained exposure to DPDPE is not altered (Qiu et al., 2007). This is in good agreement with the observation of Marie et al. who showed that hypertonic sucrose solution totally blocks hDOR endocytosis without any impact on DPDPE- and deltorphin I-induced desensitization (Marie et al., 2003b). Likewise, UFP-512 promotes a strong DOR endocytosis after 15 min exposure without significant desensitization on the cAMP pathway (Aguila et al., 2007). However, upon etorphine exposure a partial reduction of hDOR desensitization is measured when internalization is inhibited.

In contrast, the abolition of rKOR internalization by the S369A substitution also inhibits receptor desensitization on potassium currents (Mclaughlin et al., 2003).

Those data demonstrate that desensitization and internalization are usually two independent processes although in some situations a close relationship could be evidenced. Those apparent discrepancies may be related to the different behavior of MOR and DOR in terms of trafficking (see below). For MOR, internalization would rather promotes recycling and resensitization; when blocking endocytosis, desensitization would be increased. In contrast, DOR are preferentially targeted to degradation, and inhibition of endocytosis would reduce their desensitization; however, this assumption assumes that the receptor at the plasma membrane is not uncoupled from G proteins and it's not always the case.

OR trafficking

Once internalized, the OR can follow different routes: sequestration into endosomes, recycling back to the cell surface or targeting to degradation.

The group of Von Zastrow was the first to identify a protein, named GASP for GPCR associated sorting protein, which could actively target DOR to lysosome (Whistler et al., 2002). This protein selectively interacts with the C terminal region of DOR, not MOR, that could explain that under certain circumstances, DOR is degraded while MOR is recycled (Tsao and Von Zastrow, 2000; Whistler et al., 2002). The same group also identified a motif localized at the C terminal region of MOR that enables an active recycling (Tanowitz and Von Zastrow, 2003). This sequence is lacking in DOR but the chimeric DOR containing the last 17 amino acids of MOR recycles after DADLE activation in contrast to wild type. Arrestin 3, dynamin and GRK2 also participate to MOR resensitization on the activation of potassium channels in neurons from the locus coeruleus of mice treated during 6 days with morphine (Dang et al., 2011). This could suggest that those proteins would be involved in MOR trafficking after its internalization and that internalization itself contributes to resensitization (Dang and Christie, 2012). Using neurons obtained from the locus coeruleus of transgenic mice expressing a FLAG-tagged MOR, chronic morphine but not methadone during 6 days was shown to inhibit resensitization and recycling after an acute [Met^5]-enkephalin exposure (Quillinan et al., 2011). Such weak resensitization and recycling return to the level observed in naive mice when arrestin 3 was knocked-down indicating that this protein would also play a pivotal role in MOR trafficking. Arrestin 2 could regulate post-endocytic sorting of MOR upon DAMGO exposure but not morphine by enabling receptor ubiquitination, as described for different GPCRs (Marchese and Trejo, 2013), but also dephosphorylation on the S375 (Groer et al., 2011). The first hypothesis is unlikely since the sorting of the MOR either toward recycling or lysosomal degradation does not rely on receptor ubiquitination (Hislop et al., 2011). The recycling process involves protein kinases as shown by staurosporine, which increases recycling and resensitization after [Met^5]-enkephalin exposure (Arttamangkul et al., 2012). Resensitization of MOR after [Met^5]-enkephalin- or morphine-induced acute desensitization but not cellular tolerance involves dephosphorylation mediated by protein phosphatases sensitive to calyculin A but not okadaic acid (Levitt and Williams, 2012). Similarly, Doll and colleagues showed that the rapid MOR dephosphorylation at S375

involves the protein phosphatase 1γ which increases the recycling of receptors contained in endosomes to cell surface (Doll et al., 2012). The role of receptor dephosphorylation was also demonstrated for both recycling and resensitization of DOR after etorphine treatment (Hasbi et al., 2000).

As indicated above, DOR was initially described as a receptor sorted to lysosomal degradation (Tsao and Von Zastrow, 2000). However, etorphine, [Leu^5]- and [Met^5]-enkephalins rather promote a recycling of hDOR while DPDPE, Deltorphin I or SNC-80 induce a degradation and a down-regulation (Marie et al., 2003b; Lecoq et al., 2004). This indicates that the differential sorting of DOR either to recycling or degradation pathway depends on the agonist used and refers to the notion of biased agonism. Audet and collaborators found that DOR activated by SNC-80 strongly interacts with arrestin 3 (Audet et al., 2012). Consequently, the receptor is mainly targeted to lysosome while upon DPDPE exposure, interactions between DOR and arrestin 3 are loose allowing receptor recycling. The ability of DOR to recycle also depends on the duration of agonist exposure. For instance, after 30 min of etorphine treatment, DOR recycles while after 4 h this process is severely impaired (Hasbi et al., 2000). Zhang and collaborators showed different mechanisms to explain the differential sorting of DOR (Zhang et al., 2008): when the receptor is phosphorylated by GRK2 and internalized via arrestins it can recycle whereas in a non-phosphorylated form DOR undergoes an arrestin-independent sequestration which is followed by a degradation. As described for MOR, kinases can be involved in OR sorting. Src was shown to inhibit DOR recycling upon DPDPE treatment that would favor desensitization on the cAMP pathway (Archer-Lahlou et al., 2009). Recently, the endothelin converting enzyme-2, localized in endosomes, was shown to modulate recycling of DOR by degrading opioid peptides such as deltorphin II or the opioid peptide bovine adrenal medulla 22 (BAM22), a cleavage product of proenkephalin (Gupta et al., 2014). When this enzyme is inhibited, DOR recycling decreases and consequently, the desensitization increases. It is noteworthy that this enzyme is ineffective when DOR is activated by the endogenous peptide [Met^5]-enkephalin and has no role on receptor internalization.

MOLECULAR MECHANISMS INVOLVED IN OR DESENSITIZATION: A UNIFIED MECHANISM?

The vast majority of studies on OR desensitization demonstrated that phosphorylation of OR constitutes a rapid and ubiquitous regulatory mechanisms. However, as illustrated for MOR, quantitative (Lau et al., 2011) or qualitative (Just et al., 2013) differences in MOR phosphorylation were reported upon DAMGO and morphine exposure and those differences in multi-site phosphorylation would result in differential interactions with partners. Conversely, some studies using phosphorylation-deficient receptor challenged this paradigm (Qiu et al., 2003). OR phosphorylation should rather be viewed as a potentiating mechanism that would increase binding of regulatory proteins such as arrestins to the receptor. Mechanisms of desensitization share common features (phosphorylation, accessory proteins involvement such as arrestin, importance of endocytosis and receptor trafficking) and will dependent not only on agonist (biased agonism) but also on time exposure, cell system and receptor. All those mechanisms are depicted in **Figures 2A,B**.

OPIOID TOLERANCE

DEFINITION

Drug tolerance is the body's ability to protect itself against the presence of a drug. It is generally observed after protracted exposure but also after acute treatment (acute tolerance) and it is not observed for all the pharmacological effects. For opioids, tolerance to analgesia has been primarily studied as it is the main issue in clinical practice. In rodent, the ability of opioid to promote analgesia to different type of stimuli could be measured using numerous behavioral paradigms including hotplate test and tail-flick for thermal nociception (Barrot, 2012). Different parameters could modulate tolerance such as the opioid agonist used (Enquist et al., 2012), duration of treatment (Soignier et al., 2004), doses (Huidobro et al., 1976) and even the pharmacological effect observed (Mohammed et al., 2013). So, it is now established that tolerance to respiratory depression is lower than the tolerance to analgesia (Mohammed et al., 2013) and might explain fatal overdoses (White and Irvine, 1999).

OPIOID RECEPTOR-RELATED MECHANISMS OF TOLERANCE

Mechanisms of opioid tolerance are complex and multifaceted. We will focus on the mechanisms directly related to receptor regulation such as down-regulation, G protein uncoupling, desensitization, and internalization. Indeed, other mechanisms contribute to tolerance such as activation of anti-opioid systems (NPFF, NMDA) (Ueda and Ueda, 2009) but they are beyond the scope of this review.

Down-regulation

Down-regulation is the reduction of receptor number that may result from receptor internalization followed by their degradation, or decrease in receptor synthesis. So, one could hypothesize that it would contribute to tolerance by diminishing the quantity of available receptor. *In vivo*, chronic treatment with opioids promotes decrease (down-regulation), no change or increase (up-regulation) of OR (Bernstein and Welch, 1998; Stafford et al., 2001; Fabian et al., 2002). When downregulation is observed, tolerance might be measured (Gomes et al., 2002) however in some cases tolerance occurs without receptor downregulation (Polastron et al., 1994). These data suggest that downregulation is not mandatory for tolerance.

Desensitization

Desensitization and tolerance are very similar in their definition as they both include the notion of a reduced response after prolonged treatment. So, it is tempting to speculate that desensitization and its mechanisms would occur in tolerant animals. In chronic morphine-treated animals desensitization of OR was measured on ACase (Noble and Cox, 1996) and associated with tolerance to analgesic effects (Polastron et al., 1994). In cellular model, receptor uncoupling from G proteins was demonstrated to participate in desensitization (see above). Such uncoupling was also evidenced *in vivo* after chronic opioid agonist exposure.

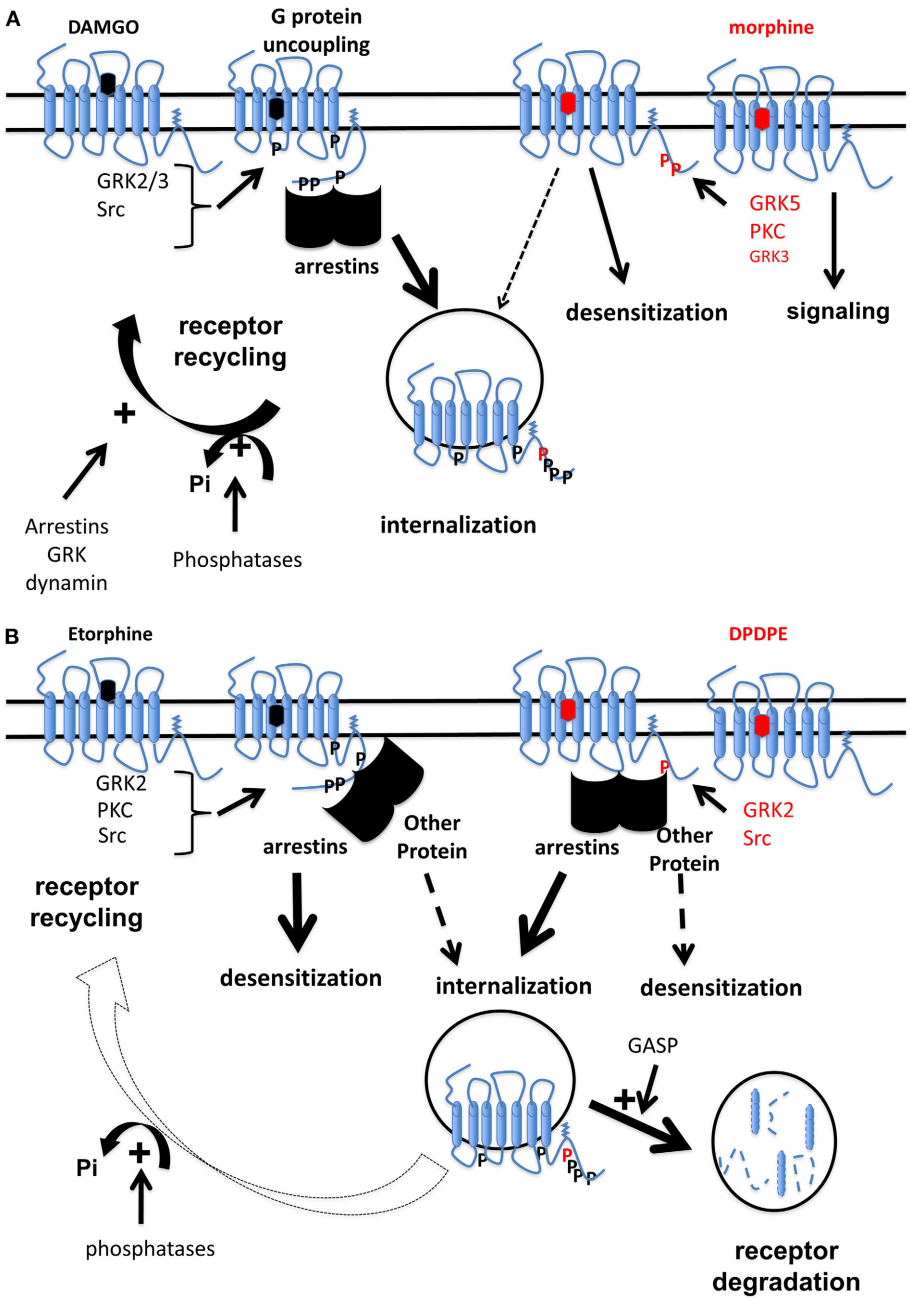

FIGURE 2 | Schematic illustration of mechanisms involved in opioid receptor desensitization by biased agonists. (A) MOR are differentially phosphorylated by different kinases upon either DAMGO or morphine exposure (Doll et al., 2011). This results in binding of arrestins to MOR upon DAMGO while this interaction is weakly detectable for morphine when a GRK is over-expressed (Groer et al., 2007). In such conditions, acute DAMGO exposure promotes G protein uncoupling from MOR while morphine does not (Whistler and Von Zastrow, 1998). However, MOR phosphorylation at S375 induced by morphine is able to promote desensitization but not internalization (Schulz et al., 2004). Some reports rather suggest that under morphine exposure, MOR is not desensitized and this continuous signaling promotes tolerance (Finn and Whistler, 2001). Even if it's now well-admitted that morphine is able to promote MOR internalization (Haberstock-Debic et al., 2005; Nowoczyn et al., 2013), DAMGO induces a stronger internalization compared to morphine (Whistler and Von Zastrow, 1998; Schulz et al., 2004). MOR is dephosphorylated by phosphatase proteins (Doll et al., 2012) then undergoes an active recycling (Tanowitz and Von Zastrow, 2003). Other proteins such as arrestins, dynamin, or GRK could participate MOR trafficking (Dang et al., 2011). In contrast, as morphine is a poor inducer of MOR internalization, receptor is maintained in a phosphorylation state at S375 for longer time compared to DAMGO. **(B)** Different kinases are involved in the regulation of hDOR (Marie et al., 2008): GRK2 plays a major role in receptor phosphorylation on S363 upon DPDPE and etorphine while other kinases are also implicated. Etorphine-induced desensitization requires arrestins but not receptor internalization. In contrast, an arrestin is involved in hDOR internalization but not desensitization upon DPDPE (Aguila et al., 2012). Once sequestrated by etorphine, hDOR is dephosphorylated and recycled back to the cell surface (Hasbi et al., 2000; Marie et al., 2003b) while upon DPDPE exposure, the receptor is mainly targeted to lysosomes for degradation (Marie et al., 2003b) probably by a mechanism involving GASP (Whistler et al., 2002).

In knock-in mice expressing DOR-eGFP, a challenge with SNC-80 but not ARM-390 induces a tolerance to analgesic response in a model of inflammatory pain with a concomitant G protein uncoupling in both brain and spinal cord homogenates (Pradhan et al., 2009). Acute and chronic treatment with morphine or fentanyl promotes a similar regulation of MOR. In parallel with analgesic tolerance, the ability of MOR to enhance [^{35}S]GTPγS binding was reduced compared to naive animals (Bohn et al., 2000; Melief et al., 2010). When arrestin 3 was knocked-out, morphine tolerance and MOR uncoupling from G proteins was reduced in chronic treated animals (Bohn et al., 2000). Interestingly, this KO did not affect tolerance induced by 5 days treatment with fentanyl, oxycodone or methadone (Raehal et al., 2011).

Phosphorylation

Anti-nociceptive tolerance induced by morphine, meperidine, and fentanyl was shown to be reduced by PKC inhibitors while DAMGO-induced tolerance and MOR desensitization was shown to rely on GRK (Hull et al., 2010). Whereas *in vitro* experiments showed that S375 is phosphorylated by GRK5 upon morphine exposure (Doll et al., 2012) and S375 phosphorylation plays a major role in MOR desensitization (Schulz et al., 2004), S375A knock-in mice still present anti-nociceptive tolerance upon acute and chronic exposure to morphine (Grecksch et al., 2011). This could indicate that MOR desensitization and tolerance are two unrelated mechanisms. Recently, the role of GRK in morphine tolerance was also questioned: while morphine predominantly promotes S375 phosphorylation by GRK5, chronic morphine treatment induced similar tolerance in wild type and in GRK5 KO mice while dependence was altered (Glück et al., 2014). Similar results were obtained in GRK3 KO mice, when morphine tolerance to analgesia was unchanged whereas tolerance to high efficacy agonists, such as fentanyl or U50,488, was reduced (Terman et al., 2004; Zhang et al., 2013). Rather than inducing desensitization, a chronic morphine treatment could promote a compensatory increase in intracellular cAMP level (also named cAMP overshoot or ACase superactivation) (Avidor-Reiss et al., 1995) and is believed to play a direct role in tolerance (Duman et al., 1988; Javed et al., 2004). In this situation, Src kinase can be recruited at the lipid raft-located MOR and phosphorylates the Y336 leading to ACase superactivation (Zhang et al., 2009). While the mechanism is still unclear, it could implicate Ras/Raf-1 which change the MOR, a GPCR, into a receptor tyrosine kinase like-complex (Zhang et al., 2013).

Endocytosis

Accumulating evidences suggest that OR endocytosis decrease opioid tolerance but by mechanisms not fully understood. The first hypothesis has been built by Whistler's group on the inability of morphine to promote MOR internalization despite its capacity to induce strong tolerance. In this case, during morphine treatment, morphine/MOR complexes would accumulate at the plasma membrane and recruit signaling pathways involved in tolerance such as ACase superactivation and NMDA receptor regulation (Finn and Whistler, 2001; He et al., 2002, 2009). In line with this hypothesis, a knock-in mice, expressing a MOR chimera where the C-terminus tail was replaced by the C-terminus tail of DOR, demonstrated less tolerance after chronic morphine treatment (Kim et al., 2008), correlated to a decrease of tolerance biomarkers (He et al., 2009). One explanation of this result is the termination of signal transduction because the DOR C-terminus tail will target the chimeric MOR to lysosomes (Finn and Whistler, 2001). Such results were confirmed when comparing other opioid agonist, buprenorphine and etonitazene. Indeed, buprenorphine, like morphine induces tolerance to analgesia without promoting MOR endocytosis, whereas etonitazene promotes less tolerance and has the ability to promote MOR internalization (Grecksch et al., 2006). Interestingly, coadministration of morphine with subactive doses of internalizing opioids, DAMGO or methadone, enables morphine-induced internalization of MOR and blocks tolerance development (He and Whistler, 2005). An alternative hypothesis was proposed by Koch and collaborators. They proposed that morphine promotes an accumulation of desensitized MOR at the plasma membrane that would result in an increase in apparent desensitization by inhibiting resensitization and would promote tolerance (Koch et al., 2001, 2005; Schulz et al., 2004). However, they found that in knock-in mice expressing MOR mutant S375A substitution, proposed by these authors to be the primary site of morphine-induced phosphorylation of MOR responsible for desensitization (Schulz et al., 2004), morphine tolerance was not affected (Grecksch et al., 2011). The RAVE (relative activity vs. endocytosis) concept proposed by Whistler et al. (1999) cannot be extended to DOR. In DOR-eGFP knock-in mice, the internalizing agonist, SNC-80 promotes acute tolerance to analgesia correlated with strong internalization whereas ARM-390 a non-internalizing agonist did not induce acute tolerance (Pradhan et al., 2009, 2010). When SNC-80 and ARM-390 are chronically administrated, tolerance to analgesia develops and is dependent on endocytosis with SNC80 but not for ARM-390. Interestingly, no tolerance for locomotor effects or anxiolysis appears in ARM-390-treated animals underlying the fact that biased agonist could be used at the behavioral level. All those data support the role of internalization and mainly recycling in reducing tolerance by allowing a sufficient quantity of functional receptors at the cell surface to produce the biological response. However, some opioid agonists such as herkinorin can promote a long lasting anti-nociception without internalization due to the absence of arrestin 3 recruitment (Lamb et al., 2012).

CONCLUSIONS

All the data presented in this review demonstrated that mechanisms of OR regulation are consistent with the model proposed by Lefkowitz (Pierce et al., 2002): agonist activation, receptor phosphorylation, arrestin binding, G protein uncoupling, desensitization, endocytosis followed by targeting to lysosomes or recycling. More interestingly, they also showed that many variations around this model exist depending on the initial conditions, revealing the complexity of OR regulation now translated to the concept of biased agonism. It's an exciting challenge to gain insight this complexity because it will offer a great opportunity to design new drugs that will be able to target a particular pharmacological effect with limited side effects.

REFERENCES

Abbadie, C., Pan, Y. X., and Pasternak, G. W. (2004). Immunohistochemical study of the expression of exon11-containing mu opioid receptor variants in mouse brain. *Neuroscience* 127, 419–430. doi: 10.1016/j.neuroscience.2004.03.033

Aguila, B., Coulbault, L., Boulouard, M., Leveille, F., Davis, A., Toth, G., et al. (2007). *In vitro* and *in vivo* pharmacological profile of UFP-512, a novel selective delta-opioid receptor agonist; correlations between desensitization and tolerance. *Br. J. Pharmacol.* 152, 1312–1324. doi: 10.1038/sj.bjp.0707497

Aguila, B., Coulbault, L., Davis, A., Marie, N., Hasbi, A., Le Bras, F., et al. (2012). ßarrestin1-biased agonism at human delta-opioid receptor by peptidic and alkaloid ligands. *Cell Signal* 24, 699–707. doi: 10.1016/j.cellsig.2011.10.018

Allouche, S., Polastron, J., Hasbi, A., Homburger, V., and Jauzac, P. (1999a). Differential G-protein activation by alkaloid and peptide opioid agonists in the human neuroblastoma cell line SK-N-BE. *Biochem. J.* 342 (Pt 1), 71–78. doi: 10.1042/0264-6021:3420071

Allouche, S., Roussel, M., Marie, N., and Jauzac, P. (1999b). Differential desensitization of human delta-opioid receptors by peptide and alkaloid agonists. *Eur. J. Pharmacol.* 371, 235–240. doi: 10.1016/S0014-2999(99)00180-6

Alonso, N., Monczor, F., Echeverria, E., Davio, C., Shayo, C., and Fernandez, N. (2014). Signal transduction mechanism of biased ligands at histamine H2 receptors. *Biochem. J.* 459, 117–126. doi: 10.1042/BJ20131226

Anselmi, L., Jaramillo, I., Palacios, M., Huynh, J., and Sternini, C. (2013). Ligand-induced mu opioid receptor internalization in enteric neurons following chronic treatment with the opiate fentanyl. *J. Neurosci. Res.* 91, 854–860. doi: 10.1002/jnr.23214

Appleyard, S. M., Celver, J., Pineda, V., Kovoor, A., Wayman, G. A., and Chavkin, C. (1999). Agonist-dependent desensitization of the kappa opioid receptor by G protein receptor kinase and beta-arrestin. *J. Biol. Chem.* 274, 23802–23807. doi: 10.1074/jbc.274.34.23802

Archer-Lahlou, E., Audet, N., Amraei, M. G., Huard, K., Paquin-Gobeil, M., and Pineyro, G. (2009). Src promotes delta opioid receptor (DOR) desensitization by interfering with receptor recycling. *J. Cell. Mol. Med.* 13, 147–163. doi: 10.1111/j.1582-4934.2008.00308.x

Arttamangkul, S., Birdsong, W., and Williams, J. T. (2014). Does PKC activation increase the homologous desensitization of μopioid receptors? *Br. J. Pharmacol.* doi: 10.1111/bph.12712

Arttamangkul, S., Lau, E. K., Lu, H.-W., and Williams, J. T. (2012). Desensitization and trafficking of μ-opioid receptors in locus ceruleus neurons: modulation by kinases. *Mol. Pharmacol.* 81, 348–355. doi: 10.1124/mol.111.076208

Arttamangkul, S., Quillinan, N., Low, M. J., Von Zastrow, M., Pintar, J., and Williams, J. T. (2008). Differential activation and trafficking of micro-opioid receptors in brain slices. *Mol. Pharmacol.* 74, 972–979. doi: 10.1124/mol.108.048512

Audet, N., Charfi, I., Mnie-Filali, O., Amraei, M., Chabot-Dore, A. J., Millecamps, M., et al. (2012). Differential association of receptor-Gbetagamma complexes with beta-arrestin2 determines recycling bias and potential for tolerance of delta opioid receptor agonists. *J. Neurosci.* 32, 4827–4840. doi: 10.1523/JNEUROSCI.3734-11.2012

Avidor-Reiss, T., Bayewitch, M., Levy, R., Matus-Leibovitch, N., Nevo, I., and Vogel, Z. (1995). Adenylylcyclase supersensitization in mu-opioid receptor-transfected Chinese hamster ovary cells following chronic opioid treatment. *J. Biol. Chem.* 270, 29732–29738. doi: 10.1074/jbc.270.50.29732

Bailey, C. P., Oldfield, S., Llorente, J., Caunt, C. J., Teschemacher, A. G., Roberts, L., et al. (2009). Involvement of PKC alpha and G-protein-coupled receptor kinase 2 in agonist-selective desensitization of mu-opioid receptors in mature brain neurons. *Br. J. Pharmacol.* 158, 157–164. doi: 10.1111/j.1476-5381.2009.00140.x

Barrot, M. (2012). Tests and models of nociception and pain in rodents. *Neuroscience* 211, 39–50. doi: 10.1016/j.neuroscience.2011.12.041

Beckett, A. H., and Casy, A. F. (1954). Stereochemistry of certain analgesics. *Nature* 173, 1231–1232. doi: 10.1038/1731231a0

Befort, K., Filliol, D., Decaillot, F. M., Gaveriaux-Ruff, C., Hoehe, M. R., and Kieffer, B. L. (2001). A single nucleotide polymorphic mutation in the human mu-opioid receptor severely impairs receptor signaling. *J. Biol. Chem.* 276, 3130–3137. doi: 10.1074/jbc.M006352200

Bernstein, M. A., and Welch, S. P. (1998). mu-Opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. *Brain Res. Mol. Brain Res.* 55, 237–242. doi: 10.1016/S0169-328X(98)00005-9

Besse, D., Lombard, M. C., Zajac, J. M., Roques, B. P., and Besson, J. M. (1990). Pre- and postsynaptic distribution of mu, delta and kappa opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord. *Brain Res* 521, 15–22. doi: 10.1016/0006-8993(90)91519-M

Bian, J. M., Wu, N., Su, R. B., and Li, J. (2012). Opioid receptor trafficking and signaling: what happens after opioid receptor activation? *Cell. Mol. Neurobiol.* 32, 167–184. doi: 10.1007/s10571-011-9755-5

Blake, A. D., Bot, G., Freeman, J. C., and Reisine, T. (1997a). Differential opioid agonist regulation of the mouse mu opioid receptor. *J. Biol. Chem.* 272, 782–790. doi: 10.1074/jbc.272.2.782

Blake, A. D., Bot, G., Li, S., Freeman, J. C., and Reisine, T. (1997b). Differential agonist regulation of the human kappa-opioid receptor. *J. Neurochem.* 68, 1846–1852. doi: 10.1046/j.1471-4159.1997.68051846.x

Blanchet, C., Sollini, M., and Luscher, C. (2003). Two distinct forms of desensitization of G-protein coupled inwardly rectifying potassium currents evoked by alkaloid and peptide mu-opioid receptor agonists. *Mol. Cell. Neurosci.* 24, 517–523. doi: 10.1016/S1044-7431(03)00173-8

Bodnar, R. J. (2014). Endogenous opiates and behavior: 2013. *Peptides* 62C, 67–136. doi: 10.1016/j.peptides.2014.09.013

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

Borgland, S. L., Connor, M., Osborne, P. B., Furness, J. B., and Christie, M. J. (2003). Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J. Biol. Chem.* 278, 18776–18784. doi: 10.1074/jbc.M300525200

Bot, G., Blake, A. D., Li, S., and Reisine, T. (1997). Opioid regulation of the mouse delta-opioid receptor expressed in human embryonic kidney 293 cells. *Mol. Pharmacol.* 52, 272–281.

Bradbury, F. A., Zelnik, J. C., and Traynor, J. R. (2009). G protein independent phosphorylation and internalization of the delta-opioid receptor. *J. Neurochem.* 109, 1526–1535. doi: 10.1111/j.1471-4159.2009.06082.x

Celver, J., Sharma, M., Thanawala, V., Christopher Octeau, J., and Kovoor, A. (2013). Arrestin-dependent but G-protein coupled receptor kinase-independent uncoupling of D2-dopamine receptors. *J. Neurochem.* 127, 57–65. doi: 10.1111/jnc.12359

Celver, J., Xu, M., Jin, W., Lowe, J., and Chavkin, C. (2004). Distinct domains of the mu-opioid receptor control uncoupling and internalization. *Mol. Pharmacol.* 65, 528–537. doi: 10.1124/mol.65.3.528

Chen, C., Shahabi, V., Xu, W., and Liu-Chen, L. Y. (1998). Palmitoylation of the rat mu opioid receptor. *FEBS Lett.* 441, 148–152. doi: 10.1016/S0014-5793(98)01547-6

Chen, Y. J., Oldfield, S., Butcher, A. J., Tobin, A. B., Saxena, K., Gurevich, V. V., et al. (2013). Identification of phosphorylation sites in the COOH-terminal tail of the mu-opioid receptor. *J. Neurochem.* 124, 189–199. doi: 10.1111/jnc.12071

Chen, Y., Mestek, A., Liu, J., Hurley, J. A., and Yu, L. (1993a). Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol. Pharmacol.* 44, 8–12.

Chen, Y., Mestek, A., Liu, J., and Yu, L. (1993b). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochem. J.* 295 (Pt 3), 625–628.

Cheng, Z. J., Yu, Q. M., Wu, Y. L., Ma, L., and Pei, G. (1998). Selective interference of beta-arrestin 1 with kappa and delta but not mu opioid receptor/G protein coupling. *J. Biol. Chem.* 273, 24328–24333. doi: 10.1074/jbc.273.38.24328

Chu, J., Zheng, H., Zhang, Y., Loh, H. H., and Law, P. Y. (2010). Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. *Cell. Signal.* 22, 684–696. doi: 10.1016/j.cellsig.2009.12.003

Clayton, C. C., Bruchas, M. R., Lee, M. L., and Chavkin, C. (2010). Phosphorylation of the mu-opioid receptor at tyrosine 166 (Tyr3.51) in the DRY motif reduces agonist efficacy. *Mol. Pharmacol.* 77, 339–347. doi: 10.1124/mol.109.060558

Connor, M., Osborne, P. B., and Christie, M. J. (2004). Mu-opioid receptor desensitization: is morphine different? *Br. J. Pharmacol.* 143, 685–696. doi: 10.1038/sj.bjp.0705938

Cox, B. M., Goldstein, A., and Hi, C. H. (1976). Opioid activity of a peptide, beta-lipotropin-(61-91), derived from beta-lipotropin. *Proc. Natl. Acad. Sci. U.S.A.* 73, 1821–1823. doi: 10.1073/pnas.73.6.1821

Dang, V. C., Chieng, B., Azriel, Y., and Christie, M. J. (2011). Cellular morphine tolerance produced by betaarrestin-2-dependent impairment

of mu-opioid receptor resensitization. *J. Neurosci.* 31, 7122–7130. doi: 10.1523/JNEUROSCI.5999-10.2011

Dang, V. C., and Christie, M. J. (2012). Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. *Br. J. Pharmacol.* 165, 1704–1716. doi: 10.1111/j.1476-5381.2011.01482.x

Dang, V. C., Napier, I. A., and Christie, M. J. (2009). Two distinct mechanisms mediate acute mu-opioid receptor desensitization in native neurons. *J. Neurosci.* 29, 3322–3327. doi: 10.1523/JNEUROSCI.4749-08.2009

Dhawan, B. N., Cesselin, F., Raghubir, R., Reisine, T., Bradley, P. B., Portoghese, P. S., et al. (1996). International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol. Rev.* 48, 567–592.

Doll, C., Konietzko, J., Poll, F., Koch, T., Hollt, V., and Schulz, S. (2011). Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br. J. Pharmacol.* 164, 298–307. doi: 10.1111/j.1476-5381.2011.01382.x

Doll, C., Poll, F., Peuker, K., Loktev, A., Gluck, L., and Schulz, S. (2012). Deciphering micro-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. *Br. J. Pharmacol.* 167, 1259–1270. doi: 10.1111/j.1476-5381.2012.02080.x

Duman, R. S., Tallman, J. F., and Nestler, E. J. (1988). Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J. Pharmacol. Exp. Ther.* 246, 1033–1039.

Ehrlich, G. K., Andria, M. L., Zheng, X., Kieffer, B., Gioannini, T. L., Hiller, J. M., et al. (1998). Functional significance of cysteine residues in the delta opioid receptor studied by site-directed mutagenesis. *Can. J. Physiol. Pharmacol.* 76, 269–277.

El Kouhen, R., Burd, A. L., Erickson-Herbrandson, L. J., Chang, C. Y., Law, P. Y., and Loh, H. H. (2001). Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. *J. Biol. Chem.* 276, 12774–12780. doi: 10.1074/jbc.M009571200

Enquist, J., Ferwerda, M., Milan-Lobo, L., and Whistler, J. L. (2012). Chronic methadone treatment shows a better cost/benefit ratio than chronic morphine in mice. *J. Pharmacol. Exp. Ther.* 340, 386–392. doi: 10.1124/jpet.111.187583

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

Fabian, G., Bozo, B., Szikszay, M., Horvath, G., Coscia, C. J., and Szucs, M. (2002). Chronic morphine-induced changes in mu-opioid receptors and G proteins of different subcellular loci in rat brain. *J. Pharmacol. Exp. Ther.* 302, 774–780. doi: 10.1124/jpet.102.036152

Feng, B., Li, Z., and Wang, J. B. (2011). Protein kinase C-mediated phosphorylation of the mu-opioid receptor and its effects on receptor signaling. *Mol. Pharmacol.* 79, 768–775. doi: 10.1124/mol.110.069096

Filizola, M., and Devi, L. A. (2013). Grand opening of structure-guided design for novel opioids. *Trends Pharmacol. Sci.* 34, 6–12. doi: 10.1016/j.tips.2012.10.002

Finn, A. K., and Whistler, J. L. (2001). Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* 32, 829–839. doi: 10.1016/S0896-6273(01)00517-7

Fujita, W., Gomes, I., and Devi, L. A. (2014). Heteromers of μ-delta opioid receptors: new pharmacology and novel therapeutic possibilities. *Br. J. Pharmacol.* doi: 10.1111/bph.12663. [Epub ahead of print].

Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., et al. (1994). cDNA cloning and regional distribution of a novel member of the opioid receptor family. *FEBS Lett.* 343, 42–46. doi: 10.1016/0014-5793(94)80603-9

Gahwiler, B. H. (1981). Development of acute tolerance during exposure of hippocampal explants to an opioid peptide. *Brain Res.* 217, 196–200. doi: 10.1016/0006-8993(81)90200-6

Gaveriaux-Ruff, C., and Kieffer, B. L. (2002). Opioid receptor genes inactivated in mice: the highlights. *Neuropeptides* 36, 62–71. doi: 10.1054/npep.2002.0900

Georgoussi, Z., Merkouris, M., Mullaney, I., Megaritis, G., Carr, C., Zioudrou, C., et al. (1997). Selective interactions of mu-opioid receptors with pertussis toxin-sensitive G proteins: involvement of the third intracellular loop and the c-terminal tail in coupling. *Biochim. Biophys. Acta* 1359, 263–274. doi: 10.1016/S0167-4889(97)00097-9

Gioannini, T. L., Onoprishvili, I., Hiller, J. M., and Simon, E. J. (1999). Inactivation of the purified bovine mu opioid receptor by sulfhydryl reagents. *Neurochem. Res.* 24, 37–42. doi: 10.1023/A:1020923928936

Glück, L., Loktev, A., Moulédous, L., Mollereau, C., Law, P.-Y., and Schulz, S. (2014). Loss of morphine reward and dependence in mice lacking G Protein-coupled receptor kinase 5. *Biol. Psychiatry*. 76, 767–774. doi: 10.1016/j.biopsych.2014.01.021

Goldstein, A., Fischli, W., Lowney, L. I., Hunkapiller, M., and Hood, L. (1981). Porcine pituitary dynorphin: complete amino acid sequence of the biologically active heptadecapeptide. *Proc. Natl. Acad. Sci. U.S.A.* 78, 7219–7223. doi: 10.1073/pnas.78.11.7219

Gomes, B. A., Shen, J., Stafford, K., Patel, M., and Yoburn, B. C. (2002). Mu-opioid receptor down-regulation and tolerance are not equally dependent upon G-protein signaling. *Pharmacol. Biochem. Behav.* 72, 273–278. doi: 10.1016/S0091-3057(01)00757-2

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

Grecksch, G., Bartzsch, K., Widera, A., Becker, A., Hollt, V., and Koch, T. (2006). Development of tolerance and sensitization to different opioid agonists in rats. *Psychopharmacology (Berl)* 186, 177–184. doi: 10.1007/s00213-006-0365-8

Grecksch, G., Just, S., Pierstorff, C., Imhof, A. K., Gluck, L., Doll, C., et al. (2011). Analgesic tolerance to high-efficacy agonists but not to morphine is diminished in phosphorylation-deficient S375A mu-opioid receptor knock-in mice. *J. Neurosci.* 31, 13890–13896. doi: 10.1523/JNEUROSCI.2304-11.2011

Groer, C. E., Schmid, C. L., Jaeger, A. M., and Bohn, L. M. (2011). Agonist-directed interactions with specific beta-arrestins determine mu-opioid receptor trafficking, ubiquitination, and dephosphorylation. *J. Biol. Chem.* 286, 31731–31741. doi: 10.1074/jbc.M111.248310

Groer, C. E., Tidgewell, K., Moyer, R. A., Harding, W. W., Rothman, R. B., Prisinzano, T. E., et al. (2007). An opioid agonist that does not induce mu-opioid receptor–arrestin interactions or receptor internalization. *Mol. Pharmacol.* 71, 549–557. doi: 10.1124/mol.106.028258

Guillemin, R., Vargo, T., Rossier, J., Minick, S., Ling, N., Rivier, C., et al. (1977). Beta-Endorphin and adrenocorticotropin are selected concomitantly by the pituitary gland. *Science* 197, 1367–1369. doi: 10.1126/science.197601

Guo, J., Wu, Y., Zhang, W., Zhao, J., Devi, L. A., Pei, G., et al. (2000). Identification of G protein-coupled receptor kinase 2 phosphorylation sites responsible for agonist-stimulated delta-opioid receptor phosphorylation. *Mol. Pharmacol.* 58, 1050–1056. doi: 10.1124/mol.58.5.1050

Gupta, A., Fujita, W., Gomes, I., Bobeck, E., and Devi, L. A. (2014). Endothelin converting enzyme-2 differentially regulates opioid receptor activity. *Br. J. Pharmacol.* doi: 10.1111/bph.12833. [Epub ahead of print].

Haberstock-Debic, H., Kim, K.-A., Yu, Y. J., and Von Zastrow, M. (2005). Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J. Neurosci.* 25, 7847–7857. doi: 10.1523/JNEUROSCI.5045-04.2005

Haberstock-Debic, H., Wein, M., Barrot, M., Colago, E. E., Rahman, Z., Neve, R. L., et al. (2003). Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. *J. Neurosci.* 23, 4324–4332.

Hasbi, A., Allouche, S., Sichel, F., Stanasila, L., Massotte, D., Landemore, G., et al. (2000). Internalization and recycling of delta-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism. *J. Pharmacol. Exp. Ther.* 293, 237–247.

He, L., Fong, J., Von Zastrow, M., and Whistler, J. L. (2002). Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 108, 271–282. doi: 10.1016/S0092-8674(02)00613-X

He, L., Kim, J. A., and Whistler, J. L. (2009). Biomarkers of morphine tolerance and dependence are prevented by morphine-induced endocytosis of a mutant mu-opioid receptor. *FASEB J.* 23, 4327–4334. doi: 10.1096/fj.09-133223

He, L., and Whistler, J. L. (2005). An opiate cocktail that reduces morphine tolerance and dependence. *Curr. Biol.* 15, 1028–1033. doi: 10.1016/j.cub.2005.04.052

Henry, A. G., Hislop, J. N., Grove, J., Thorn, K., Marsh, M., and Von Zastrow, M. (2012). Regulation of endocytic clathrin dynamics by cargo ubiquitination. *Dev. Cell* 23, 519–532. doi: 10.1016/j.devcel.2012.08.003

Hislop, J. N., Henry, A. G., and Von Zastrow, M. (2011). Ubiquitination in the first cytoplasmic loop of μ-opioid receptors reveals a hierarchical mechanism of lysosomal down-regulation. *J. Biol. Chem.* 286, 40193–40204. doi: 10.1074/jbc.M111.288555

Hong, M. H., Xu, C., Wang, Y. J., Ji, J. L., Tao, Y. M., Xu, X. J., et al. (2009). Role of Src in ligand-specific regulation of delta-opioid

receptor desensitization and internalization. *J. Neurochem*. 108, 102–114. doi: 10.1111/j.1471-4159.2008.05740.x

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258, 577–580. doi: 10.1038/258577a0

Huidobro, F., Huidobro-Toro, J. P., and Leong Way, E. (1976). Studies on tolerance development to single doses of morphine in mice. *J. Pharmacol. Exp. Ther.* 198, 318–329.

Hull, L. C., Llorente, J., Gabra, B. H., Smith, F. L., Kelly, E., Bailey, C., et al. (2010). The effect of protein kinase C and G protein-coupled receptor kinase inhibition on tolerance induced by mu-opioid agonists of different efficacy. *J. Pharmacol. Exp. Ther.* 332, 1127–1135. doi: 10.1124/jpet.109.161455

Illing, S., Mann, A., and Schulz, S. (2014). Heterologous regulation of agonist-independent mu-opioid receptor phosphorylation by protein kinase C. *Br. J. Pharmacol.* 171, 1330–1340. doi: 10.1111/bph.12546

Javed, R. R., Dewey, W. L., Smith, P. A., and Smith, F. L. (2004). PKC and PKA inhibitors reverse tolerance to morphine-induced hypothermia and supraspinal analgesia in mice. *Eur. J. Pharmacol.* 492, 149–157. doi: 10.1016/j.ejphar.2004.03.061

Johnson, E. A., Oldfield, S., Braksator, E., Gonzalez-Cuello, A., Couch, D., Hall, K. J., et al. (2006). Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Mol. Pharmacol.* 70, 676–685. doi: 10.1124/mol.106.022376

Just, S., Illing, S., Trester-Zedlitz, M., Lau, E. K., Kotowski, S. J., Miess, E., et al. (2013). Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol. Pharmacol.* 83, 633–639. doi: 10.1124/mol.112.082875

Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., et al. (1982). Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor. *Nature* 298, 245–249. doi: 10.1038/298245a0

Keith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., et al. (1998). mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. *Mol. Pharmacol.* 53, 377–384.

Kelly, E. (2013). Ligand bias at the mu-opioid receptor. *Biochem. Soc. Trans.* 41, 218–224. doi: 10.1042/BST20120331

Kenakin, T. (2011). Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* 336, 296–302. doi: 10.1124/jpet.110.173948

Kieffer, B. L. (1999). Opioids: first lessons from knockout mice. *Trends Pharmacol. Sci.* 20, 19–26. doi: 10.1016/S0165-6147(98)01279-6

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

Kim, J. A., Bartlett, S., He, L., Nielsen, C. K., Chang, A. M., Kharazia, V., et al. (2008). Morphine-induced receptor endocytosis in a novel knockin mouse reduces tolerance and dependence. *Curr. Biol.* 18, 129–135. doi: 10.1016/j.cub.2007.12.057

Knapp, R. J., Malatynska, E., Collins, N., Fang, L., Wang, J. Y., Hruby, V. J., et al. (1995). Molecular biology and pharmacology of cloned opioid receptors. *FASEB J.* 9, 516–525.

Koch, T., Schulz, S., Pfeiffer, M., Klutzny, M., Schroder, H., Kahl, E., et al. (2001). C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. *J. Biol. Chem.* 276, 31408–31414. doi: 10.1074/jbc.M100305200

Koch, T., Widera, A., Bartzsch, K., Schulz, S., Brandenburg, L. O., Wundrack, N., et al. (2005). Receptor endocytosis counteracts the development of opioid tolerance. *Mol. Pharmacol.* 67, 280–287. doi: 10.1124/mol.104.004994

Konig, C., Gavrilova-Ruch, O., Von Banchet, G. S., Bauer, R., Grun, M., Hirsch, E., et al. (2010). Modulation of mu opioid receptor desensitization in peripheral sensory neurons by phosphoinositide 3-kinase gamma. *Neuroscience* 169, 449–454. doi: 10.1016/j.neuroscience.2010.04.068

Kouhen, O. M., Wang, G., Solberg, J., Erickson, L. J., Law, P. Y., and Loh, H. H. (2000). Hierarchical phosphorylation of delta-opioid receptor regulates agonist-induced receptor desensitization and internalization. *J. Biol. Chem.* 275, 36659–36664. doi: 10.1074/jbc.M006788200

Kovoor, A., Celver, J., Abdryashitov, R. I., Chavkin, C., and Gurevich, V. V. (1999). Targeted construction of phosphorylation-independent beta-arrestin mutants with constitutive activity in cells. *J. Biol. Chem.* 274, 6831–6834. doi: 10.1074/jbc.274.11.6831

Kramer, H. K., Andria, M. L., Esposito, D. H., and Simon, E. J. (2000a). Tyrosine phosphorylation of the delta-opioid receptor. Evidence for its role in mitogen-activated protein kinase activation and receptor internalization*. *Biochem. Pharmacol.* 60, 781–792. doi: 10.1016/S0006-2952(00)00400-7

Kramer, H. K., Andria, M. L., Kushner, S. A., Esposito, D. H., Hiller, J. M., and Simon, E. J. (2000b). Mutation of tyrosine 318 (Y318F) in the delta-opioid receptor attenuates tyrosine phosphorylation, agonist-dependent receptor internalization, and mitogen-activated protein kinase activation. *Brain Res. Mol. Brain Res.* 79, 55–66. doi: 10.1016/S0169-328X(00)00097-8

Lamb, K., Tidgewell, K., Simpson, D. S., Bohn, L. M., and Prisinzano, T. E. (2012). Antinociceptive effects of herkinorin, a MOP receptor agonist derived from salvinorin A in the formalin test in rats: new concepts in mu opioid receptor pharmacology: from a symposium on new concepts in mu-opioid pharmacology. *Drug Alcohol Depend.* 121, 181–188. doi: 10.1016/j.drugalcdep.2011.10.026

Lau, E. K., Trester-Zedlitz, M., Trinidad, J. C., Kotowski, S. J., Krutchinsky, A. N., Burlingame, A. L., et al. (2011). Quantitative encoding of the effect of a partial agonist on individual opioid receptors by multisite phosphorylation and threshold detection. *Sci. Signal.* 4, ra52. doi: 10.1126/scisignal.2001748

Law, P. Y., Hom, D. S., and Loh, H. H. (1982). Loss of opiate receptor activity in neuroblastoma X glioma NG108-15 hybrid cells after chronic opiate treatment. A multiple-step process. *Mol. Pharmacol.* 22, 1–4.

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

Lecoq, I., Marie, N., Jauzac, P., and Allouche, S. (2004). Different regulation of human delta-opioid receptors by SNC-80 [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenz yl]-N,N-diethylbenzamide] and endogenous enkephalins. *J. Pharmacol. Exp. Ther.* 310, 666–677. doi: 10.1124/jpet.103.063958

Levitt, E. S., and Williams, J. T. (2012). Morphine desensitization and cellular tolerance are distinguished in rat locus ceruleus neurons. *Mol. Pharmacol.* 82, 983–992. doi: 10.1124/mol.112.081547

Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L., and Liu-Chen, L. Y. (1999). U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. *J. Biol. Chem.* 274, 12087–12094. doi: 10.1074/jbc.274.17.12087

Li, J. G., Zhang, F., Jin, X. L., and Liu-Chen, L. Y. (2003). Differential regulation of the human kappa opioid receptor by agonists: etorphine and levorphanol reduced dynorphin A- and U50,488H-induced internalization and phosphorylation. *J. Pharmacol. Exp. Ther.* 305, 531–540. doi: 10.1124/jpet.102.045559

Li, J., Li, J.-G., Chen, C., Zhang, F., and Liu-Chen, L.-Y. (2002). Molecular basis of differences in (-)(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide-induced desensitization and phosphorylation between human and rat kappa-opioid receptors expressed in Chinese hamster ovary cells. *Mol. Pharmacol.* 61, 73–84. doi: 10.1124/mol.61.1.73

Ling, K., Ma, L., and Pei, G. (1998). Differential efficacies of kappa agonists to induce homologous desensitization of human kappa opioid receptor. *Neurosci. Lett.* 240, 25–28. doi: 10.1016/S0304-3940(97)00921-X

Liu, J. G., Liao, X. P., Gong, Z. H., and Qin, B. Y. (1999a). The difference between methadone and morphine in regulation of delta-opioid receptors underlies the antagonistic effect of methadone on morphine-mediated cellular actions. *Eur. J. Pharmacol.* 373, 233–239. doi: 10.1016/S0014-2999(99)00270-8

Liu, J. G., Liao, X. P., Gong, Z. H., and Qin, B. Y. (1999b). Methadone-induced desensitization of the delta-opioid receptor is mediated by uncoupling of receptor from G protein. *Eur. J. Pharmacol.* 374, 301–308. doi: 10.1016/S0014-2999(99)00322-2

Liu, J. G., and Prather, P. L. (2001). Chronic exposure to mu-opioid agonists produces constitutive activation of mu-opioid receptors in direct proportion to the efficacy of the agonist used for pretreatment. *Mol. Pharmacol.* 60, 53–62.

Llorente, J., Lowe, J. D., Sanderson, H. S., Tsisanova, E., Kelly, E., Henderson, G., et al. (2012). mu-Opioid receptor desensitization: homologous or heterologous? *Eur. J. Neurosci.* 36, 3636–3642. doi: 10.1111/ejn.12003

Lowe, J. D., Celver, J. P., Gurevich, V. V., and Chavkin, C. (2002). mu-Opioid receptors desensitize less rapidly than delta-opioid receptors due to

less efficient activation of arrestin. *J. Biol. Chem.* 277, 15729–15735. doi: 10.1074/jbc.M200612200

Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., et al. (2012). Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature* 485, 321–326. doi: 10.1038/nature10954

Mann, A., Illing, S., Miess, E., and Schulz, S. (2014). Different mechanisms of homologous and heterologous μ-opioid receptor phosphorylation. *Br. J. Pharmacol*. doi: 10.1111/bph.12627. [Epub ahead of print].

Mansour, A., Fox, C. A., Akil, H., and Watson, S. J. (1995). Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 18, 22–29. doi: 10.1016/0166-2236(95)93946-U

Marchese, A., and Trejo, J. (2013). Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling. *Cell. Signal.* 25, 707–716. doi: 10.1016/j.cellsig.2012.11.024

Marie, N., Aguila, B., Hasbi, A., Davis, A., Jauzac, P., and Allouche, S. (2008). Different kinases desensitize the human delta-opioid receptor (hDOP-R) in the neuroblastoma cell line SK-N-BE upon peptidic and alkaloid agonists. *Cell. Signal.* 20, 1209–1220. doi: 10.1016/j.cellsig.2008.02.010

Marie, N., Landemore, G., Debout, C., Jauzac, P., and Allouche, S. (2003a). Pharmacological characterization of AR-M1000390 at human delta opioid receptors. *Life Sci.* 73, 1691–1704. doi: 10.1016/S0024-3205(03)00489-2

Marie, N., Lecoq, I., Jauzac, P., and Allouche, S. (2003b). Differential sorting of human delta-opioid receptors after internalization by peptide and alkaloid agonists. *J. Biol. Chem.* 278, 22795–22804. doi: 10.1074/jbc.M300084200

Massotte, D. (2014). *In vivo* opioid receptor heteromerization: where do we stand? *Br. J. Pharmacol*. doi: 10.1111/bph.12702. [Epub ahead of print].

Mclaughlin, J. P., Myers, L. C., Zarek, P. E., Caron, M. G., Lefkowitz, R. J., Czyzyk, T. A., et al. (2004). Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J. Biol. Chem.* 279, 1810–1818. doi: 10.1074/jbc.M305796200

Mclaughlin, J. P., Xu, M., Mackie, K., and Chavkin, C. (2003). Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. *J. Biol. Chem.* 278, 34631–34640. doi: 10.1074/jbc.M304022200

Mcpherson, J., Rivero, G., Baptist, M., Llorente, J., Al-Sabah, S., Krasel, C., et al. (2010). μ-opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol. Pharmacol.* 78, 756–766. doi: 10.1124/mol.110.066613

Megaritis, G., Merkouris, M., and Georgoussi, Z. (2000). Functional domains of delta- and mu-opioid receptors responsible for adenylyl cyclase inhibition. *Receptors Channels* 7, 199–212.

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

Meng, F., Xie, G. X., Thompson, R. C., Mansour, A., Goldstein, A., Watson, S. J., et al. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc. Natl. Acad. Sci. U.S.A.* 90, 9954–9958. doi: 10.1073/pnas.90.21.9954

Metzger, T. G., and Ferguson, D. M. (1995). On the role of extracellular loops of opioid receptors in conferring ligand selectivity. *FEBS Lett.* 375, 1–4. doi: 10.1016/0014-5793(95)01185-H

Meunier, J. C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* 377, 532–535. doi: 10.1038/377532a0

Mittal, N., Tan, M., Egbuta, O., Desai, N., Crawford, C., Xie, C. W., et al. (2012). Evidence that behavioral phenotypes of morphine in beta-arr2-/- mice are due to the unmasking of JNK signaling. *Neuropsychopharmacology* 37, 1953–1962. doi: 10.1038/npp.2012.42

Mohammed, W., Alhaddad, H., Marie, N., Tardy, F., Lamballais, F., Risede, P., et al. (2013). Comparison of tolerance to morphine-induced respiratory and analgesic effects in mice. *Toxicol. Lett.* 217, 251–259. doi: 10.1016/j.toxlet.2012.12.021

Molinari, P., Vezzi, V., Sbraccia, M., Grò, C., Riitano, D., Ambrosio, C., et al. (2010). Morphine-like opiates selectively antagonize receptor-arrestin interactions. *J. Biol. Chem.* 285, 12522–12535. doi: 10.1074/jbc.M109.059410

Mollereau, C., Parmentier, M., Mailleux, P., Butour, J. L., Moisand, C., Chalon, P., et al. (1994). ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett*. 341, 33–38. doi: 10.1016/0014-5793(94)80235-1

Morse, M., Sun, H., Tran, E., Levenson, R., and Fang, Y. (2013). Label-free integrative pharmacology on-target of opioid ligands at the opioid receptor family. *BMC Pharmacol. Toxicol.* 14:17. doi: 10.1186/2050-6511-14-17

Moulédous, L., Froment, C., Dauvillier, S., Burlet-Schiltz, O., Zajac, J.-M., and Mollereau, C. (2012). GRK2 protein-mediated transphosphorylation contributes to loss of function of μ-opioid receptors induced by neuropeptide FF (NPFF2) receptors. *J. Biol. Chem.* 287, 12736–12749. doi: 10.1074/jbc.M111.314617

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C., Cohen, S. N., et al. (1979). Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 278, 423–427. doi: 10.1038/278423a0

Namir, N., Polastron, J., Allouche, S., Hasbi, A., and Jauzac, P. (1997). The delta-opioid receptor in SK-N-BE human neuroblastoma cell line undergoes heterologous desensitization. *J. Neurochem.* 68, 1764–1772. doi: 10.1046/j.1471-4159.1997.68041764.x

Navratilova, E., Eaton, M. C., Stropova, D., Varga, E. V., Vanderah, T. W., Roeske, W. R., et al. (2005). Morphine promotes phosphorylation of the human delta-opioid receptor at serine 363. *Eur. J. Pharmacol.* 519, 212–214. doi: 10.1016/j.ejphar.2005.07.024

Navratilova, E., Waite, S., Stropova, D., Eaton, M. C., Alves, I. D., Hruby, V. J., et al. (2007). Quantitative evaluation of human delta opioid receptor desensitization using the operational model of drug action. *Mol. Pharmacol.* 71, 1416–1426. doi: 10.1124/mol.106.030023

Noble, F., and Cox, B. M. (1996). Differential desensitization of mu- and delta-opioid receptors in selected neural pathways following chronic morphine treatment. *Br. J. Pharmacol.* 117, 161–169. doi: 10.1111/j.1476-5381.1996.tb15169.x

Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., et al. (2011). Distinct phosphorylation sites on the beta(2)-adrenergic receptor establish a barcode that encodes differential functions of beta-arrestin. *Sci. Signal.* 4, ra51. doi: 10.1126/scisignal.2001707

Noda, M., Teranishi, Y., Takahashi, H., Toyosato, M., Notake, M., Nakanishi, S., et al. (1982). Isolation and structural organization of the human preproenkephalin gene. *Nature* 297, 431–434. doi: 10.1038/297431a0

Nowoczyn, M., Marie, N., Coulbault, L., Hervault, M., Davis, A., Hanouz, J. L., et al. (2013). Remifentanil produces cross-desensitization and tolerance with morphine on the mu-opioid receptor. *Neuropharmacology* 73, 368–379. doi: 10.1016/j.neuropharm.2013.06.010

Ong, E. W., and Cahill, C. M. (2014). Molecular perspectives for mu/delta opioid receptor heteromers as distinct, functional receptors. *Cells* 3, 152–179. doi: 10.3390/cells3010152

Pan, L., Xu, J., Yu, R., Xu, M. M., Pan, Y. X., and Pasternak, G. W. (2005). Identification and characterization of six new alternatively spliced variants of the human mu opioid receptor gene, Oprm. *Neuroscience* 133, 209–220. doi: 10.1016/j.neuroscience.2004.12.033

Pasternak, D. A., Pan, L., Xu, J., Yu, R., Xu, M. M., Pasternak, G. W., et al. (2004). Identification of three new alternatively spliced variants of the rat mu opioid receptor gene: dissociation of affinity and efficacy. *J. Neurochem.* 91, 881–890. doi: 10.1111/j.1471-4159.2004.02767.x

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

Patierno, S., Anselmi, L., Jaramillo, I., Scott, D., Garcia, R., and Sternini, C. (2011). Morphine induces mu opioid receptor endocytosis in guinea pig enteric neurons following prolonged receptor activation. *Gastroenterology* 140, 618–626. doi: 10.1053/j.gastro.2010.11.005

Pei, G., Kieffer, B. L., Lefkowitz, R. J., and Freedman, N. J. (1995). Agonist-dependent phosphorylation of the mouse delta-opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C. *Mol. Pharmacol.* 48, 173–177.

Pert, C. B., and Snyder, S. H. (1973). Opiate receptor: demonstration in nervous tissue. *Science* 179, 1011–1014. doi: 10.1126/science.179.4077.1011

Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000). Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. *J. Biol. Chem.* 275, 13727–13736. doi: 10.1074/jbc.275.18.13727

Petaja-Repo, U. E., Hogue, M., Leskela, T. T., Markkanen, P. M., Tuusa, J. T., and Bouvier, M. (2006). Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. *J. Biol. Chem.* 281, 15780–15789. doi: 10.1074/jbc.M602267200

Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nat. Rev. Mol. Cell. Biol.* 3, 639–650. doi: 10.1038/nrm908

Polastron, J., Meunier, J. C., and Jauzac, P. (1994). Chronic morphine induces tolerance and desensitization of mu-opioid receptor but not down-regulation in rabbit. *Eur. J. Pharmacol.* 266, 139–146. doi: 10.1016/0922-4106(94)90103-1

Portoghese, P. S. (1965). A new concept on the mode of interaction of narcotic analgesics with receptors. *J. Med. Chem.* 8, 609–616. doi: 10.1021/jm00329a013

Portoghese, P. S., Sultana, M., and Takemori, A. E. (1990). Design of peptidomimetic delta opioid receptor antagonists using the message-address concept. *J. Med. Chem.* 33, 1714–1720. doi: 10.1021/jm00168a028

Pradhan, A. A., Becker, J. A., Scherrer, G., Tryoen-Toth, P., Filliol, D., Matifas, A., et al. (2009). *In vivo* delta opioid receptor internalization controls behavioral effects of agonists. *PLoS ONE* 4:e5425. doi: 10.1371/journal.pone.0005425

Pradhan, A. A., Smith, M. L., Kieffer, B. L., and Evans, C. J. (2012). Ligand-directed signalling within the opioid receptor family. *Br. J. Pharmacol.* 167, 960–969. doi: 10.1111/j.1476-5381.2012.02075.x

Pradhan, A. A., Walwyn, W., Nozaki, C., Filliol, D., Erbs, E., Matifas, A., et al. (2010). Ligand-directed trafficking of the delta-opioid receptor *in vivo*: two paths toward analgesic tolerance. *J. Neurosci.* 30, 16459–16468. doi: 10.1523/JNEUROSCI.3748-10.2010

Qiu, Y., Law, P. Y., and Loh, H. H. (2003). Mu-opioid receptor desensitization: role of receptor phosphorylation, internalization, and representation. *J. Biol. Chem.* 278, 36733–36739. doi: 10.1074/jbc.M305857200

Qiu, Y., Loh, H. H., and Law, P. Y. (2007). Phosphorylation of the delta-opioid receptor regulates its beta-arrestins selectivity and subsequent receptor internalization and adenylyl cyclase desensitization. *J. Biol. Chem.* 282, 22315–22323. doi: 10.1074/jbc.M611258200

Quillinan, N., Lau, E. K., Virk, M., Von Zastrow, M., and Williams, J. T. (2011). Recovery from mu-opioid receptor desensitization after chronic treatment with morphine and methadone. *J. Neurosci.* 31, 4434–4443. doi: 10.1523/JNEUROSCI.4874-10.2011

Quirion, R., Zajac, J. M., Morgat, J. L., and Roques, B. P. (1983). Autoradiographic distribution of mu and delta opiate receptors in rat brain using highly selective ligands. *Life Sci.* 33 (Suppl. 1), 227–230. doi: 10.1016/0024-3205(83)90484-8

Raehal, K. M., Schmid, C. L., Groer, C. E., and Bohn, L. M. (2011). Functional selectivity at the mu-opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol. Rev.* 63, 1001–1019. doi: 10.1124/pr.111.004598

Reinscheid, R. K., Nothacker, H. P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., et al. (1995). Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* 270, 792–794. doi: 10.1126/science.270.5237.792

Rivero, G., Llorente, J., Mcpherson, J., Cooke, A., Mundell, S. J., Mcardle, C. A., et al. (2012). Endomorphin-2: a biased agonist at the mu-opioid receptor. *Mol. Pharmacol.* 82, 178–188. doi: 10.1124/mol.112.078659

Rostami, A., Rabbani, M., and Mir-Mohammad-Sadeghi, M. (2010). The role of N53Q mutation on the rat mu-opioid receptor function. *J. Biomol. Tech.* 21, 92–96.

Schulz, S., Mayer, D., Pfeiffer, M., Stumm, R., Koch, T., and Hollt, V. (2004). Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *EMBO J.* 23, 3282–3289. doi: 10.1038/sj.emboj.7600334

Simon, E. J., Hiller, J. M., and Edelman, I. (1973). Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate. *Proc. Natl. Acad. Sci. U.S.A.* 70, 1947–1949. doi: 10.1073/pnas.70.7.1947

Soignier, R. D., Vaccarino, A. L., Fanti, K. A., Wilson, A. M., and Zadina, J. E. (2004). Analgesic tolerance and cross-tolerance to i.c.v. endomorphin-1, endomorphin-2, and morphine in mice. *Neurosci. Lett.* 366, 211–214. doi: 10.1016/j.neulet.2004.05.046

Song, S. L., and Chueh, S. H. (1999). Phosphorylation promotes the desensitization of the opioid-induced Ca2+ increase in NG108-15 cells. *Brain Res.* 818, 316–325. doi: 10.1016/S0006-8993(98)01216-5

Stafford, K., Gomes, A. B., Shen, J., and Yoburn, B. C. (2001). mu-Opioid receptor downregulation contributes to opioid tolerance *in vivo*. *Pharmacol. Biochem. Behav.* 69, 233–237. doi: 10.1016/S0091-3057(01)00525-1

Tanowitz, M., and Von Zastrow, M. (2003). A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J. Biol. Chem.* 278, 45978–45986. doi: 10.1074/jbc.M304504200

Tejeda, H. A., Shippenberg, T. S., and Henriksson, R. (2012). The dynorphin/kappa-opioid receptor system and its role in psychiatric disorders. *Cell. Mol. Life Sci.* 69, 857–896. doi: 10.1007/s00018-011-0844-x

Terenius, L. (1973). Stereospecific interaction between narcotic analgesics and a synaptic plasm a membrane fraction of rat cerebral cortex. *Acta Pharmacol. Toxicol. (Copenh)* 32, 317–320. doi: 10.1111/j.1600-0773.1973.tb01477.x

Terman, G. W., Jin, W., Cheong, Y. P., Lowe, J., Caron, M. G., Lefkowitz, R. J., et al. (2004). G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. *Br. J. Pharmacol.* 141, 55–64. doi: 10.1038/sj.bjp.0705595

Thompson, R. C., Mansour, A., Akil, H., and Watson, S. J. (1993). Cloning and pharmacological characterization of a rat mu opioid receptor. *Neuron* 11, 903–913. doi: 10.1016/0896-6273(93)90120-G

Tsao, P. I., and Von Zastrow, M. (2000). Type-specific sorting of G protein-coupled receptors after endocytosis. *J. Biol. Chem.* 275, 11130–11140. doi: 10.1074/jbc.275.15.11130

Ueda, H., and Ueda, M. (2009). Mechanisms underlying morphine analgesic tolerance and dependence. *Front. Biosci. (Landmark Ed.)* 14, 5260–5272. doi: 10.2741/3596

Violin, J. D., Crombie, A. L., Soergel, D. G., and Lark, M. W. (2014). Biased ligands at G-protein-coupled receptors: promise and progress. *Trends Pharmacol. Sci.* 35, 308–316. doi: 10.1016/j.tips.2014.04.007

Walwyn, W., Evans, C. J., and Hales, T. G. (2007). Beta-arrestin2 and c-Src regulate the constitutive activity and recycling of mu opioid receptors in dorsal root ganglion neurons. *J. Neurosci.* 27, 5092–5104. doi: 10.1523/JNEUROSCI.1157-07.2007

Wang, H. L. (2000). A cluster of Ser/Thr residues at the C-terminus of mu-opioid receptor is required for G protein-coupled receptor kinase 2-mediated desensitization. *Neuropharmacology* 39, 353–363. doi: 10.1016/S0028-3908(99)00174-4

Whistler, J. L., Chuang, H. H., Chu, P., Jan, L. Y., and Von Zastrow, M. (1999). Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 23, 737–746. doi: 10.1016/S0896-6273(01)80032-5

Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., et al. (2002). Modulation of postendocytic sorting of G protein-coupled receptors. *Science* 297, 615–620. doi: 10.1126/science.1073308

Whistler, J. L., and Von Zastrow, M. (1998). Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9914–9919. doi: 10.1073/pnas.95.17.9914

White, J. M., and Irvine, R. J. (1999). Mechanisms of fatal opioid overdose. *Addiction* 94, 961–972. doi: 10.1046/j.1360-0443.1999.9479612.x

Willets, J., and Kelly, E. (2001). Desensitization of endogenously expressed delta-opioid receptors: no evidence for involvement of G protein-coupled receptor kinase 2. *Eur. J. Pharmacol.* 431, 133–141. doi: 10.1016/S0014-2999(01)01360-7

Williams, J. T. (2014). Desensitization of functional micro-opioid receptors increases agonist off-rate. *Mol. Pharmacol.* 86, 52–61. doi: 10.1124/mol.114.092098

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., Von Zastrow, M., Schulz, S., et al. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254. doi: 10.1124/pr.112.005942

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., et al. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* 485, 327–332. doi: 10.1038/nature10939

Xiang, B., Yu, G. H., Guo, J., Chen, L., Hu, W., Pei, G., et al. (2001). Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization. *J. Biol. Chem.* 276, 4709–4716. doi: 10.1074/jbc.M006187200

Xie, W. Y., He, Y., Yang, Y. R., Li, Y. F., Kang, K., Xing, B. M., et al. (2009). Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the delta-opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. *J. Neurosci.* 29, 3551–3564. doi: 10.1523/JNEUROSCI.0415-09.2009

Xu, W., Chen, C., Li, J. G., Dimattio, K., Wang, Y., Unterwald, E., et al. (2013). PKA and ERK1/2 are involved in dopamine D(1) receptor-induced heterologous

desensitization of the delta opioid receptor. *Life Sci.* 92, 1101–1109. doi: 10.1016/j.lfs.2013.04.006

Yan, Y. H., Wang, Y., Zhao, L. X., Jiang, S., Loh, H. H., Law, P. Y., et al. (2014). Role of FK506 binding protein 12 in morphine-induced mu-opioid receptor internalization and desensitization. *Neurosci. Lett.* 566, 231–235. doi: 10.1016/j.neulet.2014.02.059

Yang, L., Seifert, A., Wu, D., Wang, X., Rankovic, V., Schroder, H., et al. (2010). Role of phospholipase D2/phosphatidic acid signal transduction in micro- and delta-opioid receptor endocytosis. *Mol. Pharmacol.* 78, 105–113. doi: 10.1124/mol.109.063107

Yoon, S. H., Jin, W., Spencer, R. J., Loh, H. H., and Thayer, S. A. (1998). Desensitization of delta-opioid-induced mobilization of Ca2+ stores in NG108-15 cells. *Brain Res.* 802, 9–18. doi: 10.1016/S0006-8993(98)00531-9

Yu, Y., Zhang, L., Yin, X., Sun, H., Uhl, G. R., and Wang, J. B. (1997). Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J. Biol. Chem.* 272, 28869–28874. doi: 10.1074/jbc.272.46.28869

Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. R., Laporte, S. A., Law, P. Y., et al. (1998). Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7157–7162. doi: 10.1073/pnas.95.12.7157

Zhang, L., Loh, H. H., and Law, P. Y. (2013). A novel noncanonical signaling pathway for the mu-opioid receptor. *Mol. Pharmacol.* 84, 844–853. doi: 10.1124/mol.113.088278

Zhang, L., Zhao, H., Qiu, Y., Loh, H. H., and Law, P.-Y. (2009). Src phosphorylation of micro-receptor is responsible for the receptor switching from an inhibitory to a stimulatory signal. *J. Biol. Chem.* 284, 1990–2000. doi: 10.1074/jbc.M807971200

Zhang, X., Wang, F., Chen, X., Chen, Y., and Ma, L. (2008). Post-endocytic fates of delta-opioid receptor are regulated by GRK2-mediated receptor phosphorylation and distinct beta-arrestin isoforms. *J. Neurochem.* 106, 781–792. doi: 10.1111/j.1471-4159.2008.05431.x

Zhang, X., Wang, F., Chen, X., Li, J., Xiang, B., Zhang, Y.-Q., et al. (2005). Beta-arrestin1 and beta-arrestin2 are differentially required for phosphorylation-dependent and -independent internalization of delta-opioid receptors. *J. Neurochem.* 95, 169–178. doi: 10.1111/j.1471-4159.2005.03352.x

Zheng, H., Chu, J., Zhang, Y., Loh, H. H., and Law, P. Y. (2011). Modulating micro-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKCepsilon activation and dendritic spine stability. *J. Biol. Chem.* 286, 12724–12733. doi: 10.1074/jbc.M110.177089

Zhu, J., Luo, L. Y., Mao, G. F., Ashby, B., and Liu-Chen, L. Y. (1998). Agonist-induced desensitization and down-regulation of the human kappa opioid receptor expressed in Chinese hamster ovary cells. *J. Pharmacol. Exp. Ther.* 285, 28–36.

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