Published in final edited form as:

Trends Pharmacol Sci. 2016 April ; 37(4): 279–289. doi:10.1016/j.tips.2016.01.001.

New technologies for elucidating opioid receptor function

Michael R. Bruchas¹ and Bryan L. Roth²

¹Departments of Anesthesiology and Neurobiology, Washington University, School of Medicine, St. Louis, MO bruchasm@wustl.edu

²Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC broth@med.unc.edu

Abstract

Recent advances in technology, including high resolution crystal structures of opioid receptors, novel chemical tools, and new genetic approaches have provided an unparalleled palette of tools for deconstructing opioid receptor actions *in vitro* and *in vivo*. Here we provide a brief description of our understanding of opioid receptor function from both molecular and atomic perspectives, as well as their role in neural circuits *in vivo*. We then show how insights into the molecular details of opioid actions can facilitate the creation of functionally-selective (biased) and photoswitchable opioid ligands. Finally, we describe how newly engineered opioid receptor-based chemo- and optogenetic tools, and new mouse lines are expanding and transforming our understanding of opioid function and, perhaps, paving the way for new therapeutics.

Keywords

DREADDs; optogenetics; chemogenetics; biased signaling; functional selectivity; mouse models

New insights into the structure and function of opioid receptors facilitate small molecule and chemogenetic technologies

Although the historical aspects of opioid receptor science have been extensively summarized (1, 2), it is helpful to consider that three major classes of classical opioid receptors— μ , δ , κ —were originally identified via both pharmacological and radioligand binding approaches, without any insights into their molecular structure (see for example (3) (4) (5)). Parenthetically, it is useful to consider that, prior to the molecular cloning of the four known major opioid receptor subtypes (6) (7) (8) (9), some had even suggested that opioid receptors might not be proteins but rather cerebroside sulfate (see (10) for example).

It was with some excitement then that the inactive state structures of all four known mammalian opioid receptors were reported in 2012. Thus the structures of the mouse μ (11), human κ (12), mouse δ (13) and human nociceptin (NOP) (14) receptors appeared in the

same issue of Nature. Both the authors of the structural elucidation studies (see (11), (12), (13) (14)) and others (15, 16) have predicted that these new structures will accelerate structure-guided drug discovery. To date, modest successes have been reported for structure-guided drug discovery of new NOP (17) and κ - (18) opioid receptor (KOR) ligands, providing new chemotypes with modest potency. Additionally, nM potency, selective μ -receptor (MOR) G-protein biased agonists of novel chemotypes from structure-based screens *in silico* have been reported (Manglik et al, submitted). Given these initial successes, continuing and expanding these structure-guided approaches could provide many new opioid receptor ligands with greater therapeutic potential and reduced side-effects (Manglik et al, submitted).

Structural elucidation of opioid receptors—as might be expected—has also been useful for identifying potential modes by which ligands bind to multiple receptors. Thus, for instance, site-directed mutagenesis and structure-guided docking studies have provided novel insights into KOR binding for both conventional and novel agonists and antagonists (19). These studies have revealed that different chemotypes likely adopt different poses in the KOR binding pocket. Arylacetamides like U69,593 (Figure 1A) and diterpenes like salvinorin A (Figure 1B) are predicted to adopt both distinct and overlapping binding modes in KOR (19). Indeed, it is clear that salvinorin A, for instance, differs from all other KOR agonists in that its binding is not dependent upon a strong ionic interaction with the highly conserved aspartic acid in transmembrane domain III (TMIII; Figure 1B) (19).

Functionally-selective opioid ligands

Based on their predicted different binding poses one might suppose that salvinorin A and U69,593 might display different functional selectivity (20) or biased signaling (21). However, both U69593 (Figure 1A) and salvinorin A (Figure 1B) appear to be balanced hKOR agonists *in vitro*. By contrast, a comprehensive screen of KOR agonists and other known drugs and drug-like compounds revealed that the arylacetamide ICI 199,441 displays a modest degree of β -arrestin bias (Figure 1A), while the salvinorin A derivative RB-64 represents a highly G-protein biased agonist *in vitro* (Figure 1B; (22)). Determining the structural features responsible for biased signaling at KOR and other opioid receptors would, obviously, be transformative for structure-based design of functionally-selective ligands.

Given that prior studies revealed that RB-64 is active *in vivo* (23) the authors comprehensively studied the actions of RB-64 compared with reference KOR agonists to clarify the role(s) of G-protein vs β -arrestin-ergic signaling in mice. Initial studies indicated that RB-64 has psychotomimetic-like activity (23) in that it disrupted the pre-pulse inhibition of startle response—which is widely used to predict psychotomimetic actions of drugs (24). Next, studies in wild-type (WT) and β -arrestin2 (β ARR2) KO mice revealed that the analgesic effects of the balanced KOR agonists U69593 and salvinorin A as well as the G-protein biased agonist RB-64 were unaffected by β -arrestin2 gene disruption (25) (26), suggesting that KOR analgesia was due at least in part to G protein signaling. Similar results were recently reported for KOR-mediated inhibition of pruritus (27). Thus, a G-protein biased agonist of a different chemotype-- isoquinolinone 2.1—was as effective as the balanced agonist U50,488H for the inhibition of pruritus (27). These findings are broadly

supportive of prior studies performed with balanced KOR agonists (28) suggesting that analgesic actions of KOR agonists might be mediated by canonical G-protein signaling (Figure 1C)

KOR agonists, in addition to their analgesic (29) and psychotomimetic actions (29, 30, 31, 32), are sedative (3), aversive (33), impair coordination (3), and induce dysphoria (31) and anhedonia (28, 34). Significantly, the G-protein biased agonist RB-64 displayed a slower onset and decline of analgesia when compared with salvinorin A—as might be predicted based on its weak activity at arrestin-ergic signaling as arrestin ‘arrests’ or inhibits G-protein signaling of GPCRs (35, 36). Thus, at 30 min following administration, mice treated with RB-64 still displayed an analgesic response, while mice treated with U69593 and salvinorin A did not. RB-64 had no effect in a model of anhedonia, although it did induce conditioned place aversion in both WT and bARR2 KO mice (25). RB-64 had little effect on locomotion in the open field and treated mice showed a lower degree of motoric coordination— similar to results obtained for salvinorin A in β Arrestin2 KO mice. Taken together, these results (25) (27) support the notion that G-protein biased KOR agonists might represent novel analgesic agents with a reduced side-effect profile when compared with balanced, centrally active KOR agonists. Additionally as differences in signaling bias are but one explanation for these findings, further studies with more highly biased compounds having good drug-like properties are needed to fully test this hypothesis.

High resolution structures of opioid receptors and relevance for chemogenetics

Recently, a high resolution structure of a nanobody-stabilized state of the mouse MOR was reported, along with biochemical and molecular dynamics simulations of the MOR activation process (37, 38). Nanobodies are single-chain antibodies that are increasingly used in GPCR structural biology to stabilize various active states (39). Additionally, the highest resolution structure to date for any opioid receptor (1.8Å) was reported for the inactive state of DOR (40). Further, the first x-ray crystallographic (41) and NMR-based (42) structures of peptides in complex with opioid receptors have been recently reported. Not surprisingly, global conformational changes are evident when comparing the nanobody-stabilized conformation of MOR with the inactive state (37, 38). These are similar to those that have been seen before when comparing nanobody-stabilized active and inactive states of β 2-adrenergic (43) (44) and M2 muscarinic (45) receptors. Of note, the highly conserved sodium ion site, which stabilizes the inactive state of many GPCRs (Figure 2A, 2B) (46, 47, 48, 49) has disappeared in the active state of MOR (Figure 2D), though it is predicted to occur in the inactive MOR state (Figure 2C). Sodium ions have been demonstrated as negative allosteric modulators for opioid receptors *in situ* (50) (51), as well as cloned and purified opioid receptors *in vitro* (40).

Using the high resolution structure of KOR, along with comprehensive mutagenesis and molecular modeling (19, 52), the authors predicted that salvinorin B—an inactive metabolite of salvinorin A—would show enhanced agonist potency for the D138N mutant KOR (Figure 2E, F). Additionally, given the ubiquitous nature of the interaction of D138 with basic nitrogens seen in all endogenous opioid peptides—and as predicted by structural studies (41, 42)—the authors anticipated that the D138N mutation would also be insensitive to opioid

peptides as well as non-peptide KOR agonists. Thus, not only did the D138N hKOR mutant receptor show enhanced affinity for salvinorin B, but it also was insensitive to all tested opioid peptides and nitrogen-containing non-peptide agonists (52).

Given that the D138N mutant could be activated by the inactive KOR ligand salvinorin B, it was dubbed it κ -opioid receptor DREADD (Designer Receptor Exclusively Activated by Designer Drug (53)) or KORD (52). Several reports have now demonstrated that KORD silences neurons *in vivo* and that this silencing affects behaviors in a manner consistent with neuronal silencing (52) (54, 55). Additionally, as KORD is activated by salvinorin B, it can be used in combination with the clozapine-N-oxide (CNO)-based DREADDs (53, 56) for the multiplexed chemogenetic modulation of signaling and behavior (52, 57). Thus, based on high-resolution structures of KOR, a new DREADD-based chemogenetic tool has been developed that should be broadly useful for interrogating neural circuits and signaling.

Optogenetic tools for simulating opioid signaling *in vitro* and *in vivo*

The field of optogenetic innovation has been growing rapidly, with most of the efforts by protein engineers focused on developing novel channel opsins, with shifted kinetics, on/off rates, or ion filters (58) (59) (60). However, a few groups, including our own, have been working to develop and characterize GPCR versions of optogenetic tools, which would allow for spatiotemporal engagement of opioid signaling *in vitro* and *in vivo* (61) (62) (63) (64). Capitalizing on these recent efforts via molecular modeling in iTASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) the authors generated a chimeric receptor that contains the intracellular components (loops and C-terminus) of the rat MOR fused to the hydrophobic and extracellular components of the rat rhodopsin receptor (62). Our goal was to design and implement a photosensitive MOR-like receptor that responds to light-based stimulation by signaling to intracellular pathways with the same properties of its WT counterpart. In this report (62), the authors demonstrated that photo-stimulation results in canonical MOR signaling as measured by inhibition of cyclic AMP production, receptor desensitization, coupling to G-protein coupled inwardly-rectifying potassium (GIRK) channels, activation of mitogen-activated protein kinase cascades, and receptor internalization. Furthermore, using this approach combined with cre-loxP mouse genetic approaches, the authors expressed an AAV5-opto-MOR-YFP receptor in GABAergic neurons of the ventral tegmental area (VTA) and found that photostimulation of this pathway was rewarding, due to an opioid-like disinhibition of GABAergic tone (65, 66). Other recent reports have used expression of both adrenergic and serotonergic rhodopsin-like opto-GPCRs in structures including the dorsal raphe and basolateral amygdala to regulate anxiety behavior (61, 64, 67). Taken together, these results suggest new versions of the opto-MOR, or other opioid-receptor-rhodopsin chimeric approaches, could facilitate the millisecond control of opioid signaling, and thereby elucidate its relevance for temporally precise behaviors in defined circuits.

Additionally, the results obtained with opto-MOR (62), along with those obtained with the KOR-DREADD (52), highlight the potential to engage multiple opioid receptor signaling pathways in the same cell type. Given that many of these receptors are co-expressed, these new tools may provide a multiplexed method for dissecting receptor interactions, signaling,

and circuit level effects *in vitro* and *in vivo*. Future studies are warranted to determine how opto-opioid-like receptors function in peripheral circuits, and whether they can be further mutated or enhanced to better mimic endogenous opioid receptor function, as well as to better dissect the role of biased opioid receptor signaling *in vivo* with spatiotemporal control (28) (68) (69).

Optopharmacology for engaging opioid receptor signaling with spatiotemporal precision

Another useful advance in optical control of opioid receptor function is the recent development of photo-switchable opioid small molecules and neuropeptides. Key questions for investigating opioid receptors and their endogenous neuropeptides are “how, where, and when” endogenous peptides act within intact neural circuits. In order to begin to dissect this, the research teams of Bernardo Sabatini and John T., led by efforts of Matthew Banghart and others (70), have developed two opioid agonist peptide analogs: [Leu⁵]-enkephalin (CYLE) and the 8 amino acid form of Dynorphin A (CYDyn-8). These analogs contain a modified N-terminal carboxynitrobenzyl (CNB) chromophore, which is released at a high quantum efficiency upon photolysis. Importantly, these modified peptides are inert and functionally inactive in the absence of photolysis. However, when exposed to a pulse of ultraviolet (UV, 405nm) light, they become functional and may then activate endogenous opioid receptors. Banghart and colleagues (70) have shown robust *in vitro* data, demonstrating μ -opioid receptor-coupled GIRK channel coupling, suggesting that these compounds can be used reliably for dissecting MOR function.

Furthermore, small molecule photo-switchable opioid ligands have also been developed that can act to antagonize endogenous or exogenous opioid receptor agonists *in vitro* (71). Carboxynitroveratryl-naloxone (CNV-NLX) was generated as a caged analogue of the competitive opioid receptor antagonist naloxone (NLX). The authors (71) investigated its utility in both HEK cell and slice preparations. They reported that CNV-NLX, with dermorphin as the agonist, can block opioid receptor-mediated GIRK channel coupling after photo-uncaging. Interestingly, in this report the authors were able to utilize this novel tool to demonstrate that some MOR agonists have alternate deactivation rates, which are governed by their G-protein signaling, yet others are determined by agonist dissociation rate. In an elegant complementary study, using CYLE with CNV-NLX, two different alterations in opioid signaling were determined in MOR desensitization within locus coeruleus neurons (72). The author concludes that opioid receptor desensitization is both a reduction in “active” receptor number as well as a decrease in agonist-receptor affinity of the remaining receptor pool. The rapid spatiotemporal control of opioid ligands using photo-uncaging affords the investigator the ability to assess kinetics of association and dissociation. Further, photo-uncaging could reveal how quickly receptor-induced signaling activation/deactivation follows following receptor occupancy by ligand. These approaches are powerful additions to the opioid receptor tool box *in vitro*, and perhaps could eventually be used for behavioral and systems level experiments *in vivo*. *In vivo* photopharmacology has been a significant challenge because delivery of UV light to deep brain structures, along with pharmacological infusion is technically challenging, although new wireless devices that can co-deliver light and drug simultaneously may be promising in this respect (73). In this recent report, delivery of opioids (DAMGO) was demonstrated using a microfluidic probe.

Extensions of this technology using UV LEDs or other photo-switchable ligands could transform our understanding of the relationships between opioid ligands and receptor activity within the spatiotemporal framework of intact neural circuits (73).

Rodent genetic tools for dissecting opioid receptor function *in vivo*

Over the last decade one of the most useful animal tools utilized in the opioid field has been knockout mice of both the opioid receptors (MOR, KOR, DOR and NOP), and the peptides (POMC, Enkephalin, Preprodynorphin, Prepronociceptin) (74) (75) (76) (77) (78) (79). These global knockout mice have provided a clearer picture of the role of opioid receptors and their endogenous ligands in behavioral models of pain, analgesia, stress, depression, and anxiety (74) (75) (76) (77) (78) (79). More recently, conditional deletion approaches have been developed wherein loxP sites have been introduced flanking various opioid receptor exons, in order to utilize the power of cell-type selective cre-recombinase strategies for selective gene deletion within discrete cells and neural circuits (80) (81). Conditional knockout mice for μ , κ , and δ -opioid receptors have been developed. Initial experiments reveal discrete roles for individual neuronal populations and specific opioid receptor subtype expression (80, 81, 82). These mice have just become more widely available to the research community, and thus it is expected that future studies using them will reveal novel insights into receptor function within specific neural circuits.

Additional intersectional (e.g. using FLP recombinase) viral and knock-in approaches may further provide a powerful tool set for dissecting subsets of neurons expressing opioid receptors and their ligands (83, 84) (85). A recent example showed that selective deletion of KOR from dopamine neurons (DATcre+) has anxiolytic-like properties and alters cocaine-induced plasticity (81). In a complimentary study, Erlich and colleagues showed the rescue of KOR in KOR knockout mice (using a new Cre-dependent KOR virus) only in DATcre+ cells restores KOR-mediated conditioned place aversion (86). These types of experiments highlight both the genetic specificity and defined neural circuit contributions of distinct opioid receptors.

Rodent genetic tools for dissecting the roles of opioid peptides *in vivo*

Extending these conditional approaches for examining receptor function within discrete cell types have been efforts to develop conditional knockout mice for each endogenous opioid peptide, along with Cre-driver mice for each, so that neurons containing these peptides can be targeted using chemogenetic and optogenetic approaches (56) (Figure 3). Conditional knockout mice for proopiomelanocortin, proenkephalin, and prodynorphin, have each been developed and are in the initial testing phases for viability and phenotyping (87) (88) (89). Since each of these pre-pro-peptides generate a multiplicity of active peptide species, deleting the precursors will result in the deletion of many active peptides. Viral cre-recombinase, or INTERSECT (85) approaches are likely to have important implications when isolating the contributions of these neuropeptides to behavioral systems-level questions. In parallel, cre-driver mice of each opioid peptide will become more widely available. These will allow for expression of opto- and chemogenetic actuators in neurons expressing opioid peptides. Such Cre-driver mice would be useful for: dissecting the role of endogenous opioid tone, circuit-based opioid receptor function, how opioids are released,

and whether there are promiscuous opioid peptide-receptor interactions, as have been hypothesized and suggested over the past several years. Finally, several laboratories are also developing receptor-based cre-driver mice (*NOP-cre*, *MOR-cre*, *KOR-cre*, *DOR-cre*) for isolating populations of cells that express opioid receptors.

Recent efforts have begun to use dynorphin-cre driver mice (87) (88) in both chemo- and optogenetic experiments to define the endogenous nature of dynorphinergic tone on feeding behavior, reward, and aversion. The surprising findings thus far include the observation of optogenetically-evoked opioid neuropeptide release after relatively mild stimulation, as well as non-canonical roles for kappa-opioid mediated behavioral effects (Al-Hasani et al., 2015) (Figure 3). In contrast, POMC-cre driver animals have been useful for targeting the arcuate nucleus, and hippocampus (90), yet little is known about whether these neurons can be evoked to release proenkephalin, the endogenous MOR agonist, in an activity-dependent manner. Enkephalin-cre driver mice have recently been generated by the Allen Brain Institute, and are likely to be widely used in studies of basal ganglia function, as well as in reward neurobiology and neuropharmacology.

Finally, targeted GFP-, YFP-, and mCherry-opioid-receptor fusion mice are now being used to examine endogenous receptor localization, trafficking, and expression (91) (92) (93). Due to the constraints and limitations of opioid receptor antibodies, these mice have proven useful in determining the localization and kinetics of receptors within specific neuronal cell types. In particular, recent efforts using MOR-mCherry, and DOR-eGFP have determined that these receptors are expressed in distinct but overlapping populations dependent on the cell type and location. Furthermore, findings in NOPR-eGFP mice were recently published (91), and it was revealed that this receptor is expressed in a subpopulation of DRG neurons. Additionally, initial and intriguing data were reported on internalization properties of NOPR within endogenous neuronal cultures (91). These types of receptor-fusion fluorophore approaches do come with caveats, given that the GFP/mCherry tag could interfere with receptor trafficking and function. Additionally, background fluorescence can impede rigorous conclusions about subcellular localization of fusion proteins. Nevertheless, taken together with the other approaches described above, they provide another novel avenue of resolution that tells us how and where, opioid receptors are functional within intact neuronal circuits.

Concluding Remarks

In summary, we have outlined some of the recent advances in technology that have allowed for a deeper and more rigorous understanding of opioid receptor neurobiology and pharmacology. We have by no means provided a comprehensive survey of this rapidly progressing field, but instead we have focused on some of the emerging techniques, tools, and approaches, which have the potential to unravel historically critical mysteries in the field (see Outstanding Questions Box)

Clearly, improvements in high resolution structural determination of opioid receptors and complexes via crystallography along with enhanced GPCR modeling and docking approaches (94) will provide powerful templates for the structure-guided discovery of novel

opioid receptor ligands. Additional refinements of both chemo- and optogenetics, and viral delivery platforms will provide the technologies for resolving long-standing issues related to cell-type specific actions of opioid ligands and receptors. Finally, development of suitably drug-like and highly G-protein and β -arrestin biased ligands for all four opioid receptors will be extraordinarily valuable for elucidating the relative roles of canonical versus non-canonical signaling for the many actions mediated by exogenous and endogenous opioids. Although the field of opioid receptor pharmacology will always rest on pharmacological methods and concepts for its foundation, these emerging technologies provide an unparalleled opportunity for addressing key enigmas in opioid receptor structure and function.

References

1. Waldhoer M, et al. Opioid receptors. *Annu Rev Biochem.* 2004; 73:953–990. [PubMed: 15189164]
2. Pasternak GW, Pan YX. Mu opioids and their receptors: evolution of a concept. *Pharmacol Rev.* 2013; 65:1257–1317. [PubMed: 24076545]
3. Martin WR, et al. The effects of morphine and morphine-like drugs in the non-dependent and morphine-dependent chronic spinal dog. *Journal of Pharmacology and Experimental Therapeutics.* 1976; 197:517–532. [PubMed: 945347]
4. Robson LE, Kosterlitz HW. Specific protection of the binding sites of D-Ala²-D-Leu⁵-enkephalin (delta-receptors) and dihydromorphine (mu-receptors). *Proc R Soc Lond B Biol Sci.* 1979; 205:425–432. [PubMed: 41257]
5. Kosterlitz HW, Leslie FM. Comparison of the receptor binding characteristics of opiate agonists interacting with mu- or kappa-receptors. *Br J Pharmacol.* 1978; 64:607–614. [PubMed: 215262]
6. Evans CJ, et al. Cloning of a delta opioid receptor by functional expression. *Science.* 1992; 258:1952–1955. [PubMed: 1335167]
7. Chen Y, et al. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol Pharmacol.* 1993; 44:8–12. [PubMed: 8393525]
8. Li S, et al. Molecular cloning and expression of a rat kappa opioid receptor. *Biochem J.* 1993; 295(Pt 3):629–633. [PubMed: 8240268]
9. Mollereau C, et al. ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* 1994; 341:33–38. [PubMed: 8137918]
10. Loh HH, et al. Opiate binding to cerebroside sulfate: a model system for opiate-receptor interaction. *Life Sci.* 1975; 16:1811–1817. [PubMed: 1152609]
11. Manglik A, et al. Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature.* 2012; 485:321–326. [PubMed: 22437502]
12. Wu H, et al. Structure of the human kappa-opioid receptor in complex with JD₁Tic. *Nature.* 2012; 485:327–332. [PubMed: 22437504]
13. Granier S, et al. Structure of the delta-opioid receptor bound to naltrindole. *Nature.* 2012; 485:400–404. [PubMed: 22596164]
14. Thompson AA, et al. Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature.* 2012; 485:395–399. [PubMed: 22596163]
15. Shoichet BK, Kobilka BK. Structure-based drug screening for G-protein-coupled receptors. *Trends Pharmacol Sci.* 2012; 33:268–272. [PubMed: 22503476]
16. Filizola M, Devi LA. Grand opening of structure-guided design for novel opioids. *Trends Pharmacol Sci.* 2013; 34:6–12. [PubMed: 23127545]
17. Daga PR, et al. Structure-based virtual screening of the nociceptin receptor: hybrid docking and shape-based approaches for improved hit identification. *J Chem Inf Model.* 2014; 54:2732–2743. [PubMed: 25148595]
18. Negri A, et al. Discovery of a novel selective kappa-opioid receptor agonist using crystal structure-based virtual screening. *J Chem Inf Model.* 2013; 53:521–526. [PubMed: 23461591]

19. Vardy E, et al. Chemotype-selective modes of action of kappa-opioid receptor agonists. *J Biol Chem.* 2013; 288:34470–34483. [PubMed: 24121503]
20. Urban JD, et al. Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther.* 2007; 320:1–13. [PubMed: 16803859]
21. Kenakin T, Christopoulos A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov.* 2013; 12:205–216. [PubMed: 23411724]
22. White KL, et al. Identification of novel functionally selective kappa-opioid receptor scaffolds. *Mol Pharmacol.* 2014; 85:83–90. [PubMed: 24113749]
23. Yan F, et al. Structure-based design, synthesis, and biochemical and pharmacological characterization of novel salvinorin A analogues as active state probes of the kappa-opioid receptor. *Biochemistry.* 2009; 48:6898–6908. [PubMed: 19555087]
24. Geyer MA, Braff DL. Startle habituation and sensorimotor gating in schizophrenia and related animal models. *Schizophr Bull.* 1987; 13:643–668. [PubMed: 3438708]
25. White KL, et al. The G protein-biased kappa-opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities in vivo. *J Pharmacol Exp Ther.* 2015; 352:98–109. [PubMed: 25320048]
26. Kroeze WK, et al. PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat Struct Mol Biol.* 2015; 22:362–369. [PubMed: 25895059]
27. Morgenweck J, et al. Investigation of the role of betaarrestin2 in kappa opioid receptor modulation in a mouse model of pruritus. *Neuropharmacology.* 2015; 99:600–609. [PubMed: 26318102]
28. Bruchas MR, Chavkin C. Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology (Berl).* 2010; 210:137–147. [PubMed: 20401607]
29. Martin WR. History and development of mixed opioid agonists, partial agonists and antagonists. *Br J Clin Pharmacol.* 1979; 7:273S–279S. [PubMed: 380616]
30. Pfeiffer A, et al. Psychotomimesis mediated by kappa opiate receptors. *Science.* 1986; 233:774–776. [PubMed: 3016896]
31. Kumor KM, et al. Human psychopharmacology of ketocyclazocine as compared with cyclazocine, morphine and placebo. *J Pharmacol Exp Ther.* 1986; 238:960–968. [PubMed: 3018228]
32. Roth BL, et al. Salvinorin A: a potent naturally occurring nonnitrogenous kappa opioid selective agonist. *Proc Natl Acad Sci U S A.* 2002; 99:11934–11939. [PubMed: 12192085]
33. Iwamoto ET. Place-conditioning properties of mu, kappa, and sigma opioid agonists. *Alcohol Drug Res.* 1985; 6:327–339. [PubMed: 3011025]
34. Todtenkopf MS, et al. Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats. *Psychopharmacology (Berl).* 2004; 172:463–470. [PubMed: 14727002]
35. Lohse MJ, et al. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science.* 1990; 248:1547–1550. [PubMed: 2163110]
36. Lohse MJ, et al. Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *J Biol Chem.* 1992; 267:8558–8564. [PubMed: 1349018]
37. Huang W, et al. Structural insights into micro-opioid receptor activation. *Nature.* 2015; 524:315–321. [PubMed: 26245379]
38. Sounier R, et al. Propagation of conformational changes during mu-opioid receptor activation. *Nature.* 2015; 524:375–378. [PubMed: 26245377]
39. Steyaert J, Kobilka BK. Nanobody stabilization of G protein-coupled receptor conformational states. *Curr Opin Struct Biol.* 2011; 21:567–572. [PubMed: 21782416]
40. Fenalti G, et al. Molecular control of delta-opioid receptor signalling. *Nature.* 2014; 506:191–196. [PubMed: 24413399]
41. Fenalti G, et al. Structural basis for bifunctional peptide recognition at human delta-opioid receptor. *Nat Struct Mol Biol.* 2015; 22:265–268. [PubMed: 25686086]
42. O'Connor C, et al. NMR structure and dynamics of the agonist dynorphin peptide bound to the human kappa opioid receptor. *Proc Natl Acad Sci U S A.* 2015; 112:11852–11857. [PubMed: 26372966]

43. Rasmussen SG, et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature*. 2011; 477:549–555. [PubMed: 21772288]
44. Vardy E, Roth BL. Conformational ensembles in GPCR activation. *Cell*. 2013; 152:385–386. [PubMed: 23374334]
45. Kruse AC, et al. Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature*. 2013; 504:101–106. [PubMed: 24256733]
46. Katritch V, et al. Allosteric sodium in class A GPCR signaling. *Trends Biochem Sci*. 2014; 39:233–244. [PubMed: 24767681]
47. Gutierrez-de-Teran H, et al. The role of a sodium ion binding site in the allosteric modulation of the A(2A) adenosine G protein-coupled receptor. *Structure*. 2013; 21:2175–2185. [PubMed: 24210756]
48. Zhang C, et al. High-resolution crystal structure of human protease-activated receptor 1. *Nature*. 2012; 492:387–392. [PubMed: 23222541]
49. Vardy E, et al. Single Amino Acid Variation Underlies Species-Specific Sensitivity to Amphibian Skin-Derived Opioid-like Peptides. *Chem Biol*. 2015; 22:764–775. [PubMed: 26091169]
50. Pert CB, et al. Opiate agonists and antagonists discriminated by receptor binding in brain. *Science*. 1973; 182:1359–1361. [PubMed: 4128222]
51. Simon EJ, Groth J. Kinetics of opiate receptor inactivation by sulfhydryl reagents: evidence for conformational change in presence of sodium ions. *Proc Natl Acad Sci U S A*. 1975; 72:2404–2407. [PubMed: 1056036]
52. Vardy E, et al. A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior. *Neuron*. 2015; 86:936–946. [PubMed: 25937170]
53. Armbruster BN, et al. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A*. 2007; 104:5163–5168. [PubMed: 17360345]
54. Marchant NJ, et al. Behavioral and Physiological Effects of a Novel Kappa-Opioid Receptor-Based DREADD in Rats. *Neuropsychopharmacology*. 2015
55. Denis RG, et al. Palatability Can Drive Feeding Independent of AgRP Neurons. *Cell Metab*. 2015
56. Urban DJ, Roth BL. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annu Rev Pharmacol Toxicol*. 2015; 55:399–417. [PubMed: 25292433]
57. English JG, Roth BL. Chemogenetics-A Transformational and Translational Platform. *JAMA Neurol*. 2015;1–6. [PubMed: 26659895]
58. Deisseroth K. Optogenetics. *Nat Methods*. 2011; 8:26–29. [PubMed: 21191368]
59. Berndt A, Deisseroth K. OPTOGENETICS. Expanding the optogenetics toolkit. *Science*. 2015; 349:590–591. [PubMed: 26250674]
60. Berndt A, et al. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science*. 2014; 344:420–424. [PubMed: 24763591]
61. Siuda ER, et al. Optodynamic simulation of beta-adrenergic receptor signalling. *Nat Commun*. 2015; 6:8480. [PubMed: 26412387]
62. Siuda ER, et al. Spatiotemporal control of opioid signaling and behavior. *Neuron*. 2015; 86:923–935. [PubMed: 25937173]
63. Airan RD, et al. Temporally precise in vivo control of intracellular signalling. *Nature*. 2009; 458:1025–1029. [PubMed: 19295515]
64. Maseck OA, et al. Vertebrate cone opsins enable sustained and highly sensitive rapid control of Gi/o signaling in anxiety circuitry. *Neuron*. 2014; 81:1263–1273. [PubMed: 24656249]
65. Tan KR, et al. GABA neurons of the VTA drive conditioned place aversion. *Neuron*. 2012; 73:1173–1183. [PubMed: 22445344]
66. van Zessen R, et al. Activation of VTA GABA neurons disrupts reward consumption. *Neuron*. 2012; 73:1184–1194. [PubMed: 22445345]
67. McCall JG, et al. CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron*. 2015; 87:605–620. [PubMed: 26212712]

68. Bohn LM, et al. Exploring the Biology of G Protein-Coupled Receptors from In Vitro to In Vivo. *Mol Pharmacol*. 2015; 88:534–535. [PubMed: 26162863]
69. Roth BL, Kroeze WK. Integrated Approaches for Genome-wide Interrogation of the Druggable Non-olfactory G Protein-coupled Receptor Superfamily. *J Biol Chem*. 2015; 290:19471–19477. [PubMed: 26100629]
70. Banghart MR, Sabatini BL. Photoactivatable neuropeptides for spatiotemporally precise delivery of opioids in neural tissue. *Neuron*. 2012; 73:249–259. [PubMed: 22284180]
71. Banghart MR, et al. Caged naloxone reveals opioid signaling deactivation kinetics. *Mol Pharmacol*. 2013; 84:687–695. [PubMed: 23960100]
72. Williams JT. Desensitization of functional micro-opioid receptors increases agonist off-rate. *Mol Pharmacol*. 2014; 86:52–61. [PubMed: 24748657]
73. Jeong JW, et al. Wireless Optofluidic Systems for Programmable In Vivo Pharmacology and Optogenetics. *Cell*. 2015; 162:662–674. [PubMed: 26189679]
74. Ansonoff MA, et al. Antinociceptive and hypothermic effects of Salvinorin A are abolished in a novel strain of kappa-opioid receptor-1 knockout mice. *J Pharmacol Exp Ther*. 2006; 318:641–648. [PubMed: 16672569]
75. Kas MJ, et al. Mu-opioid receptor knockout mice show diminished food-anticipatory activity. *Eur J Neurosci*. 2004; 20:1624–1632. [PubMed: 15355329]
76. Contet C, et al. Dissociation of analgesic and hormonal responses to forced swim stress using opioid receptor knockout mice. *Neuropsychopharmacology*. 2006; 31:1733–1744. [PubMed: 16237385]
77. Sharifi N, et al. Generation of dynorphin knockout mice. *Brain Res Mol Brain Res*. 2001; 86:70–75. [PubMed: 11165373]
78. Koster A, et al. Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice. *Proc Natl Acad Sci U S A*. 1999; 96:10444–10449. [PubMed: 10468628]
79. Brady LS, et al. Region-specific up-regulation of opioid receptor binding in enkephalin knockout mice. *Brain Res Mol Brain Res*. 1999; 68:193–197. [PubMed: 10320798]
80. Gaveriaux-Ruff C, et al. Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain*. 2011; 152:1238–1248. [PubMed: 21295407]
81. Van't Veer A, et al. Ablation of kappa-opioid receptors from brain dopamine neurons has anxiolytic-like effects and enhances cocaine-induced plasticity. *Neuropsychopharmacology*. 2013; 38:1585–1597. [PubMed: 23446450]
82. Weibel R, et al. Mu opioid receptors on primary afferent nav1.8 neurons contribute to opiate-induced analgesia: insight from conditional knockout mice. *PLoS One*. 2013; 8:e74706. [PubMed: 24069332]
83. Ray RS, et al. Impaired respiratory and body temperature control upon acute serotonergic neuron inhibition. *Science*. 2011; 333:637–642. [PubMed: 21798952]
84. Dymecki SM, et al. Mapping cell fate and function using recombinase-based intersectional strategies. *Methods Enzymol*. 2010; 477:183–213. [PubMed: 20699143]
85. Fenno LE, et al. Targeting cells with single vectors using multiple-feature Boolean logic. *Nat Methods*. 2014; 11:763–772. [PubMed: 24908100]
86. Ehrich JM, et al. Kappa Opioid Receptor-Induced Aversion Requires p38 MAPK Activation in VTA Dopamine Neurons. *J Neurosci*. 2015; 35:12917–12931. [PubMed: 26377476]
87. Al-Hasani R, et al. Distinct Subpopulations of Nucleus Accumbens Dynorphin Neurons Drive Aversion and Reward. *Neuron*. 2015; 87:1063–1077. [PubMed: 26335648]
88. Krashes MJ, et al. An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. *Nature*. 2014; 507:238–242. [PubMed: 24487620]
89. McHugh TJ, et al. Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network. *Science*. 2007; 317:94–99. [PubMed: 17556551]
90. Garfield AS, et al. A neural basis for melanocortin-4 receptor-regulated appetite. *Nat Neurosci*. 2015; 18:863–871. [PubMed: 25915476]

91. Ozawa A, et al. Knock-In Mice with NOP-eGFP Receptors Identify Receptor Cellular and Regional Localization. *J Neurosci.* 2015; 35:11682–11693. [PubMed: 26290245]
92. Scherrer G, et al. Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell.* 2009; 137:1148–1159. [PubMed: 19524516]
93. Bardoni R, et al. Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. *Neuron.* 2014; 81:1312–1327. [PubMed: 24583022]
94. Huang XP, et al. Allosteric ligands for the pharmacologically dark receptors GPR68 and GPR65. *Nature.* 2015; 527:477–483. [PubMed: 26550826]
95. Ballesteros JA, Weinstein H. Integrated Methods for the Construction of Three-Dimensional Models and Computational Probing of Structure-Function Relations in G Protein-Coupled Receptors. *METHODS IN NEUROSCIENCES.* 1995; 25:366.

Glossary Box

<i>Chemogenetics</i>	The term has been used to describe the processes by which macromolecules (proteins such as receptors) can be engineered to interact with previously unrecognized small molecules. Designer Receptors Exclusively Activated by Designer Drugs (DREADDS) are a commonly used example in which G-protein coupled receptors have been engineered to respond to inert ligands CNO or Salvinorin B.
<i>DREADD</i>	Designer Receptors Exclusively Activated by Designer Drugs represent a typical GPCR-based chemogenetic tool.
<i>Cre-recombinase</i>	Cre recombinase is an enzyme derived from the P1 Bacteriophage. The enzyme is a member of the integrase family of site specific recombinases and it is known to catalyse the site specific recombination event between two DNA recognition sites (loxP sites). This 34 base pair (bp) loxP recognition site consists of two 13 bp palindromic sequences flanking an 8bp spacer region. The products of Cre-mediated recombination at loxP sites are dependent upon the location and relative orientation of the loxP sites.
<i>FLP-recombinase</i>	Similar to cre, is a site-directed recombination technology, to manipulate an organism's DNA under controlled conditions in vivo. It is analogous to Cre-lox recombination, but involves the recombination of sequences between short flippase recognition target (FRT) sites by the recombinase (Flp) derived from the 2µm plasmid of baker's yeast.
<i>Optogenetics</i>	a technique which involves the use of light to control cells in living tissue, typically neurons, that have been genetically modified to express light-sensitive proteins.
<i>Opto-XR</i>	Based on the wording of Gobind Khorana and others, chimeric GPCRs have been developed that replace the intracellular loops of bovine rhodopsin with specific intracellular components of GPCRs, including opioid, adrenergic, adenosine, and serotonergic versions.

Photostimulation

involves two methods to engage biological processes. One utilizes an uncaging process to make a compound biologically active in response to light; the other uses light-sensitive proteins such as rhodopsin that can excite, inhibit or engage a particular cell type.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Outstanding Questions

- What are the molecular details of opioid receptor activation?
- Do the activation states differ depending upon binding by peptide or small molecule ligands?
- How do different ligands engage diverse intracellular signaling pathways, and what is the structural basis for functional selectivity?
- How, where and what peptide species are released upon neuronal stimulation and are different species differentially released?
- Where are opioid receptors expressed, and how do they regulate neuronal activity *in vivo* to impact behavior?

Trends Box (684 characters with spaces; 775 max)**Trends**

- Crystal structures of the inactive states for all 4 receptors (μ , δ , κ and nociception) and the active state of μ have been elucidated and these structures are accelerating the structure-guided design of novel opioid ligands
- Functionally-selective, or biased, opioid ligands for several opioid receptors exist and hold promise as improved therapeutics with fewer liabilities
- New chemo- and optogenetic opioid receptors hold promise for transforming basic and translational opioid receptor research
- Genetically-engineered mice and photocaged opioid ligands allow unprecedented spatio-temporal control of opioid receptors, opioid peptide release and opioid ligand expression

Figure 1. Identification of G-protein and β -arrestin biased κ -opioid agonists

A. Molecular model of docking pose of U69,593 to KOR and structures of U69593 and ICI 199,441. ICI 199,441 was identified as a β -arrestin biased agonist **B.** Docking pose of salvinorin A to KOR, structure of salvinorin A and RB-64. RB-64 was identified as a G-protein biased KOR agonist **C.** Salvinorin A shows all of the prototypical actions of KOR agonists in wild-type (WT) mice although certain side-effects (sedation, impaired coordination and anhedonia) are reduced in β -arrestin2 knock-out (KO mice). RB-64 has analgesic actions and mildly impairs coordination, but is apparently devoid of anhedonia and

sedation in WT mice. RB-64 has no effect in KOR KO mice indicating its effects are likely due to KOR agonism.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Molecular insights into opioid receptor actions yield structure based design of new DREADD

A and **B** show overview and close-up view of the sodium (Na^+) site in the δ -opioid receptor (40). In **B**, residues are enumerated according to the Ballousteros-Weinstein convention (95). **C** shows the putative location of the Na^+ site in the inactive state of the μ -opioid receptor (11), while **D** shows structural rearrangements leading to the loss of this site in the activated and presumably G-protein coupled state (37). **E** shows docking results for salvinorin B—an inactive metabolite of salvinorin A to the wild-type κ -opioid receptor (52). **F** shows how

salvinorin B is predicted to interact with the D3.32N mutant opioid receptor the κ -opioid DREADD (KORD)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Summary of modern optogenetic approaches for dissecting opioid peptide and receptor function *in vitro* and *in vivo*

A. Cartoon depicting chimeric “Opto-XR” approach in which rhodopsin cDNA is fused with wildtype GPCR cDNA intracellular loops and tail to generate a photo-sensitive receptor system capable of spatiotemporal engagement of canonical GPCR signaling pathways such as Gq, Gs, and Gi or arrestin recruitment in selected cell types when combined with viral and genetic approaches *in vivo*. Opto-MOR receptors (62) take advantage of similarities between RO4 Gi-coupled opsins and Mu-opioid receptors. **B.** Adapted from (70) Left, One-letter amino-acid sequences of LE, Dyn-8 and their corresponding photo-switchable CNB-modified analogues CYLE and CYD8. Right, the chemical structure of CYLE. The CNB moiety (photoswitch) is highlighted in red. **C.** Summary of circuit-based chemogenetic and optogenetic targeting approach and available endogenous opioid cre-driver mice. Strategy is provided using a double-inverted open reading frame (DIO) construct, and fiber optics for optogenetic manipulation. For DREADD-based approaches, injection and then CNO

manipulations would occur *in vivo* or *in vitro*. Extensions of this approach are also possible, whereby other opsins or DREADDs can be used for multiplexing or inhibition experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript