New Technologies for Elucidating Opioid Receptor Function

Michael R. Bruchas[1],* and Bryan L. Roth[2],*

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 chemogenetic and optogenetic tools, and new mouse lines, are expanding and transforming our understanding of opioid function and, perhaps, paving the way for new therapeutics.

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, [3–5]). Parenthetically, it is useful to consider that, prior to the molecular cloning of the four known major opioid receptor subtypes [6–9], 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 [11–14]) 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-opioid receptor (KOR) [18] ligands, providing new chemotypes with modest potency. Additionally, nM potency, selective μ-opioid receptor (MOR) G-protein-biased agonists of novel chemotypes from structure-based screens *in silico* have been reported (A. Manglik *et al.*, unpublished). 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 (A. Manglik *et al.*, unpublished).

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

Trends

Crystal structures of the inactive states for all four receptors (μ, δ, κ, and nociceptin) 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 chemogenetic and optogenetic opioid receptors hold promise for transforming basic and translational opioid receptor research.

Genetically engineered mice and photocaged opioid ligands allow unprecedented spatiotemporal control of opioid receptors, opioid peptide release, and opioid ligand expression.

[1]Departments of Anesthesiology and Neuroscience, Washington University, School of Medicine, St Louis, MO, USA
[2]Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC, USA

*Correspondence:
bruchasm@wustl.edu (M.R. Bruchas) and bryan_roth@med.unc.edu (B.L. Roth).
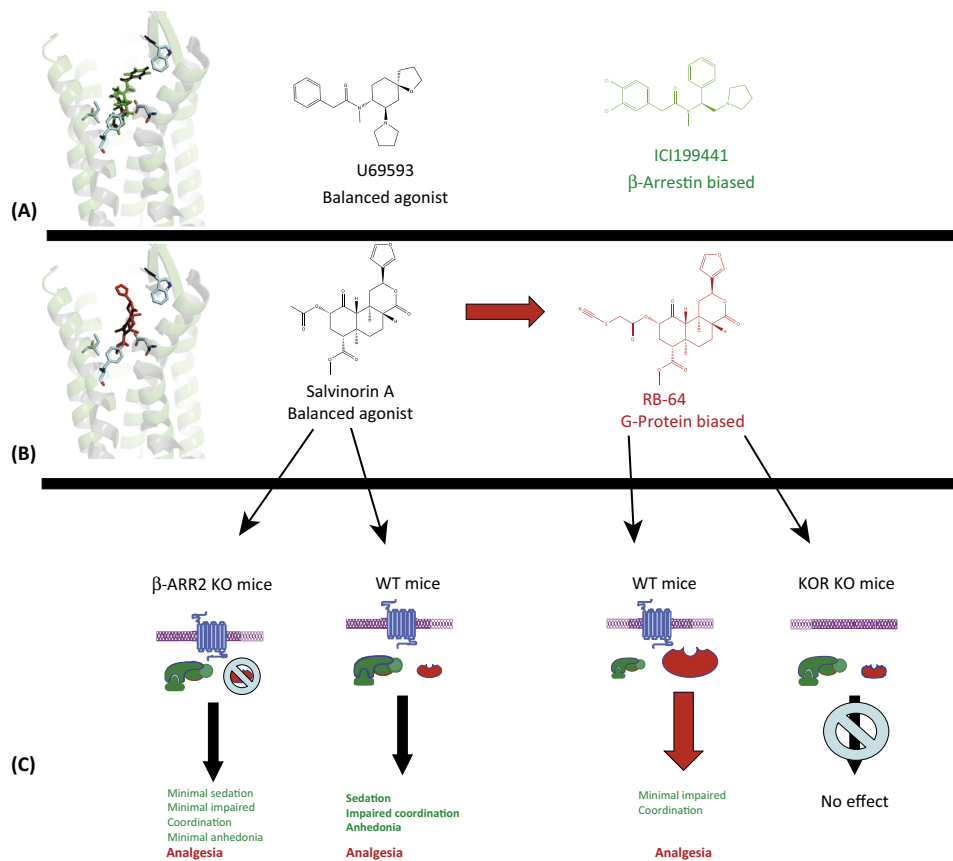
Figure 1. Identification of G-Protein- and β-Arrestin-Biased κ-Opioid Agonists. (A) Molecular model of docking pose of U69593 to κ-opioid receptor (KOR) and structures of U69593 and ICI199441. ICI199441 was identified as a β-arrestin-biased agonist. (B) Docking pose of salvinorin A to KOR, structure 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 knockout (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 agonists.

revealed that different chemotypes likely adopt different poses in the KOR binding pocket. Arylacetamides such as U69593 (Figure 1A) and diterpenes such as 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 U69593 might display different functional selectivity [20] or biased signaling [21]. However, both U69593 (Figure 1A) and salvinorin A (Figure 1B) appear to be balanced human (hKOR) agonists *in vitro.* By contrast, a comprehensive screen of KOR agonists and other known drugs and drug-like compounds revealed that the arylacetamide ICI199441 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.

Glossary

Chemogenetics: 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 GPCRs have been engineered to respond to inert ligands CNO or salvinorin B.

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 catalyze 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 8 bp spacer region. The products of Cre-mediated recombination at loxP sites are dependent upon the location and relative orientation of the loxP sites.

DREADD: Designer Receptors Exclusively Activated by Designer Drugs represent a typical GPCR-based chemogenetic tool.

FLP-recombinase: 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 2 μm plasmid of baker's yeast.

Optogenetics: a technique that involves the use of light to control cells in living tissue, typically neurons, which have been genetically modified to express light-sensitive proteins.

Opto-XR: based on the wording of Har 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.

Given that previous 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 versus β-arrestin-ergic signaling in mice. Initial studies indicated that RB-64 has psychotomimetic-like activity [23] in that it disrupted the prepulse inhibition of startle response, which is widely used to predict psychotomimetic actions of drugs [24]. Next, studies in wild-type (WT) and β-arrestin2 (βARR2) knockout (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 U50488H for the inhibition of pruritus [27]. These findings are broadly supportive of previous 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 [29–32], 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 G-protein-coupled receptors (GPCRs) [35,36]. Thus, 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 βARR2 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 βArrestin2 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 (1.8 Å) was reported for the inactive state of δ-opioid receptor (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 previously 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,B) [46–49] has disappeared in the active state of MOR (Figure 2D), although 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,
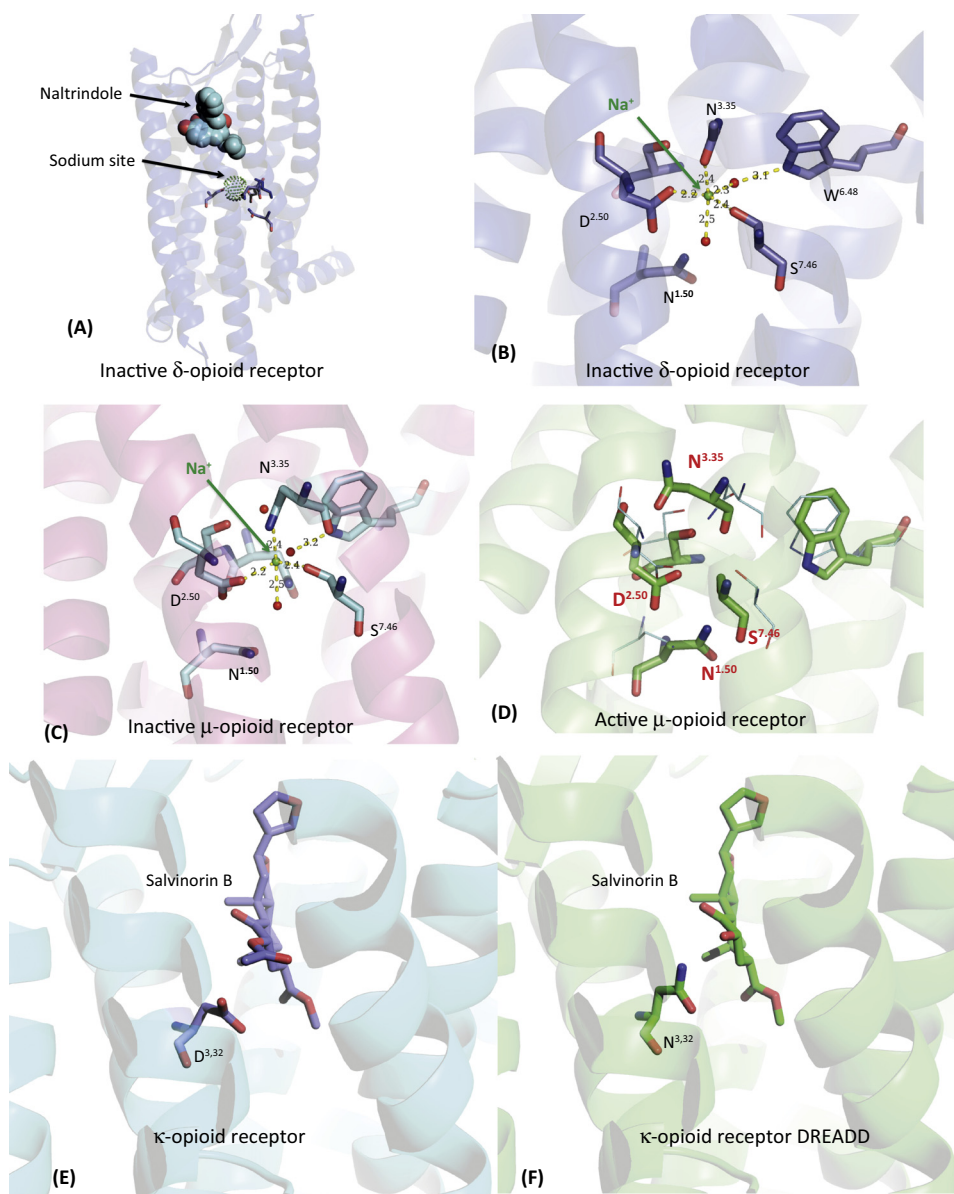
Figure 2. Molecular Insights into Opioid Receptor Actions Yield Structure-Based Design of New DREADD. (A, B) Overview and close-up view of the sodium (Na$^+$) site in the δ-opioid receptor [40]. In (B) residues are numbered according to the Ballesteros–Weinstein convention [95]. Panel (C) shows the putative 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]. Panel (E) shows docking results for salvinorin B – an inactive metabolite of salvinorin A to the wild-type κ-opioid receptor [52]. Panel (F) shows how salvinorin B is predicted to interact with the D3.32N mutant opioid receptor, the κ-opioid DREADD (KORD). Abbreviation: DREADD, Designer Receptors Exclusively Activated by Designer Drugs.

given the ubiquitous nature of the interaction of D138 with basic nitrogen 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

was also 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 κ-opioid receptor **DREADD** (see Glossary) (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 [58–60]. 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* [61–64]. 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 **photostimulation** 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 serotoninergic 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 coexpressed, 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 photoswitchable 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. To begin to dissect this, the research teams of Bernardo Sabatini and John T. Williams, led by efforts of Matthew Banghart and others [70], have developed two opioid agonist peptide analogs: [Leu^5]-enkephalin (CYLE) and the eight 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 UV light (405 nm), they become functional and may then activate endogenous opioid receptors. Banghart and Sabatini [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 photoswitchable 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 analog 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 photoswitchable 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 past decade one of the most useful animal tools utilized in the opioid field has been KO mice of both opioid receptors (MOR, KOR, DOR, and NOP) and peptides (proopiomelanocortin, enkephalin, preprodynorphin, prepronociceptin) [74–79]. These global KO 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 [74–79]. More recently, conditional deletion approaches have been developed wherein loxP sites have been introduced flanking various opioid receptor exons, to utilize the power of cell type selective **cre-recombinase** strategies for selective gene deletion within discrete cells and neural circuits [80,81]. Conditional KO 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 [80–82]. 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.
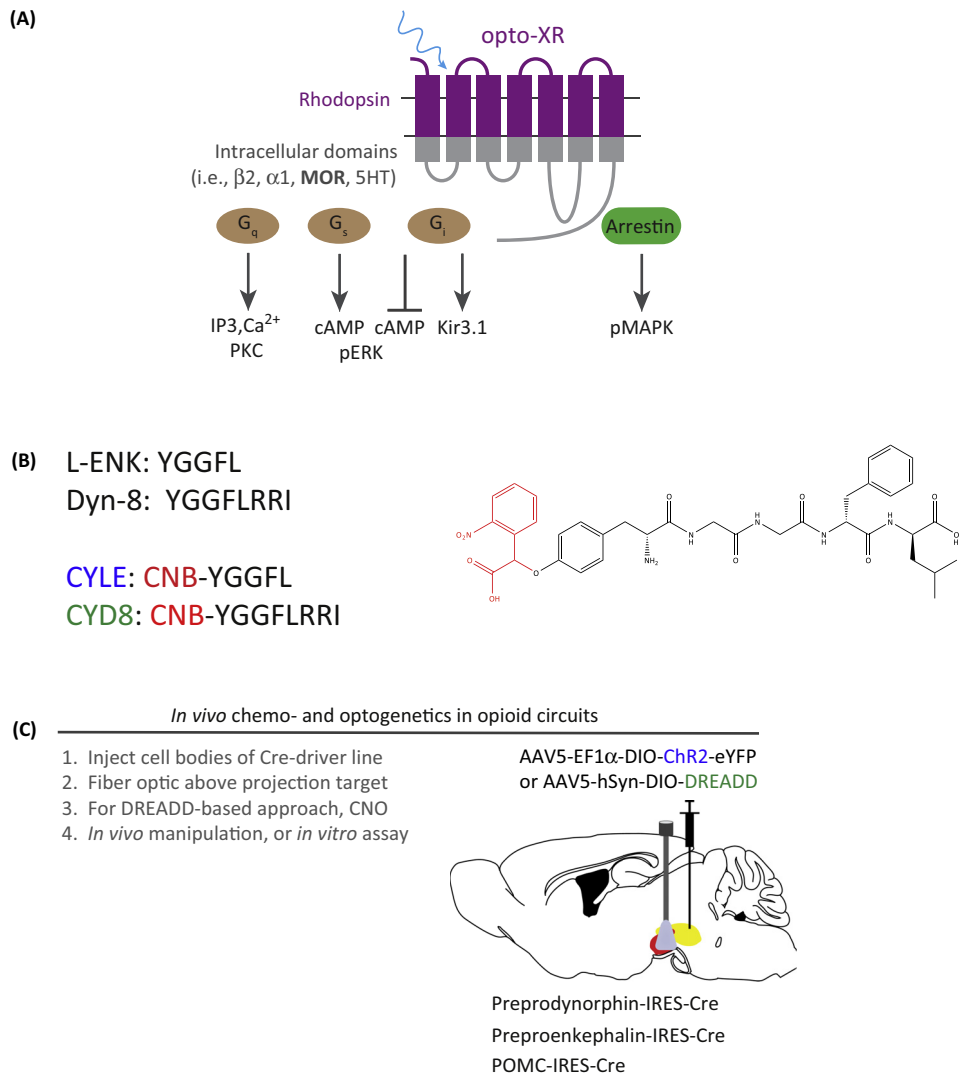
Additional intersectional (e.g., using **FLP-recombinase**) viral and knock-in approaches may further provide a powerful tool set for dissecting the functions of subsets of neurons expressing opioid receptors and their ligands [83–85]. A recent example showed that selective deletion of KOR from dopamine neurons (DATcre+) has anxiolytic-like properties and alters cocaine-induced plasticity [81]. In a complementary study, Ehrich and colleagues showed the rescue of KOR in KOR KO 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 KO 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 KO mice for proopiome-lanocortin, proenkephalin, and prodynorphin have each been developed and are in the initial testing phases for viability and phenotyping [87–89]. Since each of these prepropeptides 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 promis-cuous 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 **chemogenetic** 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 noncanonical roles for κ-opioid-mediated behavioral effects [87] (Figure 3). By contrast, proo-piomelanocortin-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 proendorphin, 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 [91–93]. Owing 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 NOP–eGFP mice were recently published [91], and it was revealed that this receptor is expressed in a subpopulation of dorsal root ganglion neurons. Additionally, initial and intriguing data were reported on internalization properties of NOP 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

(A)

opto-XR

Rhodopsin

Intracellular domains
(i.e., β2, α1, **MOR**, 5HT)

G_q G_s G_i Arrestin

IP3,Ca^{2+} cAMP cAMP Kir3.1 pMAPK
PKC pERK

(B) L-ENK: YGGFL
Dyn-8: YGGFLRRI

CYLE: CNB-YGGFL
CYD8: CNB-YGGFLRRI

(C) *In vivo* chemo- and optogenetics in opioid circuits

1. Inject cell bodies of Cre-driver line
2. Fiber optic above projection target
3. For DREADD-based approach, CNO
4. *In vivo* manipulation, or *in vitro* assay

AAV5-EF1α-DIO-ChR2-eYFP
or AAV5-hSyn-DIO-DREADD

Preprodynorphin-IRES-Cre
Preproenkephalin-IRES-Cre
POMC-IRES-Cre

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 wild-type G-protein-coupled receptor (GPCR) cDNA intracellular loops and tail to generate a photosensitive 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 μ-opioid receptors. (B) Left, One-letter amino acid sequences of LE, Dyn-8, and their corresponding photoswitchable CNB-modified analogs CYLE and CYD8. Right, The chemical structure of CYLE. The CNB moiety (photoswitch) is highlighted in red. Adapted from [70]. (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. Abbreviations: CNB, carboxynitrobenzyl; CNO, clozapine-N-oxide; CYLE, [Leu^5]-enkephalin; DREADD, Designer Receptors Exclusively Activated by Designer Drugs; Dyn-8, eight amino acid form of Dynorphin A; MOR, μ-opioid receptor.

function. Additionally, background fluorescence can impede rigorous conclusions about sub-cellular localization of fusion proteins. Nevertheless, taken together with the other approaches described earlier, they provide another novel avenue of resolution that reveals 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).

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 chemogenetics and optogenetics as well as 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 noncanonical 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.

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?

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