

Opioid pharmacology under the microscope

Damien Jullié^{1,2}, Arisbel B Gondin³, Mark von Zastrow^{1,2,*}, and Meritxell Canals^{4,5,*}

Department of Psychiatry and Department of Cellular and Molecular Pharmacology, University of California, San Francisco School of Medicine, San Francisco, California (DJ and MvZ); Drug Discovery Biology Theme, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia (ABG); Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, United Kingdom and Centre of Membrane Protein and Receptors, Universities of Birmingham and Nottingham, The Midlands, United Kingdom (MC).

¹ Department of Psychiatry, University of California, San Francisco School of Medicine, San Francisco, CA 94158, USA

² Department of Cellular and Molecular Pharmacology, University of California, San Francisco School of Medicine, San Francisco, CA 94158, USA

³ Drug Discovery Biology Theme, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC 3052 Australia

⁴ Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, United Kingdom

⁵ Centre of Membrane Protein and Receptors, Universities of Birmingham and Nottingham, The Midlands, United Kingdom

Work in the authors' labs was supported by research grants from the United States National Institutes of Health / National Institute on Drug Abuse (DA010711 and DA012864 to MvZ), the UCSF Program for Breakthrough Biomedical Research (D.J.).

Running title: Cellular basis of opioid action

Correspondence:

Address correspondence to: Meritxell Canals, PhD, COMPARE Office C101a, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK, E-mail: m.canals@nottingham.ac.uk; or Mark von Zastrow, MD, PhD, UCSF Mission Bay, Genentech Hall Room N212E, 600 16th Street, San Francisco, CA 94143, USA, E-mail: mark@vzlab.org.

Numbers:

Number of text pages: 16

Number of figures: 3

Number of references: 96

Number of words in abstract: 126

Number of words in introduction: none

Number of words in discussion: none

Nonstandard abbreviations used in this paper:

β -FNA: Beta-funaltrexamine

β -CNA: Beta-chlornaltrexamine

DOP-R: δ -opioid receptor

KOP-R: κ -opioid receptor

MOP-R: μ -opioid receptor

GIRK: G protein-coupled inwardly-rectifying potassium channel

GPCR: G protein-coupled receptor

GRK: GPCR kinases

SEP: Superecliptic pHluorin

TIR-FM: total internal reflection fluorescence microscopy

Abstract

The powerful analgesic effects of opioid drugs have captivated the interest of physicians and scientists for millennia, and the ability of opioid drugs to produce serious undesired effects has been recognized for a similar period of time (Kieffer and Evans, 2009). Many of these develop progressively with prolonged or repeated drug use and then persist, motivating particular interest in understanding how opioid drugs initiate adaptive or maladaptive modifications in neural function or regulation. Exciting advances have been made over the past several years in elucidating drug-induced changes at molecular, cellular and physiological scales of analysis. The present review will highlight some recent cellular studies that we believe bridge across scales, and focus on optical imaging approaches which put opioid drug action 'under the microscope'.

Significance statement

Opioid receptors are major pharmacological targets but their signaling at the cellular level results from a complex interplay between pharmacology, regulation, subcellular localization and membrane trafficking.

This minireview discusses recent advances in understanding the cellular biology of opioid receptors, emphasizing particular topics discussed at the 50th anniversary of the INRC meeting. Our goal is to highlight distinct signaling and regulatory properties emerging from the cellular biology of opioid receptors, and discuss potential relevance to therapeutics.

Manuscript text

Cellular basis of opioid receptor regulation

Early interest in a cellular basis for opioid adaptations was motivated by the ability of prolonged morphine exposure to produce complex regulatory effects on signaling in neuroblastoma cells (Sharma *et al.*, 1975) and to reduce receptor reserve in tissue explants (Chavkin and Goldstein, 1982). This was followed by the identification of agonist-induced down-regulation of opioid receptors associated with endocytic delivery to lysosomes (Law *et al.*, 1984). A more rapid and nondestructive process of receptor internalization was later detected, first pharmacologically and then using cellular imaging (Von Zastrow *et al.*, 1993; von Zastrow *et al.*, 1994). This led to the delineation of clathrin and dynamin -dependent endocytosis of opioid receptors promoted by agonist-induced receptor phosphorylation followed by engagement of a class of cytoplasmic adaptor and scaffolding proteins called β -arrestins. Regulated endocytosis by this conserved mechanism has now been demonstrated for all three opioid receptor types— μ (MOP-R), δ (DOP-R) and κ (KOP-R) (Keith *et al.*, 1996; Zhang *et al.*, 1996, 1998; Whistler and von Zastrow, 1998; Li *et al.*, 1999).

Once internalized, receptors can recycle non-destructively to the plasma membrane or traffic to lysosomes for proteolytic down-regulation. The decision of which route receptors take is made by receptor-specific molecular sorting operations which occur in endosomes (Tsao and von Zastrow, 2000; Whistler *et al.*, 2002). Internalized MOP-R and KOP-R typically recycle efficiently and down-regulate slowly whereas DOP-R, although also capable of non-destructive recycling, traffics preferentially to lysosomes and down-regulates more rapidly (Keith *et al.*, 1996; Li *et al.*, 1999; Tsao and von Zastrow, 2000; Tanowitz and von Zastrow, 2003). Such molecular sorting is highly

selective and directed by discrete structural determinants in the receptor. For example, a short sequence present in the cytoplasmic tail of MOP-R is necessary for efficient recycling of this opioid receptor and, when fused to the cytoplasmic tail of DOP-R, it is sufficient to redirect receptors from lysosomal to recycling pathways. Further, a MOP-R splice variant which naturally lacks this sequence, generated by alternative processing of the receptor transcript, recycles less efficiently and down-regulates more rapidly during prolonged agonist exposure (Tanowitz and von Zastrow, 2003; Tanowitz *et al.*, 2008). Additional sorting occurs later during transit to lysosomes, through translocation of receptors from the endosome limiting membrane to small membrane vesicles that are formed within the endosome lumen. This process is promoted by ubiquitination of receptors on lysine residues in the first cytoplasmic loop (Hislop *et al.*, 2011).

Such observations delineated a basic framework for the cellular regulation of opioid receptors (**Figure 1**) which largely comports with a conserved paradigm of receptor desensitization established through studies of a variety of GPCRs (DeWire *et al.*, 2007). While much of the original work elaborating this regulatory framework relied on recombinant receptor expression and the use of simplified model systems, key elements of it are now validated in physiological context. In particular, agonist-induced endocytosis of MOP-R and DOP-R has been directly demonstrated in native neurons (Sternini *et al.*, 1996; Scherrer *et al.*, 2006), and endocytic delivery of DOP-R to lysosomes has been convincingly associated with long-term down-regulation of functional antinociception in vivo (Pradhan *et al.*, 2009). Further, the same phosphorylation sites defined initially by phosphoproteomic mapping of heterologously expressed MOP-R (Lau *et al.*, 2011) have been shown to be essential for functional desensitization of postsynaptic signaling and agonist-induced internalization of MOP-R in acute brain slices, as well as for antinociceptive tolerance assessed behaviorally in vivo after repeated administration of morphine (Arttamangkul *et al.*, 2018; Kliewer *et al.*, 2019).

A main take-away from the cellular regulatory framework summarized above is that receptor trafficking and signaling operations are intricately interconnected. A critical group of enzymes which regulate opioid receptors (and many other GPCRs) are the so-called GPCR kinases or GRKs (Komolov and Benovic, 2018). Phosphorylation by isoforms of protein kinase C has also been shown to produce significant effects on MOP-R trafficking and signaling, and contributes to differentiating regulatory effects of morphine relative to opioid peptide agonists on this receptor (Bailey *et al.*, 2009; Bowman *et al.*, 2015; Civciristov *et al.*, 2019; Kunselman *et al.*, 2019). GRK-mediated phosphorylation is a general mechanism for inhibiting the ability of GPCRs to engage G proteins, and for promoting binding to β -arrestins (Shenoy and Lefkowitz, 2011; Williams *et al.*, 2013; Kang *et al.*, 2014). Accordingly, phosphorylation promotes two processes which appear to act redundantly to assure that opioid signaling from the plasma membrane is terminated—functional uncoupling of receptors from G proteins followed by physical removal of receptors from the cell surface (**Figure 1**).

Towards an expanded understanding of the signaling - trafficking relationship

According to the conserved regulatory framework summarized above, endocytosis of opioid receptors is inextricably associated with signal inactivation, effectively operating as a redundant

mechanism to assure that surface-delimited signaling is terminated after receptor phosphorylation and engagement of β -arrestin in the plasma membrane. However, it is increasingly clear that opioid receptors can produce additional signaling effects which are promoted, rather than inhibited, by receptor engagement with β -arrestin. The potential of β -arrestins to act as regulated scaffold proteins promoting the activation of kinase cascades by various GPCRs has been well described (DeWire *et al.*, 2007), and studies of KOP-R provide strong support for the physiological relevance of such signaling to opioid receptors (Bruchas and Chavkin, 2010). Recent studies of MOP-R and DOP-R suggest, in addition, that opioid receptors use β -arrestin -promoted endocytosis to enable G protein signaling from internal membrane compartments. Moreover, studies of MOP-R suggest a discrete regulatory scheme which enables opioid receptors to maintain sensitive signaling via G proteins at the plasma membrane. Key observations supporting each of these emerging views are summarized below.

Evidence for endosomal activation of opioid receptor signaling via G proteins emerged from the development of conformational biosensors which detect and localize activated MOP-R and DOP-R in living cells. Single-domain antibodies (nanobodies), generated initially as tools to stabilize active-conformation receptors for structural studies (Huang *et al.*, 2015; Manglik *et al.*, 2017), were later adapted to detect conformational activation of receptors in living cells when expressed as fluorescent fusion proteins in the cytoplasm (Stoeber *et al.*, 2018). Imaging live cells using total internal reflection fluorescence microscopy (TIR-FM), a method useful for examining events close to the cell surface, demonstrated agonist-induced recruitment of biosensor to the plasma membrane as expected. Imaging by confocal microscopy, enabling intracellular compartments to be resolved in optical sections, revealed biosensor recruitment also to endosome membranes after receptor internalization. Experiments contrasting the effects of membrane -permeant and -impermeant antagonists provided functional evidence supporting the ability of DOP-R activation in internal membrane compartments to contribute to a relatively sustained component of opioid receptor-mediated inhibition of cellular adenylyl cyclase activity. These observations add support to the overall hypothesis that phosphorylation and β -arrestin -promoted endocytosis of opioid receptors is not associated exclusively with signal termination. Rather, the conserved phosphorylation-endocytosis machinery appears to enable a second 'wave' of G protein-mediated signaling that is initiated from the endosome limiting membrane (**Figure 1**).

Evidence suggesting a distinct effect of opioid receptor endocytosis on G protein signaling from the plasma membrane emerged through studies using a combination of fluorescence imaging methods to investigate the relationship between MOP-R trafficking and signaling in neurons, and specifically focusing on this relationship in axons. MOP-R mediates both postsynaptic and presynaptic neuromodulatory effects through G proteins (Williams *et al.*, 2013). At the postsynaptic plasma membrane, MOP-R stimulates a hyperpolarizing current through G protein-dependent activation of G protein-coupled inwardly-rectifying potassium (GIRK) channels (Williams *et al.*, 1982). This response normally desensitizes over several minutes in the continuous presence of agonist and then recovers after agonist washout (Dang and Williams, 2004). Desensitization of the postsynaptic GIRK response occurs even when internalization of MOP-R is prevented (Arttamangkul *et al.*, 2006), supporting the hypothesis that endocytosis acts redundantly in surface-delimited signal termination, and receptor recycling after endocytosis

parallels recovery of MOP-R signaling from the desensitized state (Arttamangkul *et al.*, 2008; Yu *et al.*, 2010; Quillinan *et al.*, 2011). Further, mutation of phosphorylation sites in the MOP-R cytoplasmic tail that are required for agonist-induced internalization of receptors in the somatodendritic compartment blocks functional desensitization of postsynaptic GIRK signaling measured in brain slice preparations (Alvarez *et al.*, 2002; Just *et al.*, 2013; Yousuf *et al.*, 2015; Arttamangkul *et al.*, 2018). MOP-R is also known to inhibit postsynaptic calcium transients but, interestingly, this response does not rapidly desensitize. This difference appears to occur as a consequence of classical receptor reserve because reducing the overall number of functional receptors, by pre-exposing tissue slices to the irreversible opioid antagonist β -CNA, unmasks rapid desensitization of this postsynaptic response as well (Fox and Hentges, 2017). Accordingly, MOP-R signaling which occurs in the somatodendritic plasma membrane of neurons conforms to the classical cellular regulatory paradigm, with receptor phosphorylation followed by internalization producing a net loss of functional signaling responsiveness.

However, there is now evidence for a different relationship between MOP-R signaling and trafficking in axons. MOP-R inhibits transmitter release at presynaptic terminals by local G protein-dependent inhibition of voltage-gated calcium channels and components of the vesicular release machinery (Wilding *et al.*, 1995; Bourinet *et al.*, 1996; Zurawski *et al.*, 2019). This response is more highly sensitive to opioid peptides than postsynaptic GIRK signaling mediated by MOP-R, and it remains highly sensitive even under conditions of prolonged agonist exposure that strongly desensitize the postsynaptic GIRK response (Fyfe *et al.*, 2010; Pennock *et al.*, 2012; Lowe and Bailey, 2015). Further, in contrast to what is observed for postsynaptic calcium transients, reducing the overall number of functional opioid receptors by irreversible antagonist pre-exposure fails to unmask desensitization of presynaptic inhibition mediated by MOP-R (Pennock *et al.*, 2012). Accordingly, MOP-R regulation in the presynaptic compartment appears to differ fundamentally from that occurring in the postsynaptic compartment, based on its higher overall ligand sensitivity and resistance to desensitization even when overall receptor reserve is reduced.

Despite this distinction, live cell imaging of tagged MOP-R in neurons revealed agonist-induced phosphorylation and phosphorylation-dependent endocytosis in axons, with these events mediated by similar machinery as that mediating rapid desensitization of postsynaptic MOP-R signaling (Jullié *et al.*, 2019). A clue to how these conserved cellular regulatory events are compatible with a lack of functional desensitization at the presynapse became apparent from experiments using various imaging methods to examine in detail the localization and dynamics of MOP-R in axons. To visualize sites of endocytic uptake of MOP-R in axons of living neurons, a pH-sensitive green fluorescent protein variant (SEP) was fused to the extracellular domain of MOP-R. Taking advantage of rapid quenching of surface-accessible SEP fluorescence by perfusion of an acidic buffer, newly formed endocytic vesicles containing SEP-labeled MOP-R were visualized in live image series as abruptly appearing acid-resistant fluorescent punctae (Merrifield *et al.*, 2005). In contrast to clathrin-mediated endocytosis at the somatodendritic plasma membrane that occurs throughout the membrane surface at densely distributed sites (Rosendale *et al.*, 2017), endocytosis of MOP-R in axons was found to be restricted to sparsely distributed sites which are localized almost exclusively within terminals.

One might expect such local endocytosis to cause even stronger desensitization at the presynapse than at the postsynaptic plasma membrane. However, when individual MOP-R proteins were imaged using single-molecule localization microscopy, receptors were found to be uniformly distributed and not detectably accumulated at presynaptic terminals. Moreover, analysis of single-receptor trajectories revealed that MOP-R is freely mobile and rapidly diffuses on the axon surface. Confocal fluorescence microscopy and wide field imaging of axons under oblique illumination (a method that is useful for examining organelle dynamics in axons), revealed that MOP-R-containing endosomes accumulate in terminals but also move bidirectionally on the axon shaft. These endosomes support local surface recycling of MOP-R because membrane fusion events which insert receptors into the axon plasma membrane, imaged as local bursts of increased fluorescence intensity (due to dequenching of SEP upon receptor exposure at the axon surface), appear adjacent to individual endosomes both in terminals and the axon shaft domain. Together these observations suggest that local recycling enables axons to maintain a diffusive surface pool of MOP-R even after prolonged agonist exposure, and that receptors present on the axon shaft but outside of terminals have the potential to support local signaling at terminals through lateral diffusion and collisional coupling (**Figure 2**).

Indeed, the measured diffusion rate of MOP-R on the axon surface is sufficient to enable agonist-receptor complexes formed outside of synapses to diffuse into an adjacent active zone before the agonist dissociates. This suggests that resistance to rapid desensitization, a distinguishing characteristic of MOP-R signaling at the presynapse, arises from a different type of receptor reserve. In the classical model, resistance to desensitization is achieved by an overall excess of receptors. In axons, the extrasynaptic receptor fraction is protected because the conserved phosphorylation-endocytosis machinery is restricted to terminals. Presynaptic inhibition by opioids thus appears to resist desensitization by leveraging a discrete principle of 'lateral' receptor reserve, based on ligand-receptor complexes signaling after diffusing into the synapse and being protected from inactivation when outside of it. Lateral receptor reserve, unlike classical receptor reserve, enables the presynapse to remain opioid-responsive and resist desensitization with low receptor number. It also carries other new physiological implications which remain to be investigated. These include (1) enhancing reliability of presynaptic neuromodulation by increasing the rate at which individual terminals can sample independent agonist-receptor binding events, (2) achieving high absolute sensitivity to opioid ligands by using the axon as an extended 'antenna' to increase the extracellular volume that an individual presynaptic terminal can effectively sample, and (3) enabling the same active zone to be regulated by multiple GPCR types without steric inhibition, despite the typically small size of terminals and limited number of relevant effectors immobilized at individual active zones (Jullié *et al.*, 2019).

Insight into the molecular and cellular specificity of opioid drug effects

In addition to its impact on physiological desensitization, the principle of receptor reserve (in its classical meaning) is important for understanding opioid drug action, in particular agonist efficacy and partial agonism. Opioid receptors have been prototypical receptors to illustrate experimentally the impact of receptor reserve on the efficacy of drugs (Borgland *et al.*, 2003; McPherson *et al.*, 2010). Reduction of receptor reserve using irreversible antagonists, such as β -FNA or CNA, has been used extensively to identify partial agonists that show full maximal responses in highly

amplified or very efficiently coupled signaling pathways. Morphine, for example, shows lower intrinsic efficacy at MOP-R than enkephalins or fentanyl yet can produce maximal responses in systems with high receptor reserve (Borgland *et al.*, 2003). According to classical receptor theory, partial agonism is explained by the degree to which the agonist stabilizes a single activated GPCR state. However, recent biophysical evidence indicates that GPCRs dynamically fluctuate between multiple conformational states (Weis and Kobilka, 2018), and conformational heterogeneity has been explicitly demonstrated for MOP-R (Sounier *et al.*, 2015). The significance of multiple receptor states to physiological signaling by opioid peptides or the effects of drugs remains unclear, and defines an exciting area of current investigation.

Interest in partial agonism has experienced a recent renaissance partially motivated by such emerging biophysical evidence, and preceded by various functional and cellular observations which, together, suggest that agonists have the potential to differ in more than one 'dimension' of intrinsic efficacy at a given GPCR (Galadrin *et al.*, 2007; Urban *et al.*, 2007; Kenakin, 2017). A current elaboration of this concept is the proposed pharmacological paradigm of biased agonism, determined by agonists differentially promoting receptor coupling to G protein relative to β -arrestin-mediated pathways. This paradigm gained particular traction through studies of β -arrestin knockout mice, in which morphine administration resulted in enhanced antinociception but diminished respiratory depression and constipation (Bohn *et al.*, 1999; Raehal *et al.*, 2005). Based on this, G protein biased agonists – namely agonists that selectively promote opioid receptors to engage G proteins over β -arrestins – were proposed to provide a new avenue for safer opioid analgesics. This hypothesis motivated the development of compounds such as TRV130 (oliceridine) (DeWire *et al.*, 2013), PZM21 (Manglik *et al.*, 2016) and SR-17018 (Schmid *et al.*, 2017) as putative G protein-biased opioid receptor agonists with improved therapeutic profiles. The physiological validity of the bias hypothesis, in its present elaboration, is still being evaluated (Hill *et al.*, 2018). In particular, the presence of reduced respiratory depression in β -arrestin -knockout mice was recently challenged (Kliwer *et al.*, 2020). Further, the degree to which physiologically relevant differences between opioid drugs truly result from G protein bias, or reflect low agonist efficacy overall (typically lower than morphine), remains a matter of active investigation (Conibear and Kelly, 2019). Moreover, there is accumulating evidence for additional factors impacting bias profile, particularly kinetic effects on trafficking and signaling (Thompson *et al.*, 2016; Weinberg *et al.*, 2017). In the future, we anticipate that structural, biophysical and computational approaches will provide increasingly precise understanding of the underpinnings of agonist efficacy, bias and allosteric modulation leading to drugs with improved therapeutic window (Filizola, 2019; Hu, Provasi, *et al.*, 2019; Hu, Wang, *et al.*, 2019; Zarzycka *et al.*, 2019).

Another facet of bias has recently emerged thanks, in part, to the new applications of genetically encoded biosensors and advanced imaging approaches that allow unprecedented spatiotemporal resolution of receptor localization and signaling events. As mentioned above, these sensors have facilitated the detection of receptor signals in distinct parts of the cell, from diffusion across the plasma membrane (Gondin *et al.*, 2019; Metz *et al.*, 2019; Tobin *et al.*, 2019) to different subcellular compartments including endosomes and Golgi membranes (**Figure 1**) (Stoeber *et al.*, 2018). The molecular environment surrounding receptors is already well known to contribute to cell and tissue type-specific differences in signaling and drug effects. Location bias adds another

dimension of selectivity based on differences in the molecular environment around receptors at a subcellular level (Lobingier *et al.*, 2017), suggesting interesting new directions for improving drug efficacy and selectivity. This concept is relevant not only to trafficking of receptors between membrane compartments, but also to the lateral redistribution of opioid receptors between plasma membrane domains. For example, morphine and an opioid peptide full agonist were shown to produce different spatiotemporal signaling profiles by changing the selectivity with which MOP-R engages distinct protein networks, mediated by a phosphorylation-dependent change in the surface distribution of receptors (**Figure 1**) (Civciristov *et al.*, 2019).

New approaches to investigate opioid receptor biology in vivo

Ultimately, for these new directions to be exploited, it is key to better understand the physiological context in which opioid receptors operate. Cells expressing opioid receptors (and GPCRs in general) are the nodes where physiology, cell biology and biophysical concepts need to converge. Traditional approaches such as electrophysiology, immunohistochemistry and behavioral pharmacology, together with more recently developed genetic strategies to ablate or modify expression of opioid receptors in different parts of the nervous system, have paved the way for incisive studies of native tissue preparations and are beginning to illustrate the complexity of opioid drug action in vivo (Corder *et al.*, 2018). Transgenic expression of mutant MOP-R in knockout animals is also revealing additional complexity of opioid receptor regulation by phosphorylation in vivo (Arttamangkul, Leff, *et al.*, 2019). In particular, knock-in mice expressing mutant or tagged receptors are unveiling physiological regulation of opioid receptors in their native environment and delineating functional consequences in vivo (Scherrer *et al.*, 2006; Wang *et al.*, 2018; Ehrlich *et al.*, 2019; Kliewer *et al.*, 2019). Further, engineered opioid receptors that are activated by light allow precise spatiotemporal control of opioid signaling in defined neural circuits and relevance to behavior (Siuda *et al.*, 2015). Moreover, new chemical tools are being developed to enable the detection of endogenous receptors in vivo without genetic modification. For example, an elegant chemical method was described recently that exploits the selectivity of drug-receptor interactions as a pharmacological 'guide' to covalently label endogenous receptors without impairing receptor activity (**Figure 3**). Using this method, opioid receptors were imaged live in native brain slices using two-photon fluorescence microscopy, without the need for genetically encoded tags or indirect (*e.g.*, antibody) labeling methods (Arttamangkul, Plazek, *et al.*, 2019).

Conclusion and outlook

Many of the imaging methods highlighted above have focused on studies of MOP-R. Given the distinct and important effects of DOP-R and KOP-R, we anticipate that future studies using these methods will provide additional insights into the cellular basis of opioid receptor function and drug action. We also anticipate that advances in the study of other GPCRs will find application to the study of opioid receptors. For example, single molecule imaging has been used to detect 'hot spots' on the plasma membrane at which GPCRs interact with cognate G proteins (Sungkaworn *et al.*, 2017). Such imaging approaches can be used to examine GPCR interactions with other proteins as well, including receptor-receptor interactions (Calebiro and Grimes, 2020). We note that interesting progress has already been made on this front for opioid receptors, with single-

molecule imaging of receptors bound to a high-affinity fluorescent ligand revealing short-lived MOP-R homodimers in the plasma membrane (Gentsch *et al.*, 2019).

We also discussed above how structural studies enabled the development of conformational biosensors to detect opioid receptor activation in living cells. In particular, cryo-electron microscopy has emerged as a powerful approach for resolving structural details of GPCRs in complex with other proteins. A pioneering example was the determination of a structure of agonist-activated MOP-R in complex with Gi (Koehl *et al.*, 2018). Recently, cryo-electron microscopy was used successfully for structural determination of agonist-activated GPCRs in association with β -arrestin, a physiologically important complex that has eluded conventional x-ray crystallography (Zhou *et al.*, 2016; Yin *et al.*, 2019; Huang *et al.*, 2020; Staus *et al.*, 2020). It has also enabled structural determination of a GPCR bound to both β -arrestin and G protein simultaneously, a complex which has been proposed to form on the endosome membrane and transduce a sustained form of signaling by certain GPCRs after they undergo ligand-induced internalization (Vilardaga *et al.*, 2012; Nguyen *et al.*, 2019). Such structural and biophysical advances will undoubtedly fuel new progress at the cellular level. For example, cryo-electron microscopy revealed a subtle difference between the conformation of activated MOP-R when in complex with Gi relative to when in complex with a nanobody (Huang *et al.*, 2015; Koehl *et al.*, 2018). Inspired by this, a recent cell-based study compared recruitment by opioid receptors of engineered protein probes derived from corresponding G protein or nanobody folds. Considerable selectivity of protein probe recruitment to MOP-R and KOP-R was observed in living cells, depending on receptor activation by partial, biased or full agonists (Stoeber *et al.*, 2020).

In sum, the past few years have seen major advances in illuminating opioid pharmacology under the microscope. The field is now poised to resolve with unprecedented clarity fundamental receptor signaling and regulatory processes across molecular, cellular and physiological scales. Such approaches have already yielded new insight into the actions of existing drugs, are motivating new therapeutic hypotheses that are presently under investigation, and may enable future development of improved opioid therapies. In addition, because opioid receptors are widely considered prototypes for the large GPCR family, we anticipate that such efforts will continue to drive GPCR-directed therapeutic innovation more broadly.

Acknowledgements

We thank many colleagues for their valuable contributions to the field and critical discussion, and regret that we were able to highlight only a limited subset of relevant studies in the present brief review. Work in the authors' labs was supported by research grants from the United States National Institutes of Health / National Institute on Drug Abuse (DA010711 and DA012864 to MvZ), the UCSF Program for Breakthrough Biomedical Research (to D.J.) and the Academy of Medical Sciences Professorship Scheme (to M.C.).

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Jullié, Gondin, von Zastrow, and Canals.

References

- Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, von Zastrow M, Grandy DK, and Williams JT (2002) μ -Opioid Receptors: Ligand-Dependent Activation of Potassium Conductance, Desensitization, and Internalization. *J Neurosci* **22**:5769–5776, Society for Neuroscience.
- Arttamangkul S, Heinz DA, Bunzow JR, Song X, and Williams JT (2018) Cellular tolerance at the μ -opioid receptor is phosphorylation dependent. *Elife* **7**.
- Arttamangkul S, Leff ER, Koita O, Birdsong WT, and Williams JT (2019) Separation of Acute Desensitization and Long-Term Tolerance of μ -Opioid Receptors Is Determined by the Degree of C-Terminal Phosphorylation. *Mol Pharmacol* **96**:505–514.
- Arttamangkul S, Plazek A, Platt EJ, Jin H, Murray TF, Birdsong WT, Rice KC, Farrens DL, and Williams JT (2019) Visualizing endogenous opioid receptors in living neurons using ligand-directed chemistry. *Elife* **8**.
- Arttamangkul S, Quillinan N, Low MJ, von Zastrow M, Pintar J, and Williams JT (2008) Differential activation and trafficking of micro-opioid receptors in brain slices. *Mol Pharmacol* **74**:972–979.
- Arttamangkul S, Torrecilla M, Kobayashi K, Okano H, and Williams JT (2006) Separation of μ -opioid receptor desensitization and internalization: endogenous receptors in primary neuronal cultures. *J Neurosci* **26**:4118–4125.
- Bailey CP, Oldfield S, Llorente J, Caunt CJ, Teschemacher AG, Roberts L, McArdle CA, Smith FL, Dewey WL, Kelly E, and Henderson G (2009) Involvement of PKC α and G-protein-coupled receptor kinase 2 in agonist-selective desensitization of μ -opioid receptors in mature brain neurons. *Br J Pharmacol* **158**:157–164.
- Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, and Lin FT (1999) Enhanced morphine analgesia in mice lacking β -arrestin 2. *Science* **286**:2495–2498.
- Borgland SL, Connor M, Osborne PB, Furness JB, and Christie MJ (2003) Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of μ -opioid receptors. *J Biol Chem* **278**:18776–18784.
- Bourinet E, Soong TW, Stea A, and Snutch TP (1996) Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proc Natl Acad Sci U S A* **93**:1486–1491.
- Bowman SL, Soohoo AL, Shiwarski DJ, Schulz S, Pradhan AA, and Puthenveedu MA (2015) Cell-autonomous regulation of μ -opioid receptor recycling by substance P. *Cell Rep* **10**:1925–1936.
- Bruchas MR, and Chavkin C (2010) Kinase cascades and ligand-directed signaling at the κ opioid receptor. *Psychopharmacology* **210**:137–147.
- Calebiro D, and Grimes J (2020) G Protein-Coupled Receptor Pharmacology at the Single-Molecule Level. *Annu Rev Pharmacol Toxicol* **60**:73–87.

- Chavkin C, and Goldstein A (1982) Reduction in opiate receptor reserve in morphine tolerant guinea pig ilea. *Life Sci* **31**:1687–1690.
- Civciristov S, Huang C, Liu B, Marquez EA, Gondin AB, Schittenhelm RB, Ellisdon AM, Canals M, and Halls ML (2019) Ligand-dependent spatiotemporal signaling profiles of the μ -opioid receptor are controlled by distinct protein-interaction networks. *J Biol Chem* **294**:16198–16213.
- Conibear AE, and Kelly E (2019) A biased view of μ opioid receptors? *Mol Pharmacol*, doi: 10.1124/mol.119.115956.
- Corder G, Castro DC, Bruchas MR, and Scherrer G (2018) Endogenous and Exogenous Opioids in Pain. *Annu Rev Neurosci* **41**:453–473.
- Dang VC, and Williams JT (2004) Chronic morphine treatment reduces recovery from opioid desensitization. *J Neurosci* **24**:7699–7706.
- DeWire SM, Ahn S, Lefkowitz RJ, and Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* **69**:483–510.
- DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, Chen X-T, Pitis PM, Gotchev D, Yuan C, Koblish M, Lark MW, and Violin JD (2013) A G protein-biased ligand at the μ -opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* **344**:708–717.
- Ehrlich AT, Semache M, Gross F, Da Fonte DF, Runtz L, Colley C, Mezni A, Le Gouill C, Lukasheva V, Hogue M, Darcq E, Bouvier M, and Kieffer BL (2019) Biased Signaling of the Mu Opioid Receptor Revealed in Native Neurons. *iScience* **14**:47–57.
- Filizola M (2019) Insights from molecular dynamics simulations to exploit new trends for the development of improved opioid drugs. *Neurosci Lett* **700**:50–55.
- Fox PD, and Hentges ST (2017) Differential Desensitization Observed at Multiple Effectors of Somatic μ -Opioid Receptors Underlies Sustained Agonist-Mediated Inhibition of Proopiomelanocortin Neuron Activity. *J Neurosci* **37**:8667–8677.
- Fyfe LW, Cleary DR, Macey TA, Morgan MM, and Ingram SL (2010) Tolerance to the antinociceptive effect of morphine in the absence of short-term presynaptic desensitization in rat periaqueductal gray neurons. *J Pharmacol Exp Ther* **335**:674–680.
- Galandrin S, Oligny-Longpré G, and Bouvier M (2007) The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* **28**:423–430.
- Gentsch C, Seier K, Drakopoulos A, Jobin M-L, Lanoiselée Y, Koszegi Z, Maurel D, Sounier R, Hübner H, Gmeiner P, Granier S, Calebiro D, and Decker M (2019) Selective and Wash-Resistant Fluorescent Dihydrocodeinone-Derivatives Allow Single-Molecule Imaging of μ -Opioid Receptor Dimerisation. *Angew Chem Int Ed Engl*, doi: 10.1002/anie.201912683.
- Gondin AB, Halls ML, Canals M, and Bridson SJ (2019) GRK Mediates μ -Opioid Receptor Plasma Membrane Reorganization. *Front Mol Neurosci* **12**:104.
- Hill R, Disney A, Conibear A, Sutcliffe K, Dewey W, Husbands S, Bailey C, Kelly E, and Henderson G (2018) The novel μ -opioid receptor agonist PZM21 depresses respiration and

- induces tolerance to antinociception. *Br J Pharmacol* **175**:2653–2661.
- Hislop JN, Henry AG, 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.
- Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, Sanborn AL, Kato HE, Livingston KE, Thorsen TS, Kling RC, Granier S, Gmeiner P, Husbands SM, Traynor JR, Weis WI, Steyaert J, Dror RO, and Kobilka BK (2015) Structural insights into μ -opioid receptor activation. *Nature* **524**:315–321.
- Huang W, Masureel M, Qianhui Q, Janetzko J, Inoue A, Kato HE, Robertson MJ, Nguyen KC, Glenn JS, Skiniotis G, and Kobilka BK (2020) Structure of the neurotensin receptor 1 in complex with β -arrestin 1. *Nature*, doi: 10.1038/s41586-020-1953-1.
- Hu X, Provasi D, Ramsey S, and Filizola M (2019) Mechanism of μ -Opioid Receptor-Magnesium Interaction and Positive Allosteric Modulation. *Biophys J*, doi: 10.1016/j.bpj.2019.10.007.
- Hu X, Wang Y, Hunkele A, Provasi D, Pasternak GW, and Filizola M (2019) Kinetic and thermodynamic insights into sodium ion translocation through the μ -opioid receptor from molecular dynamics and machine learning analysis. *PLoS Comput Biol* **15**:e1006689.
- Jullié D, Stoeber M, Sibarita J-B, Zieger HL, Bartol TM, Arttamangkul S, Sejnowski TJ, Hosy E, and von Zastrow M (2019) A Discrete Presynaptic Vesicle Cycle for Neuromodulator Receptors. *Neuron*, doi: 10.1016/j.neuron.2019.11.016.
- Just S, Illing S, Trester-Zedlitz M, Lau EK, Kotowski SJ, Miess E, Mann A, Doll C, Trinidad JC, Burlingame AL, von Zastrow M, and Schulz S (2013) Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol Pharmacol* **83**:633–639.
- Kang DS, Tian X, and Benovic JL (2014) Role of β -arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. *Curr Opin Cell Biol* **27**:63–71.
- Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, and von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **271**:19021–19024.
- Kenakin T (2017) Signaling bias in drug discovery. *Expert Opin Drug Discov* **12**:321–333.
- Kieffer BL, and Evans CJ (2009) Opioid receptors: from binding sites to visible molecules in vivo. *Neuropharmacology* **56 Suppl 1**:205–212.
- Kliwer A, Gillis A, Hill R, Schmiedel F, Bailey C, Kelly E, Henderson G, Christie MJ, and Schulz S (2020) Morphine-induced respiratory depression is independent of β -arrestin2 signalling. *Br J Pharmacol*, doi: 10.1111/bph.15004.
- Kliwer A, Schmiedel F, Sianati S, Bailey A, Bateman JT, Levitt ES, Williams JT, Christie MJ, and Schulz S (2019) Phosphorylation-deficient G-protein-biased μ -opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat Commun* **10**:367.
- Koehl A, Hu H, Maeda S, Zhang Y, Qu Q, Paggi JM, Latorraca NR, Hilger D, Dawson R, Matile H, Schertler GFX, Granier S, Weis WI, Dror RO, Manglik A, Skiniotis G, and Kobilka BK

- (2018) Structure of the μ -opioid receptor-Gi protein complex. *Nature* **558**:547–552.
- Komolov KE, and Benovic JL (2018) G protein-coupled receptor kinases: Past, present and future. *Cell Signal* **41**:17–24.
- Kunselman JM, Zajac AS, Weinberg ZY, and Puthenveedu MA (2019) Homologous Regulation of Mu Opioid Receptor Recycling by G $\beta\gamma$, Protein Kinase C, and Receptor Phosphorylation. *Mol Pharmacol* **96**:702–710.
- Lau EK, Trester-Zedlitz M, Trinidad JC, Kotowski SJ, Krutchinsky AN, Burlingame AL, and von Zastrow M (2011) Quantitative encoding of the effect of a partial agonist on individual opioid receptors by multisite phosphorylation and threshold detection. *Sci Signal* **4**:ra52.
- Law PY, Hom DS, and Loh HH (1984) Down-regulation of opiate receptor in neuroblastoma x glioma NG108-15 hybrid cells. Chloroquine promotes accumulation of tritiated enkephalin in the lysosomes. *J Biol Chem* **259**:4096–4104.
- Li JG, Luo LY, Krupnick JG, Benovic JL, and Liu-Chen LY (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.
- Lobingier BT, Hüttenhain R, Eichel K, Miller KB, Ting AY, von Zastrow M, and Krogan NJ (2017) An Approach to Spatiotemporally Resolve Protein Interaction Networks in Living Cells. *Cell* **169**:350–360.e12.
- Lowe JD, and Bailey CP (2015) Functional selectivity and time-dependence of μ -opioid receptor desensitization at nerve terminals in the mouse ventral tegmental area. *Br J Pharmacol* **172**:469–481.
- Manglik A, Kobilka BK, and Steyaert J (2017) Nanobodies to Study G Protein-Coupled Receptor Structure and Function. *Annu Rev Pharmacol Toxicol* **57**:19–37.
- Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, Levit A, Kling RC, Bernat V, Hübner H, Huang X-P, Sassano MF, Giguère PM, Löber S, Da Duan, Scherrer G, Kobilka BK, Gmeiner P, Roth BL, and Shoichet BK (2016) Structure-based discovery of opioid analgesics with reduced side effects. *Nature* **537**:185–190.
- McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, Krasel C, Dewey WL, Bailey CP, Rosethorne EM, Charlton SJ, Henderson G, and Kelly E (2010) μ -opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol Pharmacol* **78**:756–766.
- Merrifield CJ, Perrais D, and Zenisek D (2005) Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. *Cell* **121**:593–606.
- Metz MJ, Pennock RL, Krapf D, and Hentges ST (2019) Temporal dependence of shifts in mu opioid receptor mobility at the cell surface after agonist binding observed by single-particle tracking. *Sci Rep* **9**:7297.
- Nguyen AH, Thomsen ARB, Cahill TJ 3rd, Huang R, Huang L-Y, Marcink T, Clarke OB, Heissel

- S, Masoudi A, Ben-Hail D, Samaan F, Dandey VP, Tan YZ, Hong C, Mahoney JP, Triest S, Little J 4th, Chen X, Sunahara R, Steyaert J, Molina H, Yu Z, des Georges A, and Lefkowitz RJ (2019) Structure of an endosomal signaling GPCR-G protein- β -arrestin megacomplex. *Nat Struct Mol Biol* **26**:1123–1131.
- Pennock RL, Dicken MS, and Hentges ST (2012) Multiple inhibitory G-protein-coupled receptors resist acute desensitization in the presynaptic but not postsynaptic compartments of neurons. *J Neurosci* **32**:10192–10200.
- Pradhan AAA, Becker JAJ, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gavériaux-Ruff C, and Kieffer BL (2009) In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* **4**:e5425.
- Quillinan N, Lau EK, Virk M, von Zastrow M, and Williams JT (2011) Recovery from mu-opioid receptor desensitization after chronic treatment with morphine and methadone. *J Neurosci* **31**:4434–4443.
- Raehal KM, Walker JKL, and Bohn LM (2005) Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther* **314**:1195–1201.
- Rosendale M, Jullié D, Choquet D, and Perrais D (2017) Spatial and Temporal Regulation of Receptor Endocytosis in Neuronal Dendrites Revealed by Imaging of Single Vesicle Formation. *Cell Rep* **18**:1840–1847.
- Scherrer G, Tryoen-Tóth P, Filliol D, Matifas A, Laustriat D, Cao YQ, Basbaum AI, Dierich A, Vonesh J-L, Gavériaux-Ruff C, and Kieffer BL (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.
- Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue Z, Morgenweck J, Cameron MD, Bannister TD, and Bohn LM (2017) Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics. *Cell* **171**:1165–1175.e13.
- Sharma SK, Klee WA, 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.
- Shenoy SK, and Lefkowitz RJ (2011) β -Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci* **32**:521–533.
- Siuda ER, Copits BA, Schmidt MJ, Baird MA, Al-Hasani R, Planer WJ, Funderburk SC, McCall JG, Gereau RW 4th, and Bruchas MR (2015) Spatiotemporal control of opioid signaling and behavior. *Neuron* **86**:923–935.
- Sounier R, Mas C, Steyaert J, Laeremans T, Manglik A, Huang W, Kobilka BK, Déméné H, and Granier S (2015) Propagation of conformational changes during μ -opioid receptor activation. *Nature* **524**:375–378.
- Staus DP, Hu H, Robertson MJ, Kleinhenz ALW, Wingler LM, Capel WD, Latorraca NR, Lefkowitz RJ, and Skiniotis G (2020) Structure of the M2 muscarinic receptor- β -arrestin complex in a lipid nanodisc. *Nature*, doi: 10.1038/s41586-020-1954-0.
- Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, von Zastrow M, Evans C, and Brecha

- NC (1996) Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. *Proc Natl Acad Sci U S A* **93**:9241–9246.
- Stoeber M, Jullié D, Li J, Chakraborty S, Majumdar S, Lambert NA, Manglik A, and von Zastrow M (2020) Agonist-selective recruitment of engineered protein probes and of GRK2 by opioid receptors in living cells. *Elife* **9**.
- Stoeber M, Jullié D, Lobingier BT, Laeremans T, Steyaert J, Schiller PW, Manglik A, and von Zastrow M (2018) A Genetically Encoded Biosensor Reveals Location Bias of Opioid Drug Action. *Neuron* **98**:963–976.e5.
- Sungkaworn T, Jobin M-L, Burnecki K, Weron A, Lohse MJ, and Calebiro D (2017) Single-molecule imaging reveals receptor-G protein interactions at cell surface hot spots. *Nature* **550**:543–547.
- Tanowitz M, Hislop JN, and von Zastrow M (2008) Alternative splicing determines the post-endocytic sorting fate of G-protein-coupled receptors. *J Biol Chem* **283**:35614–35621.
- 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.
- Thompson GL, Lane JR, Coudrat T, Sexton PM, Christopoulos A, and Canals M (2016) Systematic analysis of factors influencing observations of biased agonism at the mu-opioid receptor. *Biochem Pharmacol* **113**:70–87.
- Tobin SJ, Wakefield DL, Terenius L, Vukojević V, and Jovanović-Talisman T (2019) Ethanol and Naltrexone Have Distinct Effects on the Lateral Nano-organization of Mu and Kappa Opioid Receptors in the Plasma Membrane. *ACS Chem Neurosci* **10**:667–676.
- Tsao PI, and von Zastrow M (2000) Type-specific sorting of G protein-coupled receptors after endocytosis. *J Biol Chem* **275**:11130–11140.
- Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, and Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1–13.
- Vilardaga J-P, Gardella TJ, Wehbi VL, and Feinstein TN (2012) Non-canonical signaling of the PTH receptor. *Trends Pharmacol Sci* **33**:423–431.
- Von Zastrow M, Keith DE Jr, and Evans CJ (1993) Agonist-induced state of the delta-opioid receptor that discriminates between opioid peptides and opiate alkaloids. *Mol Pharmacol* **44**:166–172.
- von Zastrow M, Keith D, Zaki P, and Evans C (1994) Intracellular trafficking of epitope-tagged opioid receptors: Different effects of morphine and enkephalin. *Regul Pept* **54**:315–316.
- Wang D, Tawfik VL, Corder G, Low SA, François A, Basbaum AI, and Scherrer G (2018) Functional Divergence of Delta and Mu Opioid Receptor Organization in CNS Pain Circuits. *Neuron* **98**:90–108.e5.
- Weinberg ZY, Zajac AS, Phan T, Shiwarski DJ, and Puthenveedu MA (2017) Sequence-Specific

Regulation of Endocytic Lifetimes Modulates Arrestin-Mediated Signaling at the μ Opioid Receptor. *Mol Pharmacol* **91**:416–427.

Weis WI, and Kobilka BK (2018) The Molecular Basis of G Protein-Coupled Receptor Activation. *Annu Rev Biochem* **87**:897–919.

Whistler JL, Enquist J, Marley A, Fong J, Gladher F, Tsuruda P, Murray SR, and Von Zastrow M (2002) Modulation of postendocytic sorting of G protein-coupled receptors. *Science* **297**:615–620.

Whistler JL, and von Zastrow M (1998) Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci U S A* **95**:9914–9919.

Wilding TJ, Womack MD, and McCleskey EW (1995) Fast, local signal transduction between the mu opioid receptor and Ca²⁺ channels. *J Neurosci* **15**:4124–4132.

Williams JT, Egan TM, and North RA (1982) Enkephalin opens potassium channels on mammalian central neurones. *Nature* **299**:74–77.

Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, Koch T, Evans CJ, and Christie MJ (2013) Regulation of μ -opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* **65**:223–254.

Yin W, Li Z, Jin M, Yin Y-L, de Waal PW, Pal K, Yin Y, Gao X, He Y, Gao J, Wang X, Zhang Y, Zhou H, Melcher K, Jiang Y, Cong Y, Edward Zhou X, Yu X, and Eric Xu H (2019) A complex structure of arrestin-2 bound to a G protein-coupled receptor. *Cell Res* **29**:971–983.

Yousuf A, Miess E, Sianati S, Du Y-P, Schulz S, and Christie MJ (2015) Role of Phosphorylation Sites in Desensitization of μ -Opioid Receptor. *Mol Pharmacol* **88**:825–835.

Yu YJ, Dhavan R, Chevalier MW, Yudowski GA, and von Zastrow M (2010) Rapid delivery of internalized signaling receptors to the somatodendritic surface by sequence-specific local insertion. *J Neurosci* **30**:11703–11714.

Zarzycka B, Zaidi SA, Roth BL, and Katritch V (2019) Harnessing Ion-Binding Sites for GPCR Pharmacology. *Pharmacol Rev* **71**:571–595.

Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, and Caron MG (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.

Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, and Wang JB (1996) Differential mu opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. *J Biol Chem* **271**:11449–11454.

Zhou XE, Gao X, Barty A, Kang Y, He Y, Liu W, Ishchenko A, White TA, Yefanov O, Han GW, Xu Q, de Waal PW, Suino-Powell KM, Boutet S, Williams GJ, Wang M, Li D, Caffrey M, Chapman HN, Spence JCH, Fromme P, Weierstall U, Stevens RC, Cherezov V, Melcher K, and Xu HE (2016) X-ray laser diffraction for structure determination of the rhodopsin-arrestin complex. *Sci Data* **3**:160021.

Zurawski Z, Yim YY, Alford S, and Hamm HE (2019) The expanding roles and mechanisms of G

protein-mediated presynaptic inhibition. *J Biol Chem* **294**:1661–1670.

Figure legends

Figure 1: Agonist-induced signaling and trafficking of opioid receptors.

Inactive opioid receptors (grey) become activated (green) after binding to an agonist (A, steps 1-2). This enables signaling via G proteins (green ripples, step 2) and triggers phosphorylation of the receptor tail by GRKs followed by receptor engagement of β -arrestins (step 3) and endocytosis via clathrin-coated pits (step 4). These events inactivate G protein signaling (red receptor) and assure signal termination from the plasma membrane by endocytic removal of receptors. After receptors arrive in early endosomes, they have the capacity to signal again by engaging G proteins in the endosome membrane (green ripples, step 5). Receptors also engage molecular sorting mechanisms in the endosome limiting membrane (not shown) which determine if internalized receptors are delivered to lysosomes for proteolytic down-regulation or are nondestructively recycled to restore surface receptor responsiveness. Many non-peptide agonist drugs (drug B) are sufficiently membrane-permeant to activate a discrete pool of opioid receptors at the Golgi apparatus and activate receptors from this location (green ripples). Some agonists (drug C) induce receptor reorganization in the plasma membrane to change surface signaling (blue ripples).

Figure 2: Cellular basis for sensitive presynaptic neuromodulation by opioid receptors.

Opioid receptors inhibit synaptic vesicle (blue circles) exocytosis by locally regulating effectors that are restricted in terminals and positioned in, or adjacent to, individual presynaptic active zones. Opioid receptors are not immobilized at terminals, however, and instead are laterally mobile throughout the axon surface and collisionally couple to effectors at the presynapse. Presynaptic opioid receptors undergo phosphorylation-dependent endocytosis directly at terminals, and then are locally recycled and reinserted to the axon surface both within and outside of synapses to replenish the diffusible surface pool. Lateral diffusion of receptors is sufficiently fast for terminals to 'sample' agonist-receptor complexes formed outside of synapses. The net effect of these events is to maintain a mobile surface receptor pool which is capable of mediating sensitive signaling at the presynapse by leveraging lateral diffusion and the allosteric nature of opioid receptor signaling by heterotrimeric G proteins.

Figure 3: A non-perturbing labeling strategy for opioid receptors using ligand-guided chemistry.

Naltrexamine-acylimidazole-Alexa594 binds to the orthosteric binding site of opioid receptors, with the pharmacophore 'guiding' covalent coupling to residues located outside of the ligand binding pocket. The coupling reaction releases the pharmacophore (upon washout), leaving the native receptor fluorescently labeled and functional to undergo subsequent activation by an orthosteric agonist after labeling.

Figure 1

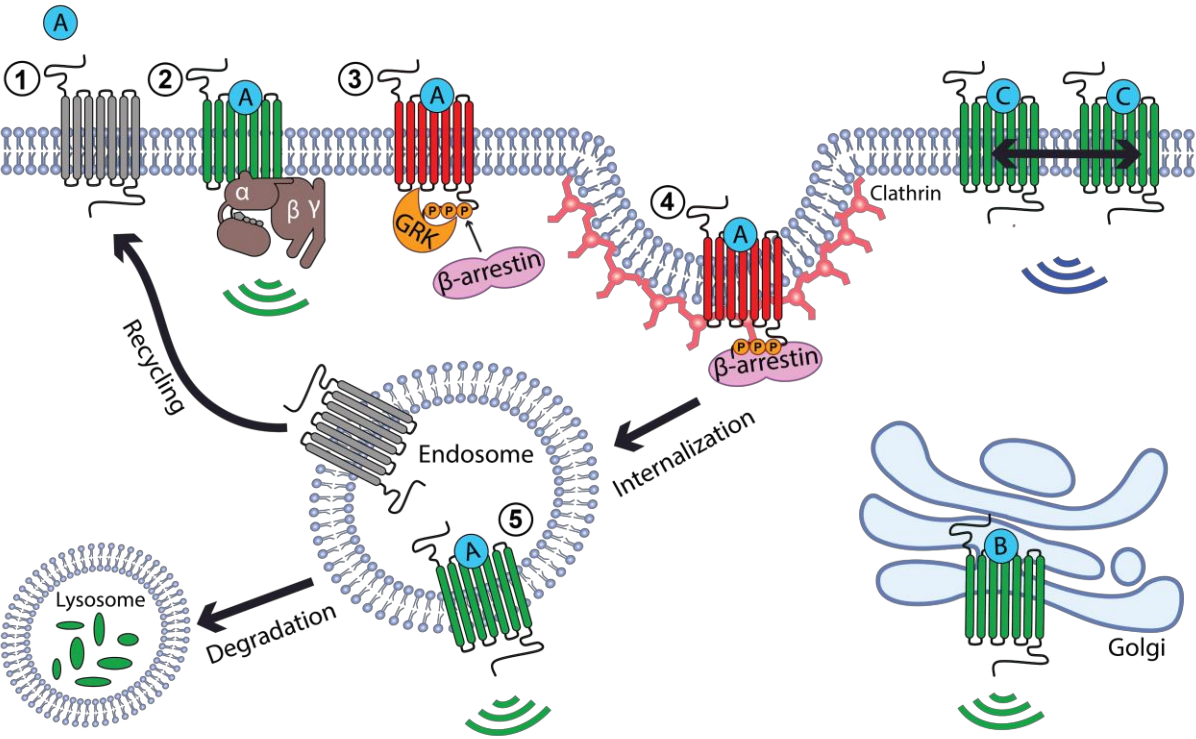


Figure 2

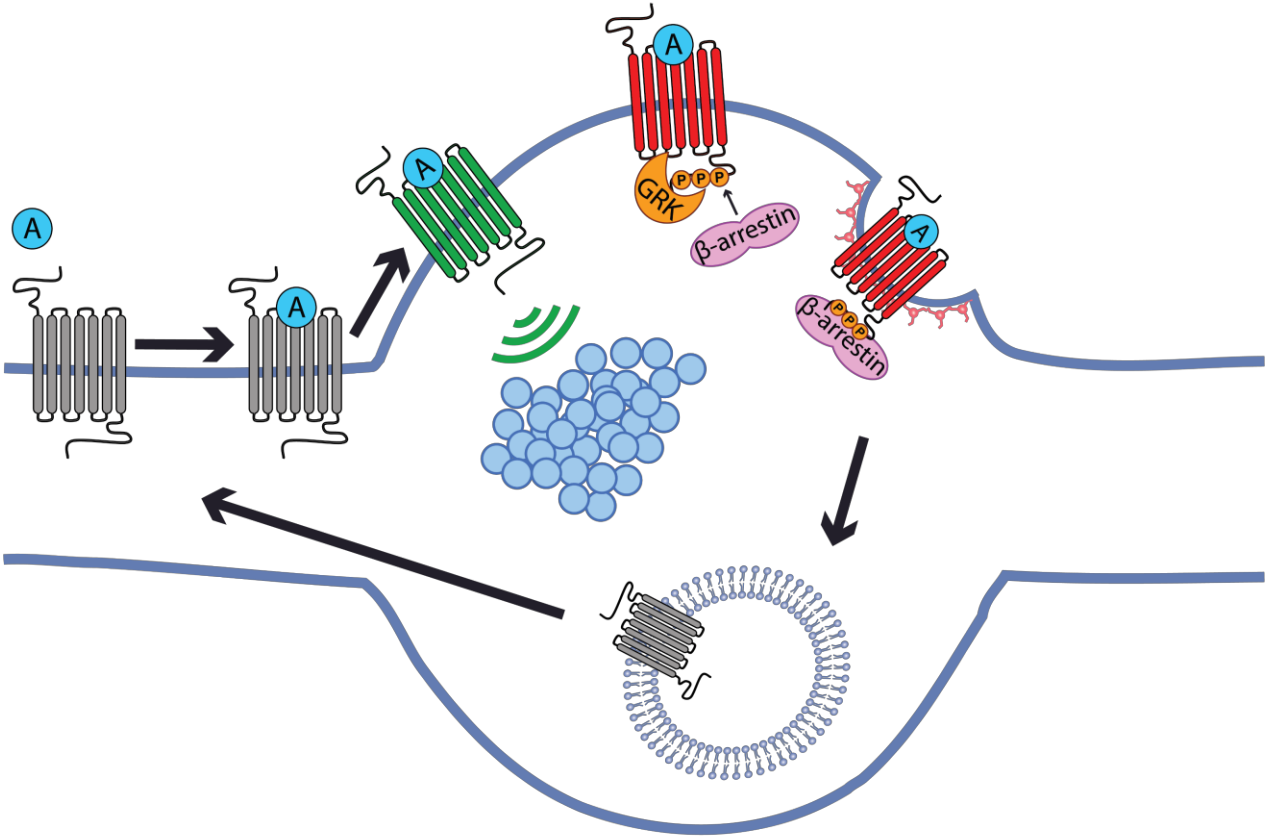


Figure 3

