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Authors

Jullié, Damien
Gondin, Arisbel B
von Zastrow, Mark
et al.

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Special Section on 50 Years of Opioid Research — Minireview

Opioid Pharmacology under the Microscope

Damien Jullié, Arisbel B. Gondin, Mark von Zastrow, and Meritxell Canals

Departments of Psychiatry and Cellular and Molecular Pharmacology, University of California, San Francisco School of Medicine, San Francisco, California (D.J., M.v.Z.); Drug Discovery Biology Theme, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Victoria, Australia (A.B.G.); Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, United Kingdom (M.C.); and Centre of Membrane Protein and Receptors, Universities of Birmingham and Nottingham, The Midlands, United Kingdom (M.C.)

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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 physiologic scales of analysis. The present review will highlight some recent cellular studies that we believe bridge across scales and will focus on optical

imaging approaches that 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 International Narcotics Research Conference. Our goal is to highlight distinct signaling and regulatory properties emerging from the cellular biology of opioid receptors and discuss potential relevance to therapeutics.

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 downregulation 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.

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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 nondestructively to the plasma membrane or traffic to lysosomes for proteolytic downregulation. The decision of which route receptors take is made by receptor-specific molecular sorting operations that occur in endosomes (Tsao and von Zastrow, 2000; Whistler et al., 2002). Internalized MOP-R and KOP-R typically recycle efficiently and downregulate slowly, whereas DOP-R, although also capable of nondestructive recycling, traffics preferentially to lysosomes and downregulates 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, an MOP-R splice variant that naturally lacks this sequence,

ABBREVIATIONS: DOP-R, δ -opioid receptor; GIRK, G protein-coupled inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; GRK, GPCR kinase; KOP-R, κ -opioid receptor; MOP-R, μ -opioid receptor; SEP, supercliptic pHluorin.

generated by alternative processing of the receptor transcript, recycles less efficiently and downregulates 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 (Fig. 1) that largely comports with a conserved paradigm of receptor desensitization established through studies of a variety of GPCRs (DeWire et al., 2007). Although 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 physiologic 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 downregulation 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 takeaway from the cellular regulatory framework summarized above is that receptor trafficking and signaling

operations are intricately interconnected. A critical group of enzymes that 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 that 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 (Fig. 1).

Toward an Expanded Understanding of the Signaling-Trafficking Relationship

According to the conserved regulatory framework summarized above, endocytosis of opioid receptors is inextricably associated with receptor 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 that are promoted, rather than inhibited, by receptor engagement with β -arrestin. The potential of β -arrestins to act as regulated scaffold proteins promoting

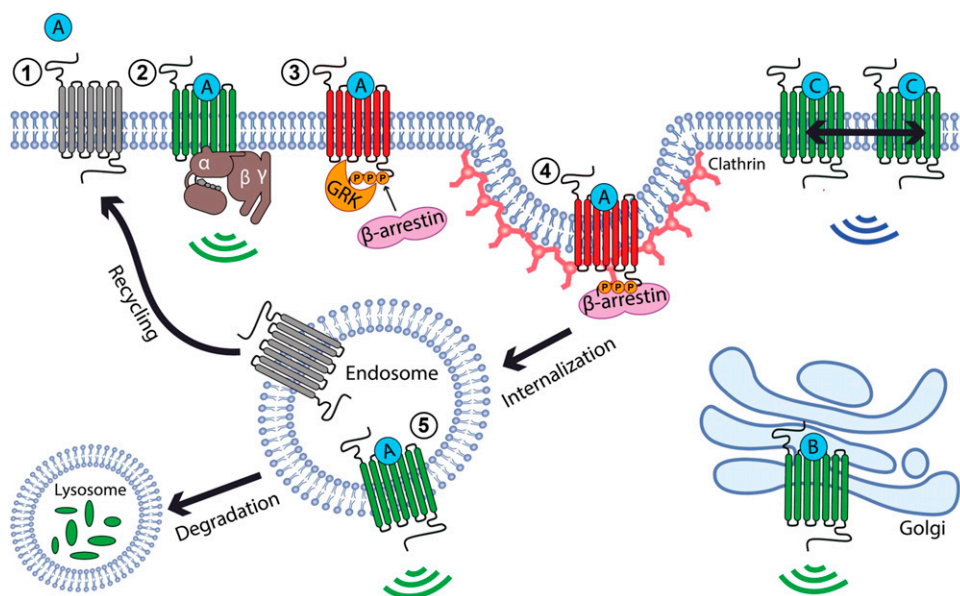


Fig. 1. Agonist-induced signaling and trafficking of opioid receptors. Inactive opioid receptors (gray) become activated (green) after binding to an agonist (A, steps 1 to 2). This enables signaling via G proteins (green ripples, step 2) and triggers phosphorylation of the receptor tail (P) by GRKs, 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 whether internalized receptors are delivered to lysosomes for proteolytic downregulation or are nondestructively recycled to restore surface receptor responsiveness. Many nonpeptide 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).

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 physiologic 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 that 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, 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, also revealed biosensor recruitment to endosome membranes after receptor internalization. Experiments contrasting the effects of membrane-permeant and membrane-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 (Fig. 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 classic receptor reserve because reducing the overall number of functional receptors by preexposing tissue slices to the irreversible opioid antagonist β -chlornaltrexamine 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 classic 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 preexposure 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., 2020). 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 GFP 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 that 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 in 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 (Fig. 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 classic 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 classic receptor reserve, enables the presynapse to

remain opioid-responsive and resist desensitization with low receptor number. It also carries other new physiologic 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., 2020).

Insight into the Molecular and Cellular Specificity of Opioid Drug Effects

In addition to its impact on physiologic desensitization, the principle of receptor reserve (in its classic meaning) is important for understanding opioid drug action, in particular agonist efficacy and partial agonism. Opioid receptors have been prototypical receptors for experimentally illustrating 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 -funaltrexamine or chlornaltrexamine, 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 classic 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 physiologic signaling by opioid peptides or the effects of drugs remains unclear and defines an exciting area of current investigation.

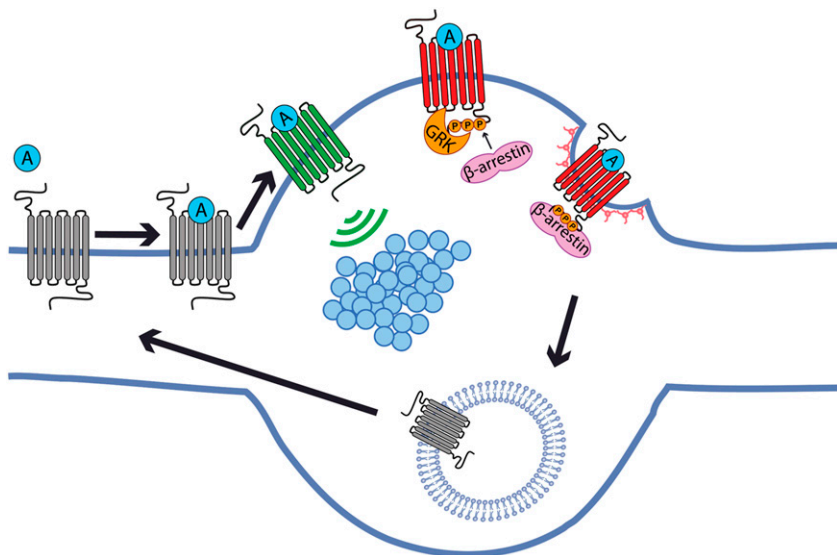


Fig. 2. Cellular basis for sensitive presynaptic neuromodulation by opioid receptors. Opioid receptors inhibit synaptic vesicle (blue circles) exocytosis by locally regulating effectors that are restricted in terminals and positioned in, or adjacent to, individual presynaptic active zones. Opioid receptors are not immobilized at terminals, however, and instead are laterally mobile throughout the axon surface and collisionally couple to effectors at the presynapse. After ligand-induced activation (A), presynaptic opioid receptors undergo phosphorylation (P) and endocytosis directly at terminals. Receptors are then locally recycled and reinserted to the axon surface both within and outside of synapses to replenish the diffusable 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 that 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.

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 physiologic 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 et al., 2019, 2020; 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 (Fig. 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 (Fig. 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 physiologic context in which opioid receptors operate. Cells expressing opioid receptors (and GPCRs in general) are the nodes at which 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 et al., 2019a). In particular, knockin mice expressing mutant or tagged receptors are unveiling physiologic 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; Kliwer 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 (Fig. 3). Using this method, opioid receptors were imaged live in native brain slices using two-photon fluorescence microscopy, without the need for genetically

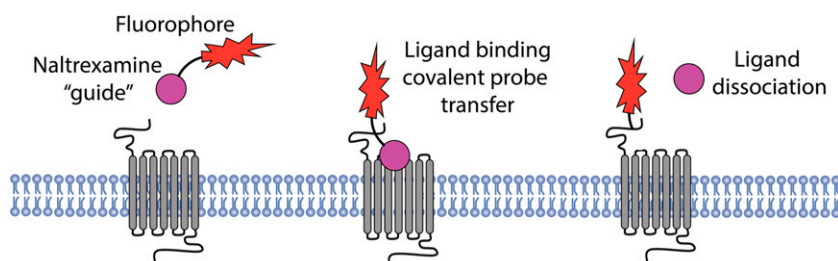


Fig. 3. A nonperturbing labeling strategy for opioid receptors using ligand-guided chemistry. Naltrexamine-acylimidazole-Alexa594 binds to the orthosteric binding site of opioid receptors, with the pharmacophore “guiding” covalent coupling to residues located outside of the ligand binding pocket. The 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.

encoded tags or indirect (e.g., antibody) labeling methods (Arttamangkul et al., 2019b).

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, cryoelectron 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, cryoelectron 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 (Villardaga et al., 2012; Nguyen et al., 2019). Such structural and biophysical advances will undoubtedly fuel new progress at the cellular level. For example, cryoelectron 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 physiologic 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.

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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.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Julli , Gondin, von Zastrow, Canals.

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Address correspondence to: Dr. Meritxell Canals, 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 Dr. Mark von Zastrow, UCSF Mission Bay, Genentech Hall Rm. N212E, 600 16th St., San Francisco, CA 94143. E-mail: mark@vzlab.org
