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Title Page:

Recent progress in opioid research from an electrophysiological perspective

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Running Title Page:

Electrophysiology in opioid research: recent progress

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Abstract: 243 words
Introduction: 440 words
Discussion: N/A minireview
Abbreviations: BRET: bioluminescent resonant energy transfer, DAMGO: [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin, DOR: delta opioid receptor, ERK: extracellular signaling-related kinase, GABA: gamma aminobutyric acid, GIRK: g-protein gated inwardly rectifying potassium channel, GRK: g protein-coupled receptor kinase, IPSC: inhibitory postsynaptic current, JUNK: c-Jun N-terminal kinase, LC: locus coeruleus, ME: [Met^5]-enkephalin, MOR: mu opioid receptor, PKC: protein kinase C, VTA: ventral tegmental area

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Abstract

Electrophysiological approaches provide powerful tools to further our understanding of how different opioids affect signaling through opioid receptors; how opioid receptors modulate circuitry involved in processes such as pain, respiration, addiction and feeding; and how receptor signaling and circuits are altered by physiological challenges such as injury, stress and chronic opioid treatment. The use of genetic manipulations to alter or remove mu opioid receptors (MORs) with anatomical and cell-type specificity and the ability to activate or inhibit specific circuits through opto- or chemo-genetic approaches are being used in combination with electrophysiological, pharmacological, and systems-level physiology experiments to expand our understanding of the beneficial and mal-adaptive roles of opioids and opioid receptor signaling. New approaches for studying endogenous opioid peptide signaling and release and the dynamics of these systems in response to chronic opioid use, pain and stress will add another layer to our understanding of the intricacies of opioid modulation of brain circuits. This understanding may lead to new targets or approaches for drug development or treatment regimens that may affect both acute and long-term effects of manipulating the activity of circuits involved in opioid-mediated physiology and behaviors. This review will discuss recent advancements in our understanding of the role of phosphorylation in regulating MOR signaling as well as our understanding of circuits and signaling pathways mediating physiological behaviors such as respiratory control and discuss how electrophysiological tools combined with new technologies have and will continue to advance the field of opioid research.

Significance Statement

This review discusses recent advancements in our understanding of mu opioid receptor function and regulation and the role of electrophysiological approaches combined with new technologies in pushing the field of opioid research forward. This covers regulation of MOR at the receptor level, adaptations induced by chronic opioid treatment, sites of action of MOR modulation of specific brain circuits and the role of the endogenous opioid system in driving physiology and behavior through modulation of these brain circuits.

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Introduction:

In the recent past, the understanding of the action of opioids on single neurons has significantly advanced in two areas, at the level of receptor modulation and in actions on opioid-sensitive pathways in the central nervous system. At the receptor level, it has been established that phosphorylation of the C-terminal tail of the mu-opioid receptor (MOR) underlies acute agonist dependent desensitization. Much of this has come from mutations of phosphorylation sites in MOR (Chen et al., 2013; Doll et al., 2011, 2012; Just et al., 2013; Lau et al., 2011; Mies et al., 2018; Moulledous et al., 2015; Wang et al., 2002) and the development of a selective and potent G protein-coupled receptor kinase (GRK) inhibitor, Compound101 (Thal et al., 2011; Lowe et al., 2015). Electrophysiological studies have been made in cell lines, neurons in brain slices with expressed receptors and in brain slices from knockin animals expressing MORs lacking regulatory phosphorylation sites in the C-terminal tail of the receptor (Yousuf et al., 2015; Miess et al., 2018; Kliewer et al., 2019; Birdsong 2013, 2015; Arttamangkul et al., 2018, 2019b). Both acute agonist-dependent desensitization and measures of long-term tolerance are reduced or eliminated in cells expressing phosphorylation-deficient mutant receptors. It has also become clear that acute desensitization is cell dependent in that the degree of acute desensitization varies with the cell type and brain region. The degree of desensitization is dependent on the effector under study as well: desensitization measured using the activation of potassium conductance at the cell body is distinctly different from the inhibition of transmitter release measured at axon terminals. Knockin animals that express fluorescently tagged opioid receptors and the ability to covalently tag endogenous opioid receptors with fluorescent dyes, has allowed appreciation of the extent of neurons and terminal fields that express opioid receptors (Arttamangkul et al., 2019; Erbs et al., 2015; Scherrer et al., 2006). Selective optical activation of neuron terminals coupled with opioid receptor pharmacology has yielded a greater understanding of opioid sensitive neural circuits. This approach in combination with the ability to knockout receptors in various brain areas using conditional MOR knockout mice has rapidly advanced the understanding of the central actions of opioids in controlling physiological processes (Birdsong et al., 2019; Charbogne et al., 2017). Finally, studies on the role of endogenous opioids in the brain are now approachable with the combination of selective activation of peptide-containing neurons and the developing area of genetically expressed peptide sensors. With these tools, a better understanding of the extent and significance of the role of the opioid system in modulating physiological and maladaptive processes such as analgesia, respiration, feeding, reward and addiction will be appreciated.

A. Receptor Phosphorylation and Acute Desensitization

Phosphorylation of G protein-coupled receptors as a mechanism that underlies acute agonist dependent desensitization has been known for decades and has now been firmly established for the mu opioid receptor (MOR). Acute agonist-

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dependent desensitization refers to a decline in signaling in the continued presence of agonist such that the cellular response to an opioid decreases over the time (Figure 1 A, B). This occurs over a timcourse of minutes to tens of minutes and can be measured in real time using electrophysiological techniques. Work with phospho-specific antibodies, quantitative mass-spectroscopy and alanine mutations of the C-terminus of MOR have indicated that multiple sites, including short cassettes, are the targets of phosphorylation in cell lines and cultured neurons (Lau et al., 2011; Just et al., 2013; Chen et. al., 2013; Miess et al., 2018; Mouledous et al., 2015). Recent physiological work on acute agonist dependent desensitization has extended the understanding of the role of C-terminus phosphorylation of MOR on downstream signaling (Arttamangkul et al 2018; 2019b; Birdsong et al., 2013; 2015; Kliewer et al., 2019).

To study the regulation of opioid receptors, cultured cells have a substantial advantage over thick preparations such as brain slices because agonists and antagonists can be readily washed from the tissue allowing rapid receptor dissociation of a variety of ligands of varying affinity and efficacy (Yousuf et al., 2015, Birdsong et al., 2013; Birdsong et al. 2015, Miess et al., 2018). The result is the ability to measure the time course of desensitization, the recovery from desensitization and changes in ligand-receptor binding kinetics. For example, a fluorescence-based assay was used to study the kinetics of ligand binding and dissociation in live cells (Birdsong et al., 2013; Birdsong et al., 2015). Fluorescently tagged ligands, dermorphin-A594 and naltrexamine-A594 were used to visualize the association and dissociation rate of binding and dissociation from the plasma membrane in HEK293 cells expressing an epitope tagged MOR (flag-MOR). Following a treatment of the cells with a saturating agonist concentration ([Met^5] enkephalin) for tens of minutes to hours, a long-lasting increase in agonist, but not antagonist, affinity was observed. The increased affinity was not seen when phosphorylation sites on the C-terminus were mutated to alanine. Surprisingly the increased affinity was found in cells where arrestin2 and arrestin3 were knocked out. The conclusion was that strongly desensitizing agonists caused a gradual increase in agonist affinity that was intrinsic to the agonist-receptor interaction. The functional consequence of the increased affinity could not be determined in this assay. When examined in brain slices there was a decrease in apparent affinity of receptors that remained functionally active following acute desensitization. This may suggest that the high affinity state observed in HEK293 cells were less or not functional with regard to activating potassium conductances (Williams, 2014). It is also possible that the difference in the time of agonist exposure in the two experiments could account for the different results (20 and 120 min in HEK293 cells, 10 min in brain slices) such that there are rapid phosphorylation events/ adaptations that occur over the timecourse of acute desensitization and slower phosphorylation events/ adaptations that occur over hours or even days that would be more akin to cellular tolerance. Nonetheless, the increased off rate in the functional receptors measured in brain slices could contribute to the decrease in signaling associated

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with acute desensitization and the development of tolerance but the precise mechanism has not been determined.

The downstream activation of potassium conductance is an ideal analog signal used to examine receptor dependent desensitization. The desensitization induced by both [Met^5]enkephalin (ME) and morphine in AtT20 cells expressing physiologically relevant levels of wild type and mutant (11S/t-A) MORs demonstrated that alanine mutations in the C-terminus eliminated desensitization induced by ME but not morphine (Yousuf et al., 2015). The desensitization induced by morphine in mice lacking all potential phosphorylation sites in the C-terminal tail (11S/T-A MOR, Figure 2) was blocked following treatment with the C-kinase inhibitor, calphostin C. Interestingly, the desensitization induced by morphine was heterologous in that the current mediated by activation of the somatostatin receptor was reduced following desensitization with morphine. Thus, in AtT20 cells, C-terminus phosphorylation of the receptor induced by ME and a secondary adaptation induced by morphine underlies acute desensitization. Similar experiments with morphine have not been possible in brain slices because of the slow washout of morphine.
The study of desensitization using neuronal recordings in brain slices has taken advantage of the ability to virally express phosphorylation-deficient mutant MOR receptors in knockout animals (mouse and rat, Birdsong et al., 2015; Arttamangkul et al., 2018; 2019b) as well as the development of knockin animals (mouse, Kliewer et al., 2019) that express alanine mutations on the MOR C-terminus. Whether virally expressed or in knockin animals, desensitization was blocked when studying receptors where all 11 phosphorylation sites were mutated to alanine. One advantage of using viral expression of mutant receptors in MOR knockout animals, both mouse and rat, is that the role of each of the phosphorylation sites in the C-terminus can be determined. Although each of the phosphorylation sites play some role in acute desensitization, mutations in the sequence from S375 to T380 resulted in a much-decreased acute desensitization (Figure 2). Additional alanine mutations resulted in greater inhibition of acute desensitization.

Cellular Tolerance and Acute Desensitization: Are they separate processes?

While acute desensitization refers to the loss of signaling over several minutes in the continued presence of an agonist, cellular tolerance refers to adaptations at the cellular level that result in reduced sensitivity to opioids that are induced over days to weeks and can last after the drug removal. Both desensitization and cellular tolerance have been measured by a decrease in receptor dependent activation of potassium conductance. The decrease in signaling has been measured in several ways. Acute desensitization has been measured first as a decline in potassium current (or membrane potential) in the continued presence of a saturating concentration of agonist (Figure 1A). The second measure is the relative decrease in current induced by a lower concentration of agonist following the application of a saturating concentration of agonist (Figure 1B) and these measures are not necessarily reflective of the same processes. Cellular tolerance

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has been measured by the decrease in the current induced by morphine (Figure 1C). This assay was developed because morphine is a partial agonist and therefore particularly sensitive to a decrease in effector coupling (Christie et al., 1987). The current induced by morphine is normalized to the current induced by the activation of a second Gi coupled receptor that activates the same potassium conductance. In the locus coeruleus the alpha-2-adrenoceptor is most commonly used. The second assay to measure tolerance uses repeated applications of a low concentration of agonist before and following treatment with a saturating concentration (Dang & Williams, 2004). This assay compares recordings from brain slices taken from naïve and chronically treated animals (Figure 1D). Signs of tolerance are (1) a larger decrease in the current induced by a saturating concentration of agonist, (2) a larger decrease in the current induced by a lower concentration of agonists following washout of the saturating concentration and (3) a decrease in the rate or extent of recovery from acute desensitization (Figure 1D).

Both knockin and virally expressed phosphorylation-deficient MORs have been utilized to characterize the role of phosphorylation sites in the C-terminal tail of MOR in regulating both acute desensitization and tolerance. Acute desensitization, cellular tolerance, and analgesic tolerance are all clearly affected by mutation of MOR phosphorylation sites (Arttamangkul et al., 2018, Arttamangkul et al., 2019b, Kliewer et al., 2019). However, the specific phosphorylation sites or overall degree of phosphorylation may affect the relative degree of acute desensitization and cellular tolerance. For example, mutation of the sequence 354TSST357 to alanine or glutamate affected some measures of tolerance without affecting the rate of acute desensitization (Arttamangkul et al., 2019b). Likewise, alanine mutation of the 375 STANT379 sequence eliminated measures of tolerance while reducing but not eliminating desensitization. Alanine mutation of other residues further decreased the degree of acute desensitization with variable effects on cellular tolerance (Arttamangkul et al., 2018, Arttamangkul et al., 2019b). Thus, there may be some specificity in which phosphorylation sites regulate tolerance and desensitization and this specificity may be agonist dependent. For example knockin mice harboring the S375A mutation display acute desensitization to ME, and cellular and analgesic tolerance to morphine that is indistinguishable from wildtype mice but these mice appear to not develop analgesic tolerance to fentanyl (Kliewer et al., 2019) suggesting that different phosphorylation sites may play a role in tolerance to different agonists.

While the sequence from S375 to T379 in MOR appears to be the primary site involved in acute desensitization, the story is likely more complicated. Using phospho-specific antibodies, it has been demonstrated that deleting phosphorylation sites of S375 to T379 (and S375 alone) prevents or greatly attenuates the rate and extent of phosphorylation at other sites (Doll et al., 2011, Miess et al., 2019). Alanine mutation of the 354TSST357 cluster also modestly decreased the rate of phosphorylation of other non-mutated residues as well. Therefore, phosphorylation and/or protein binding to serine and threonine

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residue at these phosphorylation sites likely facilitates or catalyzes phosphorylation of other residues in the C-terminus of MOR. Thus mutation of one residue could cause changes in the extent or kinetics of phosphorylation of many residues. This may be particularly important during acute desensitization when the kinetics of receptor phosphorylation are on the same time scale as the kinetics of desensitization (Doll et al., 2011; Yousuf et al., 2013).

Due to the interconnectedness of phosphorylation in the C-terminus of MOR, it has been difficult to determine whether individual phosphorylation sites regulate specific processes or whether the bulk amount of C-terminus phosphorylation is responsible for receptor desensitization, internalization and long-term tolerance. Investigating effector systems using multiple assays and timescales may be a useful approach to untangle these processes. For example, mutation of the TSST cassette blunts stable recruitment of arrestin measured with beta galactosidase complementation assay (slow time scale) but not a BRET assay (rapid timescale) while mutation of the STANT cassette decreased ß arrestin recruitment using both assays. These results suggest that individual phosphorylation sites may play specific complementary roles in regulating opioid signaling and arrestin binding (Miess et al., 2018) depending on the time course of agonist exposure and agonist efficacy. While it is becoming clear exactly what effects receptor phosphorylation have on opioid receptor signaling, progress is also being made on connecting these phosphorylation events to specific kinases.

Only recently has it been possible to pharmacologically block acute desensitization using recordings from brain slices from wild type animals. Phosphorylation of the C-terminal tail of MOR by high-efficacy agonists has been demonstrated to depend on G protein receptor kinases 2 and 3 (GRK 2/3) (Doll et al., 2012). The GRK2,3 inhibitor, compound101 effectively blocked acute opioid desensitization in locus coeruleus neurons (Lowe et al., 2015,). Although the inhibition of GRK was expected to block or reduce acute desensitization there is a substantial literature that implicates several other kinases, notably PKC, JNK and ERK1/2 (Bailey et al., 2004, 2009; Dang et al., 2009, reviewed Bailey et al., 2006; reviewed Williams et al., 2013). It is therefore somewhat surprising that inhibition of GRK is so effective. One potential explanation is that receptor phosphorylation by GRKs is rapid while kinase activation and receptor phosphorylation by other kinases is slow relative to the time course of acute desensitization.

A role of PKC in acute desensitization was proposed in locus coeruleus neurons based on an increase in the rate and extent of desensitization induced by ME and morphine following the activation of PKC by phorbol esters and muscarinic receptors (Bailey et al., 2006, 2009). Experiments in AtT20 cells using phosphorylation deficient receptors found that although acute desensitization induced by peptide agonists was blocked, morphine-induced desensitization was unaffected and only blocked by the combination of inhibition of PKC and mutation of C-terminal phosphorylation sites (Yousuf et al., 2015; Miess et al., 2018). The interpretation was that PKC mediated desensitization was mediated

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by phosphorylation of an accessory molecule rather than MOR. The increase in acute desensitization induced by phorbol esters has not been reproduced in the locus coeruleus, however there is agreement that PKC contributes to the short term tolerance observed following chronic morphine treatment (Bailey et al., 2009; Arttamangkul et al., 2014; Levitt & Williams 2012).

Recent work with brain slices taken from morphine treated animals (6-7 days) had found compound101 is less effective at blocking one component of acute desensitization following chronic morphine treatment (Leff and Williams, International Narcotics Research Conference Abstract, 2019). When the combination of kinase inhibitors to block both PKC and JNK was used, acute desensitization induced by ME was blocked in chronic morphine treated animals, suggesting that chronic morphine treatment shifts kinase regulation of acute desensitization from predominantly GRK-mediated to GRK, PKC and JNK-mediated. Unlike the heterologous morphine-induced desensitization in the AtT20 cells that was blocked by calphostin C (Yousuf et al., 2015), this mechanism was blocked in phosphorylation deficient receptors in slices from morphine treated animals suggesting a direct action on the receptor. It is also possible that the continued signaling and/or the lack of internalization of the phosphorylation deficient receptors in the brain slice experiments disrupted the activation of the PKC/JNK dependent process. The underlying mechanism for the development of PKC/JNK dependent tolerance remains to be determined. It is necessary to determine by what mechanism this adaptation is induced and for how long it persists following withdrawal. Also, does chronic treatment with other agonists of varying efficacy, such as fentanyl and buprenorphine, induce this remarkable adaptation? It has been known for some time that, following chronic treatment with morphine, acute desensitization is increased and the recovery from desensitization is prolonged (Dang & Williams, 2004; Quillinan et al., 2011; Levitt & Williams, 2012). Therefore, the induction of a GRK2/3-insensitive mechanism may account for the increased acute desensitization following chronic morphine treatment.

B. Is receptor phosphorylation the whole story?

The activation of potassium conductance in neurons of the locus coeruleus has served as a model system for the study of MOR desensitization for many years. It is now apparent that there is considerable variation in the extent of acute receptor desensitization across brain regions. In locus coeruleus, the decline in the peak outward current is about 50% during the application of a saturating concentration of an efficacious agonist. There are examples of neurons where the decline in current is nearly complete (cholinergic striatal interneurons, personal observation) and other examples such as neurons in the Kolliker-Fuse where there is little or no acute decline (Levitt & Williams, 2018). Further, in the locus coeruleus, mechanisms of desensitization may change over time as it has been observed that desensitization in older rats was homologous (occurring at MOR) while desensitization in younger rats appeared heterologous (affecting signaling through multiple receptors) (Llorente et al., 2012). It is not clear what

mechanisms underlie the differences between neuronal subtypes or developmental stages and there is little in the literature that has directly addressed the potential differences. From expression systems, it is clear that altering receptor number, signaling and regulatory components (such as GRKs and arrestins), or effectors can alter the efficacy, potency and signaling induced by opioids (Whistler and Von Zastrow 1998; Zhang et al., 1998).

There is also a rapid form of apparent desensitization that has been proposed to be mediated by the GRK dependent chelation of beta/gamma subunits resulting in dissociation from the potassium channel (Raveh et al., 2010; Yousuf et al., 2015; reviewed Gurevich et al., 2012). Thus the extent and time course of desensitization is dependent on the expression of multiple downstream processes that can vary widely among different cells.

C. Desensitization measured with different effectors

There are three electrophysiological measures that are commonly used to study the action of opioids: activation of potassium current, inhibition of calcium current, and inhibition of transmitter release. The activation of potassium current is by far the most commonly used measure to study desensitization. It is now certain that there is no detectable desensitization when one measures opioid receptor dependent inhibition of transmitter release (Blanchet & Luscher, 2002). Manipulations such as decreasing the receptor reserve with the irreversible blockade of receptors with ß-chlornaltrexamine, chronic morphine treatment, or prolonged incubation with a saturating concentration of agonist have not uncovered any evidence that the inhibition of transmitter release is affected by receptor desensitization (Fyfe et al., 2011; Pennock et al., 2012; Pennock & Hentges 2011; 2016; Fox & Hentges 2017).

The reason for the lack of presynaptic desensitization has been unknown. Based on recent receptor trafficking experiments, however, the lack of apparent presynaptic inhibition has been proposed to result from rapid diffusion of activated receptors along the cell surface from the axon to release sites, effectively buffering presynaptic inhibition from the loss of functional receptors due to internalization (Jullie et al., 2019). This work showed that MORs were internalized only in areas of transmitter release (axon varicosities) and not along the axons. This internalization was phosphorylation dependent and receptors were replenished in varicosities through local recycling and lateral diffusion of MORs from the axon to the varicosity. That work went on to show that the MOR agonist DAMGO inhibited transmitter release, measured by the imaging of synaptic vesicle exocytosis. Thus, receptor recycling and rapid lateral diffusion of receptors from the axon, where internalization does not occur, to release sites may provide a relatively constant pool of functional MOR in spite of receptor internalization and explain this apparent dichotomy between desensitization seen in postsynaptic but not presynaptic compartments (Jullie et al., 2019).

D. Floxed receptors

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The understanding of the long-distance pathways affected by opioids is now possible by combining the use of the floxed MOR animals (Weibel et al., 2013) to knockout MORs in select neuronal populations (Varga et al., 2019; Bachmutsky et al., 2019). This approach has been used to increase understanding of the action of opioids that mediate respiratory depression (Varga et al., 2019; Bachmutsky et al., 2019). The regulation of respiration is mediated by multiple nuclei. Most work on the depression of respiration induced by opioids centered on the pre-Botzinger complex. Recent work found that both the Kolliker-Fuse and pre-Botzinger complex are key sites of opioid action, that the two nuclei mediated depression in different concentration ranges, and that a very small number of neurons in the pre-Botzinger complex are opioid sensitive. Neurons in each nucleus are hyperpolarized by opioids, however these areas are highly interconnected, indicating that presynaptic inhibition of the reciprocal connections most likely plays a key role in the overall action of opioids (Varga et al., 2019). Although the regulation of respiration involves a wide range of processes spanning multiple brain regions, the selective deletion of MORs in specific brain regions is beginning to untangle the actions of opioids in this complex system.

When MORs were selectively removed in GABA forebrain neurons, largely in the dorsal striatum and nucleus accumbens, using floxed MOR: Dlx5/6-*Cre* mice, locomotor effects of heroin were abolished while measures of morphine analgesia and dependence were unaffected and motivation to obtain rewards was increased (Charbogne et al, 2017). In the VTA, GABA IPSCs measured on GABA neurons were rendered insensitive to DAMGO (whereas GABA IPSCs measured on dopamine neurons that were evoked by local stimulation remained sensitive to inhibition by DAMGO (Charbogne et al., 2017). These data demonstrate the ability to dissociate opioid effects on different behaviors and different sub-circuits that are likely to mediate these behaviors. While we are making progress in understanding how MOR in specific synapses and cell types is modulating drug-induced behavior, our understanding of the effects of the endogenous opioid system is still lagging behind.

E. Endogenous opioids

With the discovery of endogenous opioid peptides in the mid 1970s there was great excitement over the determination of the physiological role of these endogenous peptides. There is an enormity of work using exogenous application of opioid peptides to activate opioid receptors in multiple brain areas. Opioid peptides active receptors at concentrations low enough that one would expect to observe functional consequences following the release of endogenous peptides yet it has been incredibly difficult to detect release of endogenous opioids to study them at the cellular level at least in brain slices or cultured neurons. One prominent hypothesis for the inability to detect the functional actions of endogenous peptides is that the method(s) used to evoke peptide release have simply not been appropriate. Studies to date have used electrical stimulation.

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One early study came from work at the mossy fiber synapse in the hippocampus (Weiskopf et al., 1993). In that study, selective stimulation of the mossy fibers with 10 pulses at 100 Hz 4 times resulted in the release of dynorphin (presumed) that was antagonized by naloxone and nor-BNI (at what was most likely a concentration that blocked both mu and kappa opioid receptors). Likewise in the striatum, a stimulus of 5 pulses at 100 Hz evoked a transient release of presumably enkephalin that inhibited glutatmate release. The inhibition of glutamate release was occluded by the MOR agonist DAMGO and blocked by the MOR antagonist CTOP (Blomeley et al., 2011). A more recent study in the amygdala found release using pairs of stimuli (Winters et al., 2017). Peptidase inhibitors were used to prevent degradation of opioid peptides and thus prolong the presence of the peptides in the extracellular space to amplify the activation of receptors (Winters et al., 2017; Atwood et al., 2014). Following the cocktail of peptidase inhibitors, both the amplitude and duration of endogenously released opioid was increased. Given that the distribution of peptide containing neurons and projections are well known, as is the widespread distribution of opioid receptors, it is surprising that more has not been done at a cellular level, however knowledge about methods to induce peptide release and systems for rapid detection of peptides has been lacking. Discovery of the functional roles of endogenous peptides in the opioid system at the cellular level is an important step in a complete understanding of opioids in the brain.

Recent technical developments may be used to foster a better understanding of peptide release and subsequent receptor activation. Difficulties in the detection of opioid peptide release using electrical stimulation may be problematic given that this form of stimulation non-selectively activates multiple pre- and postsynaptic neurons, particularly when applied a high frequency or intensity. Recent advances in the ability to selectively activate peptide containing neurons, and specifically opioid peptide containing neurons, using optogenetics could potentially improve the ability to evoke peptide release. Dynorphin release has been measured using an optogenetic approach, however the relatively low frequency stimulation used to maintain action potential firing with channelrhodopsin necessitated long stimulation periods to measure peptide accumulation (Al-Hasani et al., 2015). The development of genetically expressed peptide sensors that increase fluorescence upon binding of peptides is a promising avenue for peptide detection. These sensors are based on G protein-coupled receptors along with a circularly permuted GFP. The binding affinity is, for the most part, similar to that of endogenous opioid receptors (Patriarchy et al., 2018). The plasma membrane localization and the similarity in affinity between these molecules and opioid receptors makes them ideally suited for the localized detection of peptide transmitter. The sensors can be expressed in a wide area such that imaging the increase in fluorescence can be examined at low magnification allowing both the site(s) of release and the extent of diffusion to be measured. Most importantly, with a rapid and robust detection method, the mechanisms that underlie the release of peptides can be determined.

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While this review focuses on mu opioid receptor signaling, endogenous enkephalins and endorphins can activate both mu and delta opioid receptors and it's possible for the kappa opioid selective peptide dynorphin to be metabolized to [Leu]$_5$enkephalin. Thus, endogenous peptides can activate multiple opioid receptors and neurons within local circuits can express various opioid receptors and serve opposing functions. Delta opioid receptors (DOR) inhibit GABA release from patches of MOR-rich neurons in striatum (Banghart et al., 2015). The activation of MORs in the striatum decreases excitatory afferents from thalamic projections whereas DOR activation disinhibits neurons in the anterior cingulate cortex to increase excitatory afferent input to the striatum (Birdsong et al, 2019). Thus the site(s) of receptor activation can have diverse effects on the final output of the medium spiny neurons in the striatum. Additionally, receptor phosphorylation, trafficking, and signaling to downstream effectors all dynamically regulate the function of opioid receptors. Therefore, understanding the temporal and spatial dynamics of how endogenous opioid peptides are sensed by both pre and postsynaptic opioid receptors will be a key step in creating a clearer picture of how these peptides mediate their diverse physiological effects.

To this end, the cellular distribution of opioid receptors has been explored using knockin animals that express receptors tagged with fluorescent ligands (Scherrer et al., 2006, Erbs et al., 2015). Early work imaging GFP-labeled delta receptors in neurons cultured from knockin mice were used to characterize the internalization of those receptors (Scherrer et al., 2006). A chemistry-based approach has been developed recently called "traceless affinity labeling" (Hayachi & Hamachi, 2012). This method used naltrexamine to guide a reactive molecule to opioid receptors. Once the naltrexamine becomes bound to the receptor the local concentration of the reactive molecule is high enough to enable a covalent reaction with the receptor. This reaction places a fluorescent tag on the receptor and at the same time cleaves the link with naltrexamine. The naltrexamine is then free to dissociate from the receptor. Thus, functional endogenous opioid receptors in wild type animals are covalently bound to fluorescent ligands (Arttamangkul et al., 2019a). This approach is ideal for the identification of opioid sensitive neurons in living brain slices. With the use of charged fluorescent molecules only plasma membrane associated receptors are labeled. Opioid receptor positive neurons can be identified in preparations of heterogeneous populations of neurons. This approach in combination with selective activation of peptide containing neurons and the detection of peptide release with the sensors has the potential for a complete characterization of endogenous opioid communication.

F. Electrophysiology of biased agonists

There has been intense interest in the development of biased agonists that can maintain the therapeutic actions of opioid while limiting on-target side effects. The differential activation of G-proteins and recruitment of arrestin by agonists has been the focus of considerable work and has been reviewed extensively (Hill et al., 2018; Rivero et al., 2012; Conibear & Kelly 2019; Gurevich & Gurevich 2019). Decades of electrophysiological work has demonstrated various

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mechanisms by which the excitability of neurons is decreased and neurotransmission is inhibited by opioid-receptor mediated activation of heterotrimeric G-proteins. From this body of work, G-protein mediated activation of potassium channels, inhibition of voltage gated calcium channel, inhibition of adenylyl cyclase and SNARE protein function have been well-established (Logothetis et al., 1987; Ikeda, 1996; Blackmer et al., 2001; Zurawski et al., 2019). However, to our knowledge, there is no indication as to how arrestin signaling could inhibit neurotransmission on the time scale that is close to G-protein dependent processes. That is not to say that arrestin signaling is not physiologically relevant but at this time there are no electrophysiological measures of arrestin dependent processes. Because the phosphorylation deletion mutants of MOR do not recruit arrestin, they can be considered "G-protein biased" receptors. Activation of these receptors still inhibits neurotransmission through activation of pre and postsynaptic mechanisms as well as or better than the wildtype receptor. Additionally, these G-protein biased receptors still effectively induce analgesia, respiratory depression, and withdrawal suggesting arrestin is not involved in these processes (Kliewer et al., 2019). In agreement with data from β–arrestin-2 knockout mice (Connor et al., 2014), it is clear, that tolerance at the electrophysiological and behavioral level is severely impaired in these mice, supporting a role for arrestin in these processes and suggesting that tolerance could be dissociated from the other effects of opioids that are associated with G-protein signaling.

Studies of agonist bias have used a combination of electrophysiological, cell biological and biochemical approaches in multiple cell based assays and have concluded that agonist efficacy, receptor reserve and the amplification of downstream signaling are important factors in the determination of receptor bias (Rivero et al., 2012; Miess et al., 2018; reviewed Conibear, Kelly 2019). Thus, downstream signaling and the particular assays studied impact the interpretation of agonist bias. The hypothesis that GPCRs move through multiple states that result in association with different downstream molecules has been tested recently (Stober et al., 2020). Using engineered molecules that mimic G-proteins (mGsi) and G-protein receptor kinases, GRKs (Nb33), this study demonstrated that different agonists resulted in distinct association of the two molecules with opioid receptors (Stoeber et al., 2020). The engineered probes were functionally inactive and known to bind selectively to agonist bound receptors such that probe binding directly reflected agonist dependent GPCR conformations. In experiments with the kappa opioid receptor there was a distinct difference in the association of the engineered molecules that was agonist dependent. Dynorphin bound kappa receptors increased association of both mGsi and Nb33 whereas etorphine bound receptors only bound mGsi. Similar differential results were obtained using MOR with DAMGO and the partial agonist mitragynine pseudoindoxyl. The demonstration of agonist dependent receptor association with downstream molecules supports the hypothesis that GPCRs move through distinct conformations that foster association with different downstream molecules. These conformations are the underlying mechanism of agonist bias

(Stoeber et al., 2020). How these conformations relate to physiological processes will be an important avenue for future research.

Summary:

Electrophysiological tools have been used for decades to complement structural, molecular, pharmacological and physiological/ behavioral methods to advance the understanding of opioid receptor function from the molecular to whole animal level. This review focused on recent advances in the understanding of MOR function using an electrophysiological approach. It is clear that receptor phosphorylation plays a key role in mediating MOR desensitization and tolerance. This appears to be mediated by phosphorylation of the C-terminus of MOR acutely by GRKs and following chronic opioid treatment by other kinases (PKC and JNK). Using optogenetics, imaging and newly generated mouse lines the modulation of specific neuronal populations by opioids has broadened the understanding of the opioid system. The knowledge of the cellular location, function and dynamics of opioid receptors and the endogenous opioid pathways are keys to the understanding of the actions of opioids on analgesia, respiration, feeding, reward and addiction. These investigations have the potential to yield improved therapeutics for pain relief and addiction treatment.

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Authorship Contributions:

Wrote or contributed to the writing of the manuscript: Birdsong, Williams

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Figure Legends:

Figure 1: Illustrations of electrophysiological experimental measures of desensitization and cellular tolerance. Desensitization is generally measured in two ways, first as in A, an acute decrease in the response, usually a current or voltage measurement, in the continued presence of a saturating concentration of agonist over a period of minutes (generally 5-10 minutes). The extent of this decrease in signaling is acute desensitization (black arrow). Reversal of this signaling by agonist washout or application of antagonist is used to ensure that the baseline measurement has not changed during the experiment. The second measure of desensitization B) is used in preparations such as cell culture or with agonists that can quickly be washed out of brain slices like [Met^5] -enkephalin. First, a moderate concentration of agonist is applied to elicit approximately a half-maximal response (EC50). Next, a saturating concentration of agonist is applied for minutes, similar to the protocol shown in "A" to desensitize the receptor (black arrow). The saturating agonist is washed out of the preparation and then the EC50 concentration of agonist is retested periodically to measure desensitization (cyan arrow) and the recovery from desensitization over time, which is nearly complete after 30-45 minutes. Cellular tolerance to chronic drug treatment is measured in several ways as well. C) First, an agonist such as morphine is applied and a response is measured, this is then reversed with an antagonist such as naloxone. Then a control agonist that activates another receptor but ultimately activates the same downstream effector is tested (red bar). The relative response to morphine vs. the control agonist is measured. This is done in preparations from naïve animals (black line) or animals that have been chronically treated with morphine or other opioids for a period of days, generally 5-7 days (dotted line). The readout of cellular tolerance is a decrease in the response of morphine following chronic morphine treatment (gray shaded box, gray arrows) when normalized to the control agonist. D) A second hallmark of opioid signaling in morphine tolerant animals is an increase in desensitization following chronic morphine treatment. A protocol identical to that done in "B" is done on chronically morphine treated mice, there is a characteristic increase in the decline in signaling in response to the saturating concentration of agonist (black arrow) as well as a smaller response to the EC50 concentration following acute desensitization (cyan arrow). Furthermore, the rate of recovery from desensitization is prolonged and the recovery from desensitization is incomplete (orange arrow).

Figure 2: Summary of electrophysiological data examining receptor desensitization, and tolerance dependence on potential C-terminal phosphorylation sites in MOR. 11 potential serine and threonine phosphorylation sites in the C-terminal tail of MOR are highlighted in yellow in the wildtype MOR (these sites are identical in rat and mouse). Potential phosphorylation sites were mutated to alanine, indicated by the red highlighting of residues, in several studies that are summarized here. These mutations are named under the Construct column. The effects of these mutations on the

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processes of receptor desensitization, internalization and cellular tolerance have been measured in various systems and have been reported. Processes that remained intact in the mutant receptor are indicated with a “Yes” while “No” indicates elimination of these processes, “Decreased” indicates a partial effect and “Mixed” indicates that different assays or ligands provided differing results. (Sources: 1 Arttamangkul et al. 2018, 2 Arttamangkul et al. 2019b, 3 Kliewer et al. 2019, 4 Miess et al., 5 Yousuf et. al, 6 Quillinan et. al., 7 John Williams, personal observation)

Figure 1

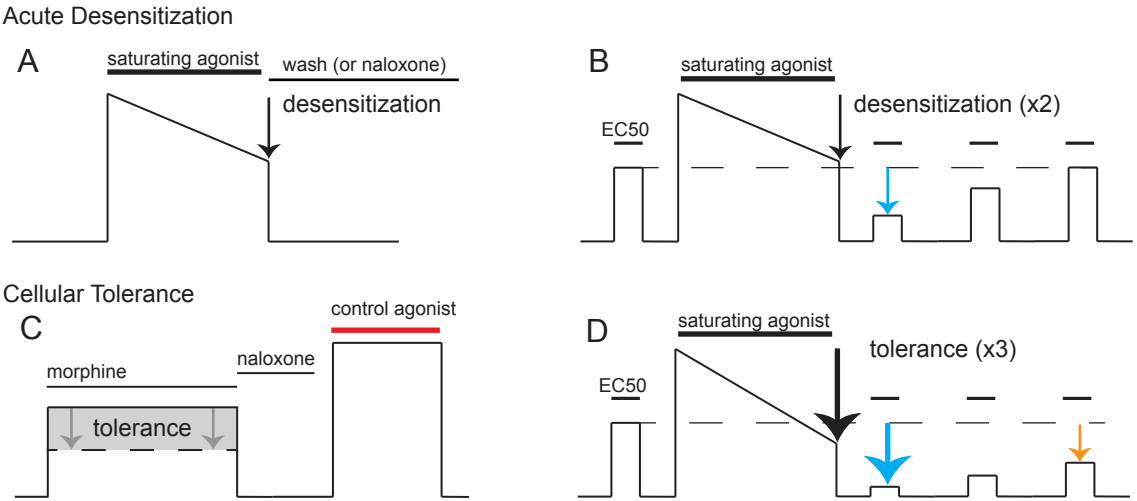
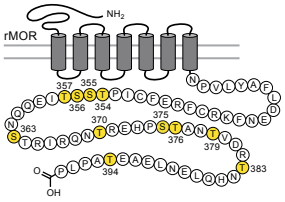
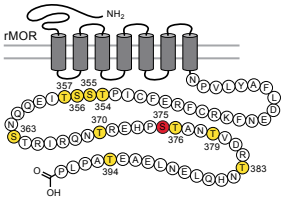
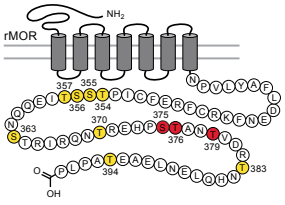
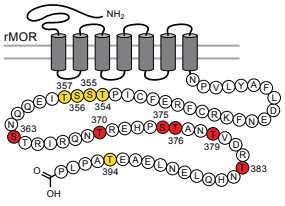
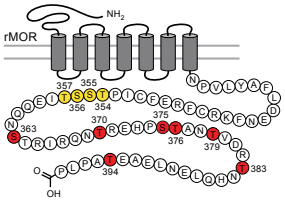
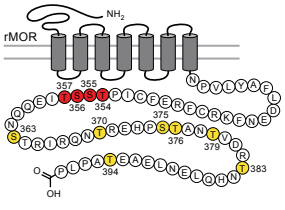
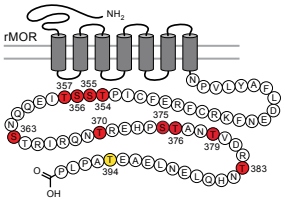
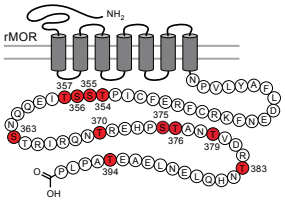

Acute Desensitization
A
saturating agonist
wash (or naloxone)
desensitization
B
saturating agonist
EC50
desensitization (x2)
Cellular Tolerance
C
control agonist
morphine
naloxone
tolerance
D
saturating agonist
tolerance (x3)
EC50

Figure 2

	Construct	Cell Type	Desensitization	Internalization	Tolerance	Reference
	Wild Type	LC Rat LC Mouse AtT20	Yes Yes Yes	Yes Yes Yes	Yes Yes	1,2 3,6,7 5
	S375A	LC Mouse	Yes		Yes	3,7
	3S/T-A	LC Rat AtT20	Decrease Yes	No	Mixed	2 4
	6S/T-A	AtT20	Yes	No		5
	7S/T-A	LC Rat	No		Decreased	2
	TSST-4A	LC Rat AtT20	Yes Yes	Yes	Mixed	2 4
	10S/T-A	LC Mouse	No		No	3
	11S/TA	LC Rat LC Mouse AtT20	No Decrease Mixed	No No No	No No	1 3,7 4