DISSERTATION

# DIFFERENTIAL DESENSITIZATION OF PRE- AND POSTSYNAPTIC MU OPIOID RECEPTORS REGULATING PROOPIOMELANOCORTIN NEURONS OF THE ARCUATE NUCLEUS

Submitted by

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ABSTRACT

DIFFERENTIAL DESENSITIZATION OF PRE- AND POSTSYNAPTIC MU OPIOID RECEPTORS REGULATING PROOPIOMELANOCORTIN NEURONS OF THE ARCUATE NUCLEUS

The mu opioid receptor (MOR) is the primary target of powerful opiate analgesics such as morphine and codeine. Repeated use of opiates, as may occur in patients with chronic pain, leads to the development of tolerance to the drugs' analgesic effects and may result in the development of dependence. This reduces the effectiveness of opiate-based treatments over extended periods of time, and can result in withdrawal when such a treatment is terminated. Many years of study have been dedicated to understanding the processes that lead to the development of tolerance, as an understanding of the mechanisms underlying tolerance could lead the development of novel therapeutic strategies that prolong the efficacy of opioid-based pain treatments. One particular area of focus has been on acute desensitization of the MOR.

Studies of acute desensitization, defined as the loss of receptor function that occurs in the seconds to minutes following activation with an agonist, largely focus on the attenuation of desensitization of desensitization-susceptible MORs found on the somato-dendritic region of neurons in various parts of the nervous system. In these studies, we will focus on characterizing desensitization-resistant MORs located on the axon terminal region of GABAergic neurons that form synapses with hypothalamic proopiomelanocortin (POMC) neurons. Activation of presynaptic MORs, as well as other $G\alpha_{i/o}$-coupled GPCRs located on presynaptic terminals, results in an inhibition of GABA release, which causes a subsequent inhibition of the amplitude or frequency of inhibitory postsynaptic currents (IPSCs). Our findings demonstrate that apparent resistance to desensitization by presynaptic MORs, measured as a sustained inhibition of IPSC amplitude or frequency, cannot be explained by a large receptor reserve, nor can

desensitization become detectable after chronic treatment with the opiate morphine. It was also found that resistance to desensitization is a common, but not universal, property of $G\alpha_{i/o}$-coupled G-protein coupled receptors located on presynaptic terminals. Comparison of desensitization-resistant MORs with desensitization-susceptible $GABA_B$ receptors revealed that both populations of receptors have similar receptor-effector coupling, and that resistance or susceptibility to desensitization is unaffected by experimental conditions that isolate either $Ca^{2+}$-independent spontaneous release or $Ca^{2+}$-dependent synchronous release. These findings provide evidence that resistance or susceptibility to desensitization is not dependent on particular receptor-effector coupling, and is likely receptor delimited.

The previous findings suggest that resistance to desensitization by the MOR may be conferred by altered physical properties of presynaptic receptors relative to their postsynaptic counterparts. A likely way that these physical differences could manifest would be through differential mobility of pre- and postsynaptic receptors. To provide proof of principle that such measurements can be made, single-particle tracking of MORs containing an N-terminal FLAG tag was performed the AtT20 cell line. MOR diffusion was measured before and after activation with a maximal, desensitizing concentration of the full MOR agonist DAMGO. In the absence of DAMGO, FLAG-MORs could be found in either a mobile or immobile state. After ten minutes in the presence of DAMGO the fraction of immobile FLAG-MORs was increased, but both mobile and immobile receptors were still present. Because ten minutes in a maximal concentration of DAMGO is sufficient to cause MOR desensitization to reach a maximum and for the internalization of most desensitized receptors to occur, the findings demonstrate that steady-state signaling of the MOR may be maintained by both mobile and immobile receptors. These findings provide a basis for future studies comparing the mobility of pre- and postsynaptic MORs in neurons, as well as determining the role of mobile and immobile MORs in signaling pathways recruited by the receptor.

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## Chapter 1: Introduction

G-protein coupled receptors (GPCRs) comprise a large family of receptors that play a critical role in a diverse range of physiological functions. These functions include, but are not limited to, modulation of the cardiovascular system, digestive system, immune system, and nervous system (Heng et al., 2013). GPCRs mediate cellular responses to a wide variety of endogenous stimuli, such as neurotransmitters and hormones, as well as exogenous stimuli, such as odorants and light. This diverse set of functions has led to GPCRs becoming a popular target for pharmaceutical interventions. Perhaps popular is an understatement since, collectively, GPCRs are targeted by approximately 40% of pharmaceuticals on the market (Overington et al., 2006; Santos et al., 2017).

One sub-family of GPCR that has been of particular interest as a drug target is the opioid receptor family. Activation of opioid receptors, and the mu opioid receptor (MOR) in particular, results in robust analgesia. This has led to opioid agonists being widely prescribed to treat both acute and chronic pain. However, repeated administration of opioid agonists results in the development of tolerance to their analgesic effects, resulting in a diminishing effectiveness of these drugs during continued usage. In addition to analgesia, use of MOR agonists is known to result in a sense of euphoria in many individuals. The analgesic and euphoric effects of these drugs produce a high potential for abuse, which can result in repeated bouts of opioid use and subsequent development of tolerance. Tolerance to the analgesic and euphoric effects of opioids results in increasing dosages, and eventually overdose. This is a rising problem in the United States, and coincides with an increase in prescriptions of opioid based medications (Chakravarthy et al., 2012).

This combination of therapeutic utility and abuse liability have led to MOR agonists being heavily studied at the clinical, behavioral, anatomic, and cellular levels. Decades of work have pointed to the development of tolerance as being a process that occurs at the cellular level, due

to signaling cascades that are activated during prolonged continuous or repeated MOR activation (Williams et al., 2013). It is also thought that desensitization of the MOR is an early step in the development of tolerance to opioid agonists (Williams et al., 2001; Dang and Christie, 2012; Williams et al., 2013). More recent work has shown that the response of these receptors to prolonged agonist treatment may vary not only on the cellular level, but within subcellular compartments (Fyfe et al., 2010). The studies presented here will address potential mechanisms underlying compartment selective resistance to desensitization by the MOR.

### 1.1 Discovery of G-protein Coupled Receptors

For over a century physiologists and pharmacologists have understood that certain chemical compounds, which we now know are selective agonists of cellular receptors, exerted their effects through mechanisms that occur on the cellular level (Langley, 1905). However, the existence of receptors that were specifically targeted by these compounds was doubted, and many viewed the concept of receptors simply as a heuristic tool (Dale, 1943; Ahlquist, 1973). This began to change when in the late 1960s groups were able to apply the concepts used in insulin-detecting radioimmunoassays (Yalow and Berson, 1960) to peptide hormones and their putative cellular receptors (Roth, 1973). Radioligand binding was first used to demonstrate selective binding of adrenocorticotropic hormone (ACTH) to its receptor (Lefkowitz et al., 1970). This study was the first of many that would demonstrate that the concept of receptors was more than just a useful heuristic model, and marked the beginning of the modern field of receptor physiology and pharmacology (Lefkowitz, 2013).

Radioligand binding studies were subsequently used to identify a host of receptors (Roth, 1973; Lefkowitz, 2013), including the putative opiate receptors (Pert and Snyder, 1973). In the decade following the identification of receptors via radioligand binding, work began to purify and clone these receptors. Early purification techniques required high concentration of protein to be successful, and thus only receptors with fortuitously high concentrations found in nature, such as rhodopsin (Irreverre et al., 1969) or the nicotinic acetylcholine receptor (Olsen et

al., 1972), were purified. Purification and reconstitution of a low density hormone receptor was first achieved with the β-adrenergic receptor (Caron et al., 1979; Cerione et al., 1983; Cerione et al., 1984). The receptor was subsequently cloned, revealing that it possessed the seven transmembrane regions and an unexpected homology to the previously characterized light-sensitive protein rhodopsin (Dixon et al., 1986). The seven transmembrane structure of these proteins would soon be known as a defining feature of G-protein coupled receptors (Dohlman et al., 1987).

**1.2 Opioid Receptor Structure and Function**

Like the adrenergic receptors, the opioid receptors had a variety of known agonists and antagonists with varying properties that were heavily studied due to their clinical significance (Portoghese, 1966; Dole, 1970; Creese and Snyder, 1975). Also like the adrenergic receptors, the opioid receptors are Class A (rhodopsin-like) GPCRs. The homology between the adrenergic and opioid receptors allowed early work on the structure and function of adrenergic receptors to lay the groundwork for similar studies on the opioid receptors. After the cloning of the opioid receptors (Evans et al., 1992; Kieffer et al., 1992; Meng et al., 1993; Thompson et al., 1993), the extracellular loops and core of the opioid receptors were determined to be critical for ligand binding, and conferring ligand selectivity. This was done via a combination of site-directed mutagenesis (Surratt et al., 1994; Bot et al., 1998; Xu et al., 1999; Chavkin et al., 2001) and through the construction of chimeric opioid receptors (Meng et al., 1995; Onogi et al., 1995; Xue et al., 1995; Chavkin et al., 2001). This mirrored the approach used to study the α and β adrenergic receptors (Lefkowitz, 2013). Mutagenesis studies also identified the intracellular loops of the opioid receptors as being critical for G-protein interactions, and the C-terminus, along with the intracellular loops, as being important sites of phosphorylation (Chavkin et al., 2001). Mutations in these sites also demonstrated that phosphorylation of a given site is dependent on the particular agonist used to activate the receptor, and that different sites can

modulate receptor desensitization and internalization in an agonist dependent manner (Law et al., 2000a; Chavkin et al., 2001; Williams et al., 2013).

Although early pharmacological experiments hinted that a plethora of opioid receptor subtypes may exist (Goldstein and James, 1984), three opioid receptors (μ [MOR] (Thompson et al., 1993), δ [DOR] (Evans et al., 1992; Kieffer et al., 1992), and κ [KOR] (Meng et al., 1993)) have been identified and cloned. A fourth opioid-like receptor, the nociceptin receptor (NOP), that is closely related to the opioid receptors evolutionarily but demonstrates very low affinity to opioid receptor agonists and antagonists, has also been identified (Fukuda et al., 1994). The amino acid sequences of these proteins show a high degree of homology, with even the most divergent of the sequences still bearing 67% homology (Knapp et al., 1995). This homology in the sequences of the opioid receptor family results in a large degree of overlap in their ability to bind agonists and antagonists, with the exception of the NOP. For example, the endogenous opioid peptide β-endorphin is a potent agonist of the MOR, and also of the MOR's closest relative the DOR. The MOR can also be activated by the endogenous peptide dynoprhin A, which is the primary endogenous ligand of the more distantly related KOR (Williams et al., 2001). In fact, opioid receptors are defined in part by their ability to bind the nonselective opioid receptor antagonist naloxone, providing evidence that the close sequence homology of the opioid receptors results in close structural homology between the receptors (Knapp et al., 1995). Solving the crystal structures of the opioid receptors in recent years has confirmed that strong structural homology exists within this family of receptors (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012).

Opioid receptors exert their cellular functions, at least in part, through activation of the canonical $G\alpha_{i/o}$ G-protein pathway (Fig. 1.1) (Burns et al., 1983; Kurose et al., 1983; Connor and Christie, 1999). In short, the receptor recruits $G\alpha_{i/o}$-GDP, along with an associated βγ subunit, after binding an agonist. GDP is then exchanged for GTP, which allows the Gα and Gβγ subunits exert their effects on the cell. For the $G\alpha_{i/o}$ pathway these effects could include

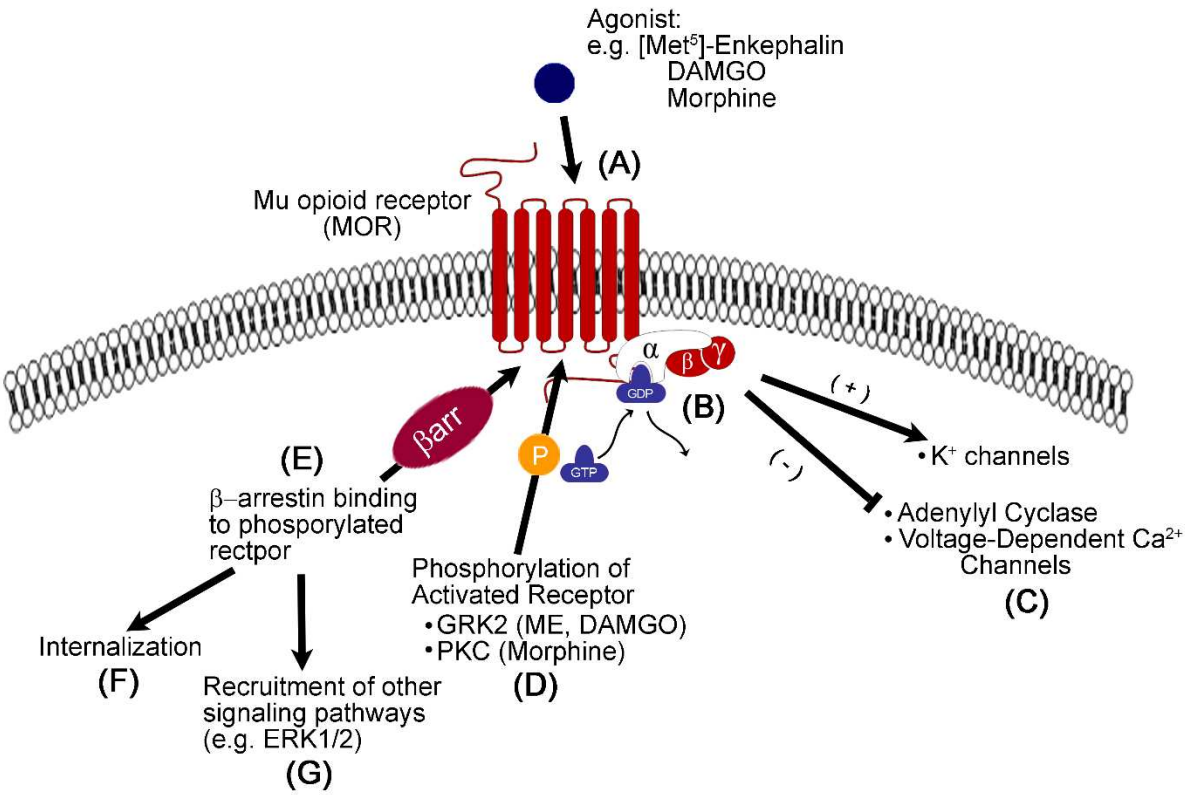


**Figure 1.2 Mu opioid receptor signaling. A)** Binding of an agonist to the MOR causes a conformational change **(B)** that allows the receptor to recruit G-proteins. **C)** The activated G-protein subunits then acutely modulate neuronal excitability by activating $K^+$ channels and inhibiting voltage-dependent $Ca^{2+}$ channels and adenylyl cyclase. Activation of the receptor also results in **(D)** phosphorylation by receptor kinases and **(E)** subsequent β-arrestin binding. **F)** β-arrestin binding allows the receptor to be internalized, but is also important in **(G)** the recruitment of G-protein-independent signaling pathways by the MOR. The recruitment of the these signaling pathways by the β-arrestin bound receptor are likely important in long-term changes in cellular regulation induced by the MOR via transcriptional regulation.

inhibition of adenylyl cyclase (Collier and Roy, 1974), inhibition of $Ca^{2+}$ channels (Gross and Macdonald, 1987), activation of $K^+$ channels (North et al., 1987), or direct inhibition of vesicular release (Capogna et al., 1993). In general, the overall effect of activation of $G\alpha_{i/o}$-coupled receptors in neurons is to inhibit the transmission of information through a neuron, whether that occurs through hyperpolarization and inhibition of firing at the somato-dendritic region of a neuron or through the inhibition of neurotransmitter release at an axon terminal. However, this generalization may be simplistic in certain cases. For example, the inhibition of glutamate

release from presynaptic terminals can alter the firing patterns of downstream neurons without causing an overall inhibition of their activity (Gerachshenko et al., 2009).

Opioid receptors also affect cellular function in more prolonged ways than the relatively acute effects produced by activation of the $G\alpha_{i/o}$-coupled pathway (Fig. 1.1). Activation of opioid receptors can result in phosphorylation of the receptor by multiple kinases which, as mentioned above, are differentially recruited by different agonists of the receptor (Williams et al., 2013). For example, G-protein-coupled receptor kinases (GRKs) are known to phosphorylate the intracellular loops and C-terminals of opioid receptors after receptor activation with a full agonist such as [$Met^5$]-Enkephalin (ME). This in turn recruits β-arrestin (βarr) binding, which promotes internalization, as well as recruitment of extracellular signal-regulated kinases (ERKs). ERKs can then act on various downstream targets including regulators of G-protein singaling (RGS proteins) and transcription factors (Williams et al., 2013). Thus, activation of opioid receptors can have long lasting effects on cellular function outside of inhibiting neuronal firing or neurotransmitter release.

**1.3 Tolerance, Dependence, and Addiction to Opioids**

Tolerance to opioids refers to the reduced response to opioid agonists exhibited after prolonged (hours, days, or weeks) exposure to opioid agonists. Tolerance manifests as a reduced sensitivity to submaximal concentrations of opioid agonists, and sometimes as a reduction in the maximal response to opioids (Dang and Christie, 2012; Williams et al., 2013). The decreased sensitivity to opioid agonists after the onset of tolerance means that larger doses of agonist are required to achieve the desired effect of the drug, whether that effect is analgesic, which is the intended effect of prescribed opioids, or euphoric, anxiolytic or anti-depressive. Prolonged use of opioids results not only in tolerance to opioids, but also in the development of dependence. Dependence is the result of a combination of cellular and circuit level adaptations

brought about by continuous activation of the opioid receptors (Williams et al., 2001; Dang and Christie, 2012; Williams et al., 2013), as well as circuit level adaptations that result from the euphoric, anxiolytic, and anti-depressive effects of the drugs (Evans and Cahill, 2016).

A notable property of opioid tolerance is that different effects of opioid drugs develop tolerance over different time courses. Of particular concern are opioid-induced respiratory depression and constipation, which develop tolerance more slowly than the analgesic effects of the drugs (Ling et al., 1989). The differential development of tolerance between the desired effects of opioids and their negative side effects can therefore lead to overdose during continuous use (White and Irvine, 1999). However, cessation of opioid use results in withdrawal, which causes a host of adverse effects such as sweating, diarrhea, shaking, hyperalgesia, and dysphoria (Benyamin et al., 2008; Evans and Cahill, 2016). This creates a dilemma for users of opioids where cessation of use will result in significant physical and psychological distress, but continued use may result in overdose. This complicated relationship between the desirable and undesirable effects of opioids, and the changes in brain circuitry that these effects induce, leads to the cycles of use seen in many addicts (Evans and Cahill, 2016).

Opioid receptors exert their effects on many different regions of the nervous system, hence the wide range of side effects produced by opioid treatment (Laschka et al., 1974). Circuit level adaptations in these various regions certainly play a role in the development and behavioral manifestations of opioid tolerance, dependence, and addiction (Christie, 2008; Evans and Cahill, 2016). However, all of these broad behavioral effects begin with activation of the opioid receptors, and in particular the MOR. Thus, the circuit level adaptations that result from continued opioid use are thought to result from cellular adaptations, which in turn originate from signaling events induced by MOR activation. An early step in the development of these cellular adaptations is desensitization of the MOR itself (Williams et al., 2001; Dang and Christie, 2012; Williams et al., 2013).

### 1.4 Overview of MOR desensitization

Prolonged or repeated activation of the MOR with a sufficiently high concentration of agonist results in desensitization of the receptor (Law et al., 1983; Connor et al., 2004; Williams et al., 2013). Acute desensitization refers to the uncoupling of a receptor from its effectors that occurs in the initial minutes of a continuous application of a sufficiently high concentration of agonist (Fig. 1.2) (Harris and Williams, 1991; Connor et al., 2004; Williams et al., 2013). Desensitization of MORs can also be measured as an attenuated response to submaximal concentrations of agonist (i.e. a right-shifted dose response) that generally reverses on a timescale of tens of minutes (Fig. 1.2) (Alvarez et al., 2002; Borgland et al., 2003; Connor et al., 2004). This loss of sensitivity to agonists is distinct from the loss of sensitivity observed during tolerance, as it occurs in the presence of tolerance, and reverses much more quickly than tolerance (Levitt and Williams, 2012; Williams et al., 2013). It also serves as a useful measure of desensitization in systems where acute desensitization is not readily observable due to complicating factors such as receptor reserve (Connor et al., 2004).

Desensitization of the MOR has also been reported to be homologous, meaning that desensitization of the MOR does not result in desensitization of other GPCRs found in the same cell (Fig. 1.2a) (Harris and Williams, 1991; Fiorillo and Williams, 1996; Bailey et al., 2004; Dang and Williams, 2005; Williams et al., 2013). In systems where heterologous desensitization occurs, recruitment of kinases and other downstream effectors are able to attenuate the activity of other receptors located on the same cell as the receptor being activated (Fig. 1.2b) (Kelly et al., 2008). Interestingly, the homologous regulation of MORs has also been reported after chronic treatment with opioids (Law et al., 1983; Kennedy and Henderson, 1991), perhaps hinting at a link between the cellular processes underlying desensitization and tolerance (Christie, 2008).

MOR desensitization occurs on a timescale that is generally slower than phosphorylation of the receptor (Doll et al., 2011), similar to βarr binding (McPherson et al., 2010; Molinari et al.,

2010), and faster than internalization (Borgland et al., 2003; Arttamangkul et al., 2006; Arttamangkul et al., 2008). These findings are consistent with an early hypothesis that MOR desensitization occurrs when phosphorylated receptor is decoupled from its effectors by β-arrestin binding and is subsequently internalized, processed in endosomes, and returned to the plasma membrane (Law and Loh, 1999). This hypothesis was consistent with the general model of GPCR desensitization developed studying the β-adrenergic receptor (Krupnick and Benovic, 1998; Luttrell and Lefkowitz, 2002).

Early experiments performed in heterologous expression systems supported the hypothesis that desensitization of the MOR was due to phosphorylation and subsequent βarr binding. Expression of both GRK2 and βarr2 was required to observe desensitization of MORs expressed in *Xenopus* oocytes by the full MOR agonist [D-Ala$^2$,N-Me-Phe$^4$,Gly$^5$-ol]-Enkephalin (DAMGO) (Kovoor et al., 1997). Similarly, expression of a dominate negative GRK2 mutant in HEK293 cells resulted in attenuation of DAMGO-induced acute desensitization (Johnson et al., 2006), and expression of a phosphorylation-deficient MOR, or knockout of βarr1/2, in mouse embryonic fibroblast (MEF) cells eliminated DAMGO-induced desensitization (Chu et al., 2008).

However, other studies, including those performed in neurons instead of heterologous expression systems, demonstrated a more complicated relationship between MOR phosphorylation, βarr binding, and desensitization (Law and Loh, 1999; Dang and Christie, 2012; Williams et al., 2013). For example, several studies performed in locus coeruleus (LC) neurons described the role of GRK2 in MOR desensitization as either being very important (Bailey et al., 2009b; Lowe et al., 2015), playing a complementary role to ERK1/2 (Dang et al., 2009), or unnecessary (Arttamangkul et al., 2012). Also, ME- and DAMGO-induced acute desensitization is observed in βarr2 knockout mice (Walwyn et al., 2007; Arttamangkul et al., 2008; Dang et al., 2009), suggesting that βarr binding to the receptor may not be required to terminate signaling. A more recent study produced more evidence that βarr recruitment is not required for ME-induced desensitization of the MOR, while also finding that phosphorylation is

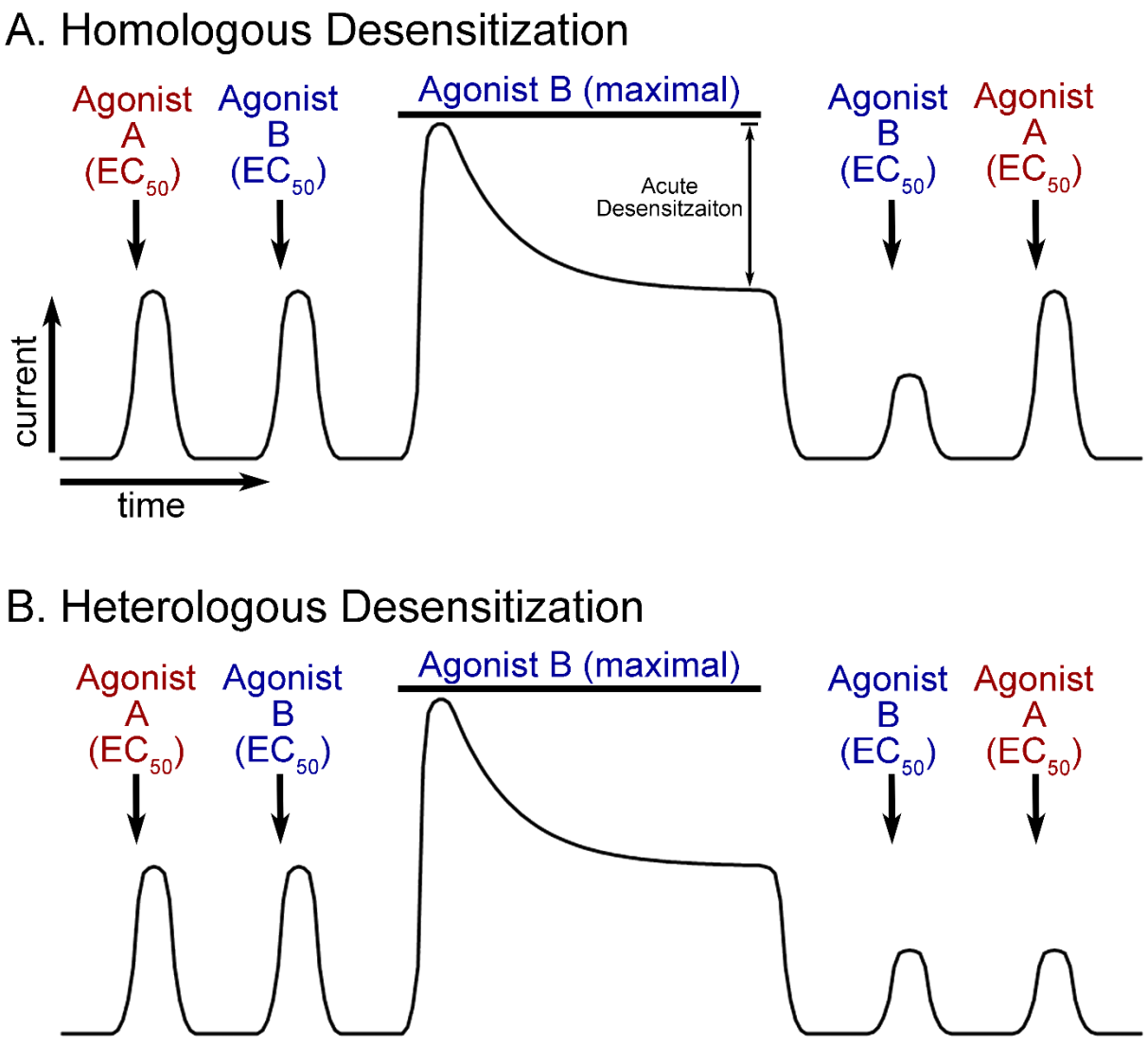


**Fig. 1.2 Acute, homologous and heterologous desensitization of GPCRs.** This figures shows mock voltage-clamp data where activation of two different GPCRs activates an outward current (upward deflections in the plots). **A)** A half-maximal concentration of an agonist ($EC_{50}$) for receptor A and receptor B was applied before a maximal concentration of the agonist for receptor B. The maximal concentration of the agonist for receptor B results in an acute reduction in the current mediated by receptor B (acute desensitization). In this example, the current decays to half of its original amplitude before reaching a steady state. Receptor B, but not receptor A, exhibits a reduced response to the previous half-maximal concentration of its agonist when reapplied after a maximal, desensitizing concentration of agonist B. Because the response of receptor A to its agonist was not affected by desensitization of receptor B we can say that desensitization of receptor B is homologous. **B)** In the second mock experiment, the desensitization of receptor B with a maximal concentration of agonist B results in a reduced response by both receptors A and B to a submaximal concentration of agonist, making desensitization of receptor B in this example heterologous.

critically important for desensitization to occur (Yousuf et al., 2015). In contrast with early hypotheses on MOR desensitization, these studies provide compelling evidence that MOR desensitization does not result from phosphorylation and subsequent βarr binding. While phosphorylation of the receptor appears to be critical, the exact kinases and phosphorylation patterns required to induce desensitization remain somewhat ambiguous. While GRK and βarr clearly play some role in MOR desensitization, it is likely that their recruitment and importance varies between cell types, and that other factors besides GRKs and βarr are recruited by the activated MOR and contribute to desensitization (Williams et al., 2013).

**1.5 Agonist-dependent Desensitization and Recruitment of Effectors**

The biochemical pathway by which MORs desensitize is also likely to depend on the agonist used to activate the receptor. The same study that demonstrated the GRK2-dependence of DAMGO-induced desensitization of the MOR in HEK293 cells also demonstrated that morphine-induced desensitization relied on PKC-mediated phosphorylation (Johnson et al., 2006). In MEF cells, morphine-induced desensitization was not eliminated by expression of phosphorylation deficient mutant MORs, nor by knockout of βarr1 and βarr2 (Chu et al., 2008). A similar result was achieved in AtT20 cells, when a phosphorylation deficient mutant MOR that did not exhibit ME-induced desensitization robustly desensitized in the presence of morphine (Yousuf et al., 2015). PKC activators have been reported to enhance morphine- and ME-induced, but not DAMGO-induced, desensitization of MORs in LC neurons (Bailey et al., 2004; Bailey et al., 2009a; Bailey et al., 2009b). A more recent study has recapitulated the enhancement of ME- and morphine-induced desensitization by the phorbol ester PKC activators phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), but found evidence consistent with phosphorylation independent effects of these drugs being responsible for their effects on desensitization (Arttamangkul et al., 2015).

Whatever the underlying mechanism of phorbol ester enhanced desensitization of the MOR, whether PKC-mediated or otherwise, it is clear that the effect is dependent on the

agonists used as DAMGO-induced desensitization is unaffected by these compounds. The ability of different agonists for a given receptor to preferentially recruit different downstream effectors is referred to as ‘biased agonism’ or ‘functional selectivity’ (Urban et al., 2007). This preferential recruitment of different downstream effectors by different agonist was recognized two decades ago, and has been a consistent topic of inquiry since then (Kelly et al., 2008; Kelly, 2013). The differential recruitment of downstream effectors by different agonists is likely due to inducing different conformational changes in the receptor upon binding (Urban et al., 2007). In fact, multiple agonist-dependent conformation states have been demonstrated for the $\beta_2$-adrenergic receptor (Kahsai et al., 2011).

**1.6 Using Opioid Structure and Function to Create Novel Opioid Agonists**

The realization that agonists induce biased recruitment of downstream effectors by the MOR has allowed researchers to screen for novel agonists with favorable pharmacological profiles, and will provide insight for rational, structure-based approaches to drug design (Raehal et al., 2011; Thompson et al., 2015a; Bruchas and Roth, 2016). These efforts have largely focused in particular on finding agonists that only weakly recruit βarr2 while still strongly coupling to G-proteins due to the relationship between βarr2 and the negative side effects associated with opioid analgesics (Raehal et al., 2005). Recent work has been performed profiling the ability of a plethora of opioid agonists to recruit G-proteins and βarr (McPherson et al., 2010; Molinari et al., 2010; Kelly, 2013; Williams et al., 2013; Thompson et al., 2015b). The agonists studied possessed a wide range of pharmacological profiles, and biased agonism was even observed amongst the endogenous peptide agonists of the MOR (Thompson et al., 2015b). Together these finding support the idea that slight alterations in receptor conformation induced by different agonists of the MOR likely result in differential recruitment of downstream effectors.

With the recent publications of the crystal structures of the DOR, MOR, NOP, and KOR (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012), studies

focusing on the specific conformational changes opioid agonists produce are now possible (Huang et al., 2015; Sounier et al., 2015). This information has led to the discovery of novel ligands for both the KOR and NOP using structure-based virtual screening (Negri et al., 2013; Daga et al., 2014), as well as a promising biased agonist of the MOR that produce analgesia with decreased respiratory depression and constipation when compared to morphine (Manglik et al., 2016).

This work has provided hope of improved, rationally designed opioid therapeutics going forward. However, it is worth noting that the work described above has focused on opioid receptors located on the soma and dendrites of neurons (or heterologous expression systems that are roughly analogous to the somatic region of neurons). Opioid receptors located on the axon terminals also provide important contributions to opioid-mediated analgesia and reward, but their coupling and desensitization has not been a focus of the opioid field up to this point.

**1.7 Importance of Presynaptic MORs**

Mu opioid receptors located on the axon terminal region of neurons are, like their postsynaptic counterparts, $G\alpha_{i/o}$-coupled, and activation of these receptors results in the inhibition of neurotransmitter release. Presynaptic MORs are known to be localized to axon terminals in variety of regions in the brain and spinal cord important in analgesic and rewarding effects of opioids. For example, MORs located on the axon terminals of GABAergic interneurons of the ventral tegmental area (VTA) play an important role in the rewarding effects of opioids by inhibiting GABA release onto VTA dopamine neurons, thus increasing dopamine neuron activity and subsequently increasing dopamine release (Johnson and North, 1992). Presynaptic MORs also inhibit glutamatergic inputs onto neurons in the marginal zone of the dorsal horn, a region important in pain and temperature sensation, thus contributing to the antinociceptive properties of opioids (Hori et al., 1992). Although the analgesic and rewarding effects of opioid receptors feature prominently in their study, presynaptic MORs are also found in brain regions important in food intake (Emmerson and Miller, 1999; Hentges et al., 2009; Pennock and Hentges, 2011;

Dicken et al., 2012), arousal (Blanchet and Luscher, 2002; Li and van den Pol, 2008), anxiety (Blaesse et al., 2015), and learning and memory (Cohen et al., 1992).

Studies performed *in vivo* demonstrate that knockout, knockdown, or pharmacological blockade of G-protein inwardly rectifying $K^+$ channels (GIRKs), which are thought to be one of the primary means of MOR-mediated inhibition of the somato-dendritic region of neurons, does not completely prevent analgesia (Mitrovic et al., 2003; Marker et al., 2004; Nakamura et al., 2014). In fact, in one of these studies morphine-induced analgesia was unchanged at lower doses (Marker et al., 2004), and in another morphine-induced analgesia was unaffected by GIRK inhibition (although analgesia produced by oxycodone was robustly inhibited) (Nakamura et al., 2014). Although alternative explanations may exist, these studies hint at the importance of presynaptic MORs in the ascending pain pathway.

In addition to their known importance in pain pathways, the persistent activation of presynaptic MORs during chronic use of opioids is likely to play a role in withdrawal when opioid use ceases (Williams et al., 2001). Multiple studies have demonstrated increased release probability of both GABA (Bonci and Williams, 1997; Chieng and Williams, 1998; Ingram et al., 1998) and glutamate (Bie et al., 2005) from MOR containing terminals during withdrawal. This compensatory increase in release probability is due to upregulation of the adenylyl cyclase/cAMP system (Bonci and Williams, 1997; Chieng and Williams, 1998; Ingram et al., 1998; Bie and Pan, 2005; Bie et al., 2005), and LTP induced by this increase in release after withdrawal may be responsible for withdrawal-induced hyperalgesia (Drdla et al., 2009). Interestingly, presynaptic MORs often exhibit an enhanced ability to inhibit release after chronic treatment with opioids (Chieng and Williams, 1998; Ingram et al., 1998; Hack et al., 2003). This is in contrast to the enhanced desensitization (Dang and Williams, 2004, 2005) and sometimes smaller maximal effect (Christie et al., 1987; Bagley et al., 2005) exhibited by postsynaptic MORs after a similar treatment.

The presynaptic actions of MOR agonists have been long recognized (Cairnie et al., 1961; Henderson et al., 1972b; Henderson et al., 1972a), even before the actual discovery of the opioid receptors. Despite this, the study of presynaptic MORs has lagged behind that of postsynaptic MORs. When compared to their postsynaptic counterparts, presynaptic MORs are relatively difficult to study due to their localization within the cell. With a few notable exceptions, such as the Calyx of Held (Borst and Soria van Hoeve, 2012) and the giant terminals of bipolar neurons in the goldfish retina (Matthews, 1999), presynaptic terminals cannot be accessed with a patch-clamp electrode. Because of this, the effect of presynaptic receptors on the membrane properties of presynaptic terminals cannot be measured directly. Nevertheless, advances in electrophysiological, pharmacological, imaging, and genetic tools have allowed significant advances in the understanding of the mechanisms underlying the actions of presynaptic MORs, and GPCRs in general.

**1.8 Mechanisms of Presynaptic Inhibition by MORs**

Presynaptic MORs can inhibit release through multiple mechanisms that can vary with between cell types and the type of release studied. Perhaps the most broadly recognized mechanism by which presynaptic GPCRs inhibit neurotransmitter release is via the inhibition of voltage-dependent $Ca^{2+}$ channels (VDCCs) that produce the $Ca^{2+}$ influx needed for synchronous release to occur. However, presynaptic GPCRs are also known to inhibit release through the activation of presynaptic $K^+$ channels, and through direct interactions with the vesicular release machinery (Miller, 1998). Presynaptic MORs can inhibit release through all of three of the above-mentioned mechanisms (Fig. 1.3), but the downstream effectors of presynaptic MORs vary by synapse.

Inhibition of glutamate release by the MOR via the inhibition of $Ca^{2+}$ influx has been reported in synapses in the dorsal horn of the spinal cord (Hori et al., 1992), while inhibition of glutamate release in the central nucleus of the amygdala (CeA) occurs via phospholipase-$A_2$ ($PLA_2$) mediated activation of 4-aminopyridine (4-AP) sensitive $K^+$ channels (Zhu and Pan,

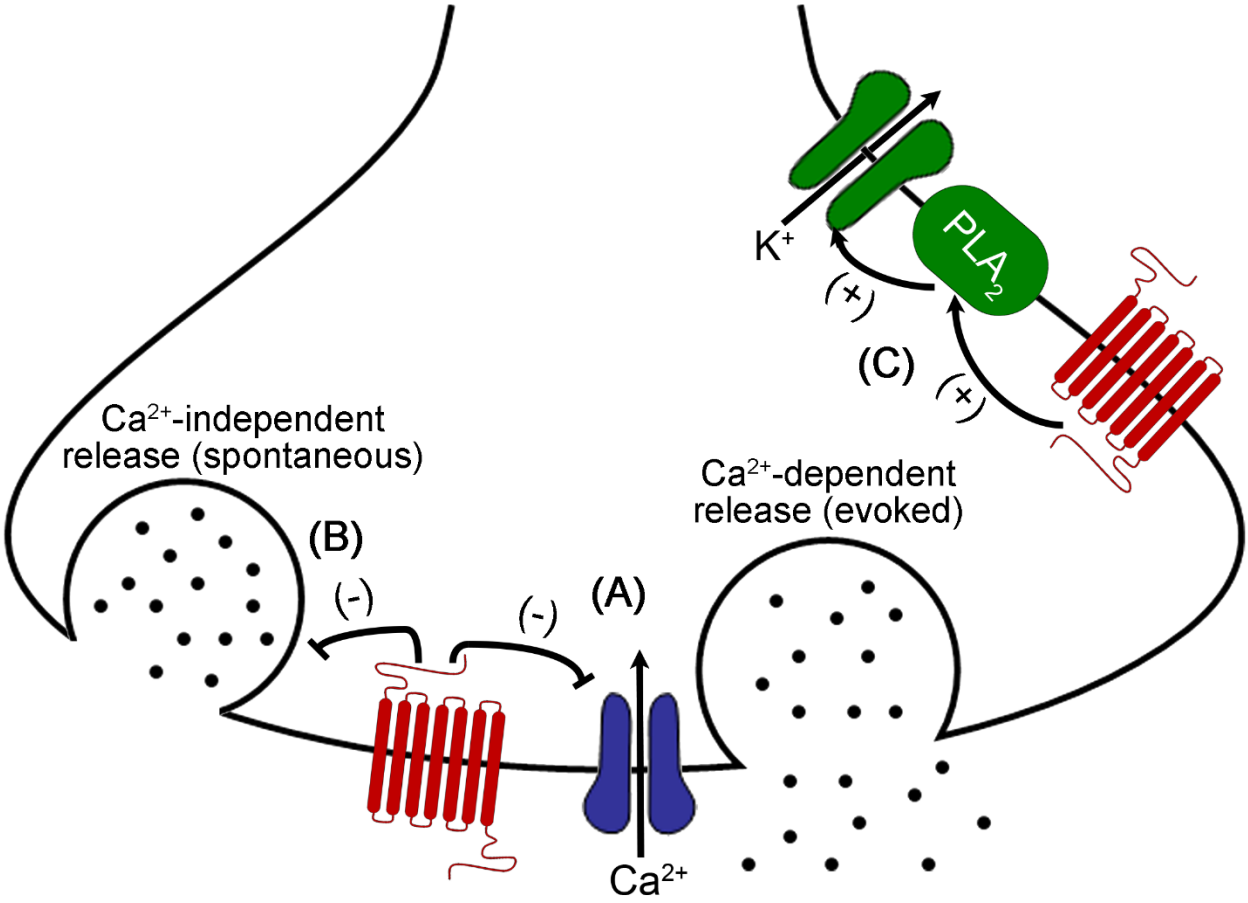


**Fig. 1.3 Coupling of presynaptic MORs.** Presynaptic MORs have been shown to inhibit neurotransmitter release from presynaptic terminals through multiple mechanisms. **A)** MORs can inhibit the $Ca^{2+}$ influx needed for neurotransmitter release via the inhibition of voltage-dependent $Ca^{2+}$ channels. **B)** They are also known to inhibit release directly through direct action at the release machinery. **C)** Presynaptic MORs can also increase phospholipase $A_2$ activity, which subsequently activates 4-AP sensitive $K^+$ channels. Differential inhibition of evoked and spontaneous release has been demonstrated for other $G\alpha_{i/o}$-coupled GPCRs, and could potentially also occur with MORs.

2005). A similar mechanism of inhibition to that found in the CeA is responsible for MOR-mediated inhibition of GABA release in the periaqueductal grey (PAG) (Vaughan et al., 1997) and VTA (Bergevin et al., 2002). Multiple studies have shown that inhibition of GABA release in the hippocampus by presynaptic MORs occurs via direct actions at the release machinery (Capogna et al., 1993; Rekling, 1993; Lupica, 1995; Capogna et al., 1996).

All of the above-mentioned studies were performed by measuring spontaneous or evoked postsynaptic currents or potentials in the presence of various pharmacological blockers

of potential MOR effectors. For example, Vaughan et al. demonstrated that MOR-mediated inhibition of GABA release in the PAG was dependent on the $PLA_2$ pathway by determining that ME-induced reductions in evoked inhibitory postsynaptic currents (eIPSCs) were absent in the presence of multiple blockers of the $PLA_2$ pathway (Vaughan et al., 1997). Copogna et al. demonstrated that MOR-mediated inhibition of IPSCs was maintained in the presence of $Ca^{2+}$ ionophores and blockers of $Ca^{2+}$ and $K^+$ channels (Capogna et al., 1996). They provide compelling, yet indirect, evidence for the mechanism of presynaptic inhibition of release by MORs.

The output measured in these studies (activation of ligand-gated ion channels on the postsynaptic membrane) is at least two steps removed from the activation of the effector used to inhibit release (e.g. G-proteins activated by the receptor acting on the vesicular release machinery). Although little work has been done on the MOR attempting to bypass this shortcoming, work performed on other presynaptic $G\alpha_{i/o}$-coupled GPCRs provide insight into precise mechanisms mediating inhibition of neurotransmitter release. Studies involving other $G\alpha_{i/o}$-coupled GPCRs have taken advantage of advancements in imaging and genetic tools to determine how GPCRs in axon terminals are organized with respect to their downstream effectors, and what interactions between the effectors, G-proteins and receptors results in inhibition of release.

The ability of the Gβγ subunit to modulate VDCCs has been understood for decades (De Waard et al., 2005), but a recent study used fluorescence resonance energy transfer (FRET) microscopy to describe the spatial relationship between $GABA_B$ receptors ($GABA_BRs$), $G\alpha_o\beta\gamma$, and the presynaptic VDCC $Ca_v2.2$. Using FRET pairing between fluorescently labeled proteins, this group was able to determine that presynaptic $GABA_BRs$ located in hippocampal axon terminals are found in small (<10 nm), preformed complexes with G-proteins and VDCCs. These complexes must be present for inhibition of the $Ca_v2.2$ to occur, and undergo rearrangement after activating the $GABA_BR$ (Laviv et al., 2011).

A string of studies over the past fifteen years has been performed to determine how GPCR-mediated inhibition of the vesicular release machinery occurs. These studies were largely performed in the lamprey spinal cord, where 5-HT receptor (5HTR)-mediated inhibition of excitatory transmission occurs through a mechanism downstream of $Ca^{2+}$ influx (Takahashi et al., 2001). Due to the large size of the axons in the lamprey spinal cord, measurements of $Ca^{2+}$ influx into terminal regions and direct manipulation of the presynaptic environment were possible. This allowed for the confirmation that 5HTR-mediated inhibition was in fact occurring at the release machinery (Blackmer et al., 2001). This effect was later attributed to interactions of the βγ subunit with the release machinery (Photowala et al., 2006), specifically the C-terminus of SNAP-25 (Gerachshenko et al., 2005). The binding of βγ to SNAP-25 was later found to exert its effects by interfering with interactions between SNAP-25 and $Ca^{2+}$ sensor synaptotagmin (Yoon et al., 2007; Wells et al., 2012), and that relatively small alterations in the structure of the SNARE complex interfered with 5HT-induced inhibition of release (Hamid et al., 2014).

Although care must be taken to not over interpret these results with regards to MOR-mediated inhibition of release, studies such the ones mentioned above provide interesting insights into how MOR-mediated inhibition is occurring. In particular, the fact that MORs in a given synapse tend to inhibit release through a particular mechanism, but usually not multiple mechanisms, would suggest that the receptors might be spatially confined in a way that prevents their interaction with other effectors. The findings of Laviv et al. may support this hypothesis.

In some cases, GPCRs may inhibit spontaneous or evoked release, but not both (Glitsch, 2006; Pennock and Hentges, 2011; Hamid et al., 2014). In addition to the evidence that GPCRs located on presynaptic terminals may be spatially confined, there is evidence in GABAergic (Mathew et al., 2008; Chung et al., 2010), glutamatergic (Sara et al., 2005; Atasoy et al., 2008; Sara et al., 2011), and *Drosophila* neuromuscular junction (Koenig and Ikeda, 1999;

Melom et al., 2013) terminals that vesicles released during spontaneous and evoked release are drawn from separate pools of vesicles. Together with findings on the specificity of GPCR-effector coupling in synaptic terminals, the ability of some receptors to act exclusively on confined pools of vesicles that are spatially segregated provides compelling evidence that the sub compartmental organization of presynaptic GPCRs is critical to their function.

**1.9 Desensitization of Presynaptic MORs**

Much like the coupling of presynaptic MORs, the desensitization of presynaptic MORs has been relatively lightly studied due to the complications that studying receptors located on presynaptic terminals presents. Because terminals generally cannot be accessed with a patch pipette, direct electrophysiological studies of MORs located on terminals are not possible. This also prevents pharmacological manipulations of the presynaptic terminal via the pipette solution. However, the unique physiological properties of these receptors will make them compelling targets for future studies. In particular, presynaptic MORs have been shown to resist acute desensitization (Blanchet and Luscher, 2002; Fyfe et al., 2010; Pennock and Hentges, 2011; Lowe and Bailey, 2015).

Resistance to acute desensitization has been reported for presynaptic MORs located in the LC (Blanchet and Luscher, 2002), PAG (Fyfe et al., 2010), arcuate nucleus (Pennock and Hentges, 2011), and VTA (Lowe and Bailey, 2015). Resistance to desensitization appears to be a general, and possibly ubiquitous, property of MORs located on presynaptic terminals. However, it is possible that resistance to desensitization varies between synapses, and may be the result of particular receptor-effector coupling (Blanchet and Luscher, 2002). This will not become clear until the coupling of presynaptic MORs and desensitization of presynaptic MORs in a larger number of brain regions has been characterized.

The mechanisms underlying resistance to desensitization by presynaptic MORs are still a mystery, and has remained scarcely studied to this point. It is not known whether resistance to desensitization is agonist dependent. If it is agonist dependent, the relationship between agonist

efficacy and desensitization is altered versus postsynaptic receptors as full agonists such as DAMGO and ME do not induce desensitization (Blanchet and Luscher, 2002; Fyfe et al., 2010; Pennock and Hentges, 2011; Lowe and Bailey, 2015). A relationship between receptor-effector coupling and resistance to desensitization has been suggested (Blanchet and Luscher, 2002), but has not been addressed in a comparative manner. A negative correlation between receptor density and the rate of desensitization has been observed (Law et al., 2000b), and high receptor reserve can mask desensitization using certain assays (Connor et al., 2004). The receptor reserve of MORs on presynaptic terminals has not been measured, thus high receptor density still remains a possible explanation for the observed resistance to desensitization by presynaptic MORs. All of these possibilities will need to be addressed.

Interestingly, resistance to acute desensitization by presynaptic MORs does not appear to be a characteristic unique of that receptor. Presynaptic $GABA_BRs$ in hippocampal cultures (Wetherington and Lambert, 2002a), the VTA (Cruz et al., 2004), and in the PAG (Liu et al., 2013), as well as $A_1$ adenosine receptors in hippocampal cultures (Wetherington and Lambert, 2002b), have been shown to be resistant to acute desensitization. These findings suggest that resistance to desensitization may be a generalizable property of $G\alpha_{i/o}$-coupled GPCRs located within axon terminals. However, acute desensitization of presynaptic $GABA_BRs$ has been reported (Tosetti et al., 2004), as well as resistance to desensitization by postsynaptic $GABA_BRs$ (Cruz et al., 2004; Liu et al., 2013). To provide insight into what confers resistance to desensitization, comparative studies will need to be undertaken to determine what differences exist between desensitization-resistant and –susceptible presynaptic GPCRs. The overall goal of the studies presented here will be to use a pharmacological and comparative approach to determine what properties of presynaptic MORs underlie their resistance to desensitization.

### 1.10 Aims of the Present Studies

In the following three chapters, studies will be described that were performed in an attempt to understand the mechanisms underlying resistance to acute desensitization by

presynaptic MORs. It is currently unknown how downstream signaling of presynaptic MORs differs from that of postsynaptic MORs. Despite the lack of a causal link between receptor desensitization and systemic tolerance in response to opioids, there is still a great deal of evidence that these processes involve common signaling pathways. If presynaptic MORs do not undergo desensitization, then perhaps they also do not recruit the downstream signaling molecules needed to induce long term cellular adaptations in response to chronic opioids. If this is the case, then presynaptic MORs may be an enticing target for future drug development due to their presence in critical points of the pain pathways. However, before such studies can be pursued we must first determine whether there is reason to believe that presynaptic MORs are uniquely regulated. In the following chapters, the overarching hypothesis that resistance to desensitization by presynaptic MORs is a receptor-delimited property that is the result of unique physiological properties of presynaptic MORs was tested. This was addressed in three ways:

1) Presynaptic MORs were subjected to pharmacological manipulations known to unmask or amplify the desensitization of postsynaptic MORs. The failure of these manipulations to unmask desensitization of presynaptic MORs would imply that pre- and postsynaptic MORs are likely differentially regulated.
2) The role of receptor-effector coupling in resistance to desensitization was addressed through a comparative study between presynaptic MORs and another presynaptic $G\alpha_{i/o}$-coupled GPCR found in the same population of axon terminals that is known to exhibit acute desensitization. If resistance to desensitization occurs independent of particular receptor-effector coupling it would provide additional evidence that resistance to desensitization is receptor-delimited, and also that resistance to desensitization is likely a common, if not ubiquitous, property of presynaptic MORs.
3) The mobility of MORs within the plasma membrane was measured in a cell line expressing an epitope-tagged MOR. These experiments determined how the mobility of

MORs relates to their activation, and in particular described the mobility of the receptors that maintain steady state signaling after desensitization has reached a plateau (see Fig. 1.2 for example of steady state signaling). These experiments provide a measure of agonist-induced changes at the receptor that does not rely on the output of an effector, as well as single molecule resolution that is lacking in electrophysiological and biochemical studies of MORs. The techniques developed in this chapter can later be applied to studies of presynaptic MORs in neurons, and will complement further studies on the downstream signaling of presynaptic MORs.

Together, the studies presented support the hypothesis that resistance to desensitization is a receptor-delimited property, and that presynaptic MORs likely act through signaling mechanisms that are distinct from their postsynaptic counterparts. These findings will lay the groundwork for future studies examining the downstream signaling of presynaptic MORs, and determining how that signaling differs from that of postsynaptic MORs (e.g. recruitment of β-arrestin, GRKs, and ERKs). If exclusive recruitment of presynaptic MORs fails to recruit these signaling pathways, which are critical in the development of tolerance, then perhaps targeting of presynaptic MORs may provide an efficacious means of producing analgesia without many of the side effects that result from continuous activation of postsynaptic MORs during chronic treatment.

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## Chapter 2: Resistance to Desensitization by Presynaptic MORs Is Not Due to High Receptor Reserve or Slow Time Course of Desensitization

### 2.1 Overview

The overarching hypothesis of the studies presented in the following chapters is that resistance to desensitization by presynaptic MORs is a receptor-delimited property that is the product of unique physiological properties of presynaptic MORs. However, it is also possible that presynaptic MORs desensitize in a manner similar to postsynaptic MORs, but this desensitization is masked by high receptor density or desensitization that is relatively slow or small in magnitude. In the experiments described in this chapter, we subjected MORs located on axon terminals presynaptic to proopiomelanocortin (POMC) neurons located in the arcuate nucleus to pharmacological manipulations known to unmask or enhance desensitization of postsynaptic MORs. If these manipulations unmask desensitization of presynaptic receptors it would suggest that presynaptic MORs are regulated in a manner similar to postsynaptic MORs. If resistance to desensitization is maintained it would provide evidence for unique regulation of presynaptic MORs.

Resistance to acute desensitization has been reported for several other $G\alpha_{i/o}$-coupled GPCRs located on presynaptic terminals, but most of the studies on this topic only focus on one type of receptor in the synapse being studied. In this chapter, we also examined multiple other $G\alpha_{i/o}$-coupled GPCRs presynaptic to POMC neurons to determine if they were also resistant to acute desensitization. Characterizing the desensitization of other $G\alpha_{i/o}$-coupled GPCRs located on these terminals will help determine whether resistance or susceptibility to desensitization is a receptor-delimited property, and will open the door for comparative studies examining the role of receptor-effector coupling in desensitization.

Overall, the findings presented in this chapter support the hypothesis that the regulation of presynaptic MORs is distinct from that of postsynaptic MORs. We also find that while

resistance to desensitization is a common property of $G\alpha_{i/o}$-coupled GPCRs presynaptic to POMC neurons, it is not ubiquitous. This will allow for comparative studies between desensitization-resistant and –susceptible receptors, which are described in chapter 3.

The following chapter has been reproduced with the permission of *The Journal of Neuroscience*[1], and was originally published on July 25, 2012 in Volume 32, Issue 30 of *The Journal of Neuroscience* under the title “Multiple Inhibitory G-Protein-Coupled Receptors Resist Acute Desensitization in the Presynaptic But Not Postsynaptic Compartments of Neurons.” I designed and executed the experiments described in this manuscript, except for the experiments measuring light-evoked transmitter release from POMC neurons (Fig. 2.3) which were carried out by Matthew S. Dicken. Shane T. Hentges provided guidance for the design of experiments in this chapter, and aided me in the drafting of the manuscript.

This chapter is accompanied by an additional manuscript located in Appendix II that was originally published on August 22, 2014 in Volume 592, Issue 19 of *The Journal of Physiology* under the title “Direct inhibition of hypothalamic proopiomelanocortin neurons by dynorphin A is mediated by the μ-opioid receptor.” I designed and executed the experiments described in this manuscript. Shane T. Hentges provided guidance for experimental design and aided me in the drafting of this manuscript.

**2.2 Summary**

Acute desensitization is a common property of $G_{i/o}$-coupled receptors. Recent data, however, suggest that, unlike μ-opioid receptors (MORs) located somatodendritically in neurons or expressed in heterologous systems, MORs in the presynaptic compartment of neurons are resistant to acute desensitization. It is not yet clear whether this differential desensitization is a shared property of many $G_{i/o}$-coupled receptors nor whether receptors located presynaptically and postsynaptically in a single cell type display differential desensitization. Here, whole-cell

[1] See Appendix I for persmissions

recordings were made from proopiomelanocortin (POMC) neurons in mouse brain slices. Agonists for μ-opioid, nociceptin, and $GABA_B$ receptors induced postsynaptic currents that desensitized within minutes, whereas inhibition of presynaptic transmitter release mediated by these receptors was maintained throughout agonist exposure. Expression of channelrhodopsin2 in POMC neurons allowed for light-evoked transmitter release from POMC neuron terminals, which was detected by recording postsynaptic currents in downstream neurons. Light-evoked currents were inhibited throughout the application of all agonists tested. Thus, the same receptors that desensitize when expressed in the postsynaptic compartment of POMC neurons resist desensitization when located in the presynaptic compartment. Pharmacologic knockdown of MORs revealed that depletion of receptor reserve does not account for presynaptic resistance to desensitization. In ~25% of recordings with $GABA_B$ agonist application, presynaptic $GABA_B$ receptors desensitized, suggesting that resistance to desensitization is not due to an intrinsic property of the terminals themselves. Together, the results indicate that a variety of presynaptic receptors can continue to function after their postsynaptic counterparts desensitize and suggest that a compartment-specific modification may confer resistance to desensitization.

**2.3 Introduction**

Among inhibitory G-protein-coupled receptors (GPCRs), μ-opioid receptor (MORs) have been the subject of particularly intense study due to the role that these receptors play in analgesia and drug abuse. Consistent with the regulation of many GPCRs, continued activation of MORs can cause a reduction in cellular responses as agonist binding leads to desensitization of the receptor (Reiter and Lefkowitz, 2006). This desensitization of the receptor is thought to be an important early step in the development of tolerance to repeated or continued exposure to MOR agonists (Martini and Whistler, 2007; Christie, 2008). Therefore, much work has focused on determining the mechanisms underlying MOR receptor desensitization since limiting this

desensitization could help preserve analgesic actions of MOR agonists during prolonged treatment.

To date, there is not a clear consensus on the mechanism of MOR desensitization. Like other GPCRs, agonist binding to MORs causes a conformational change in the receptor, activation of intracellular signaling cascades, and receptor phosphorylation with eventual β-arrestin binding and internalization, although internalization is not required for acute desensitization (Arttamangkul et al., 2006). In neurons, acute desensitization has often been studied by examining the ability of MOR agonists to activate postsynaptic receptors coupled to G-protein-coupled inwardly rectifying potassium (GIRK) channels. In many cell types, MOR agonists induce GIRK-mediated outward currents that decline within minutes of agonist exposure, representing desensitization of the receptor (Williams et al., 2001). MORs located on the presynaptic terminals of neurons can inhibit transmitter release through actions at voltage-gated potassium and calcium channels (Williams et al., 2001). Interestingly, recent studies in neurons maintained in intact brain slices indicate that presynaptic MORs can continue to inhibit transmitter release during an agonist exposure that causes desensitization of postsynaptic MORs (Blanchet and Luscher, 2002; Fyfe et al., 2010; Pennock and Hentges, 2011). The resistance to desensitization of presynaptic MORs has not been thoroughly studied.

The present study examines the nature of the presynaptic resistance to desensitization in a defined population of neurons in the arcuate nucleus of the hypothalamus. The proopiomelanocortin (POMC) neurons in this region are regulated both presynaptically and postsynaptically by MORs as well as $G_{i/o}$-coupled κ-opioid, nociceptin, and $GABA_B$ receptors, allowing for a comparative study of several receptor types. Previous work indicates that the postsynaptic MORs on POMC neurons desensitize during acute exposure to the MOR agonist [D-Ala$^2$,*N*-MePhe$^4$,Gly-ol$^5$]-enkephalin (DAMGO), whereas the MORs on terminals presynaptic to these neurons do not (Pennock and Hentges, 2011). The present results show that similar to MORs, κ, nociceptin, and $GABA_B$ receptors all display distinct resistance to desensitization in

the presynaptic but not postsynaptic compartment, suggesting that a common mechanism confers differential desensitization of many GPCRs. Current data also illustrate that presynaptic receptors can resist desensitization to various agonists and that resistance is independent of receptor reserve. The ability of presynaptic receptors to continue to signal once postsynaptic receptors have desensitized implies a functional switch from direct postsynaptic actions to presynaptic actions occurs during agonist exposure.

**2.4 Methods and Materials**

*Animals*

Transgenic mice expressing discosoma red (DsRed) (Hentges et al., 2009) or Cre recombinase (Xu et al., 2005) driven by the POMC promoter were backcrossed onto the C57BL/6 background for >11 generations. Mice were housed at controlled temperatures (22–24℃) with a constant 12 h light/dark cycle. Standard rodent chow and tap water were provided *ad libitum*. Brain slices were prepared from both male and female mice (unless otherwise indicated) between 6 and 14 weeks of age. Standard PCR genotyping was performed to identify transgenic mice. All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee and met the United States Public Health Services guidelines.

*Brain Slice Preparations*

Mice were deeply anesthetized with isoflurane, and brains were rapidly removed and placed into ice-cold artificial CSF (aCSF) solution containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 $NaH_2PO_4$, 21.4 $NaHCO_3$, and 11 glucose. All aCSF solutions were saturated with a 95% $O_2$/5% $CO_2$ mixture. Sagittal slices (240 μm) were prepared using a VT 1200S vibratome (Leica). Brain slices containing the arcuate nucleus were then transferred into warm (37℃) aCSF containing MK -801 (15 μM; Sigma-Aldrich). Slices were allowed to rest for ≥45 min before transfer to the recording chamber.

*Viral Gene Transfer*

To express channelrhodopsin-2 (ChR2) selectively in POMC neurons, a viral vector containing a floxed sequence for ChR2 with an mCherry tag [AAV2/9.EF1.dflox.hChR2(H134R)-mCherry. WPRE.hGH; obtained from the Penn Vector Core (University of Pennsylvania School of Medicine, Philadelphia, PA); 200 nl] was injected unilaterally into the arcuate nucleus of the hypothalamus of POMC-Cre transgenic mice (8 weeks of age). Brain slices were prepared 14–28 d after injection, which was a sufficient length of time to yield high levels of ChR2 expression.

*Electrophysiology*

Brain slices placed in the recording chamber were continuously perfused with warm (37℃; ~1.5 ml/min) aCSF saturated with 95% $O_2$/5% $CO_2$. Recording pipettes had tip resistances between 1.5 and 2.5 MΩ when filled with an internal solution containing the following (in mM): 57.5 K-methyl sulfate, 57.5 KCl, 20 NaCl, 1.5 $MgCl_2$, 5 HEPES ($K^+$ salt), 0.1 EGTA, 2 ATP, 0.5 GTP, 10 phosphocreatine, pH 7.3. POMC neurons were identified in the slice by the presence of DsRed or ChR2-mCherry. After obtaining a seal of >1 GΩ, negative pressure was applied to rupture the cell and enter whole-cell mode. Cells were held at −60 mV, and no series resistance compensation was applied. To electrically evoke transmitter release, a bipolar stimulating electrode was placed in the middorsal arcuate nucleus and pairs (100 ms delay between pulses) of 0.5 ms stimuli (10–800 μA as needed to reliably evoke IPSCs at ~50% maximum amplitude) were applied every 20 s. Miniature IPSCs were collected at 10 kHz and digitally filtered at 1 kHz. Events were collected for 15 s/sweep with a 15 s delay between each sweep and detected using Axograph X software based on rise time kinetics. Events with a rise time of <100 μs were rejected. All mIPSC recordings were made in the presence of tetrodotoxin (300 nM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (10 μM) and KCl and K-methylsulfate in the internal solution were replaced with CsCl and Cs-methanesulfonate.

Light-evoked transmitter release from POMC-ChR2-expressing neurons was accomplished by applying a brief (25 ms) blue light pulse to the slice every 20 s. Recordings

were made in unidentified arcuate neurons within 100 μm of mCherry-expressing cells. The intensity of the light used to evoke transmitter release was between 5 and 11 mW/mm$^2$, and was adjusted for each recording to achieve a consistent evoked PSC. All recordings were made using an Axoclamp 700B or 200B (Molecular Devices) amplifier. AxographX software (Axograph) was used for data collection. Recordings in which the series resistance exceeded 20 MΩ or changed significantly during the course of the experiment were not accepted for analysis. The AMPA receptor antagonist DNQX (10 μM) was constantly perfused onto the slice during the course of the experiment to ensure that all evoked PSCs recorded and analyzed were inhibitory (eIPSCs).

*Chronic Morphine Treatment*

To treat mice chronically with morphine, POMC-DsRed transgenic mice (8- to 10-week-old males) were anesthetized and miniosmotic pumps (Alzet; DURECT Corporation) containing morphine were placed subcutaneously. Morphine-treated mice received 50 mg · kg$^{-1}$ · d$^{-1}$ of morphine for 5–7 d before being killed. Brain slices prepared from morphine-treated mice were prepared and collected in either morphine-free aCSF or aCSF containing morphine (1 μM). Slices collected into morphine-free aCSF were left for 2 h before being transferred to the recording chamber to allow morphine to wash from the slice. Slices collected into aCSF containing morphine were given at least 45 min to rest before being transferred to the recording chamber where they were constantly perfused with aCSF containing morphine. To precipitate withdrawal, the perfusion solution was changed from aCFS with morphine (1 μM) to aCSF containing naloxone (1 μM).

*Drugs*

Stock solutions of DNQX (Sigma-Aldrich), (+)-MK-801 (Sigma-Aldrich), (+)-(5α,7α,8β)-*N*-methyl-*N*[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69593) (Biomol International), and (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP 55845) (Tocris Bioscience) were prepared in DMSO as stock solutions (at 10,000×

final concentration). [Met$^5$]-enkephalin (Sigma-Aldrich), baclofen (Sigma-Aldrich), nociceptin (Sigma-Aldrich), the cyclized peptide D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ (CTAP) (Tocris Bioscience), nor-binaltorphimine (nor-BNI) (Sigma-Aldrich), morphine (Sigma-Aldrich), tetrodotoxin (TTX) (Tocris Bioscience), and β-chlornaltrexamine (β-CNA) (Sigma-Aldrich) were prepared as stock solutions in distilled water. All drugs were diluted in aCSF to achieve the working concentrations. β-CNA was divided into aliquots, and then stored at −80°C. For each experiment performed using β-CNA, an individual aliquot was thawed, diluted, and perfused onto the slice within 5 min to ensure no degradation of the compound before use. Dose–response curves were made for opioid and $GABA_B$ receptor agonists to determine the maximal and $EC_{50}$ concentrations as used in the present studies.

*Estimating Presynaptic MOR Receptor Reserve*

Dose–response curves representing the inhibition of eIPSC amplitude by MORs presynaptic to POMC neurons before and after treatment with an irreversible antagonist (β-CNA) were fitted using GraphPad Prism software. Values for the predicted concentration of ME necessary to reduce eIPSC amplitude by given values between 20 and 41% were obtained from these curves. Concentrations that were predicted to achieve an equivalent inhibition of eIPSC amplitude before ($[ME]_{control}$) and after ($[ME]_{\beta\text{-CNA}}$) β-CNA exposure were plotted against one another as a double reciprocal plot. The plot was fitted according to Furchgott's method (Furchgott, 1966) using the following equation:

$$\frac{1}{[ME]_{Control}} = \frac{1-q}{qK_A} + \frac{1}{q}\frac{1}{[ME]_{\beta-\mathrm{CNA}}} \quad (Eq.1)$$

where $q$ is equal to the fraction of MORs still functional after β-CNA treatment and $K_A$ is the dissociation constant of ME at MORs presynaptic to POMC neurons. Estimated values for $q$ and $K_A$ were obtained using GraphPad Prism.

*Statistics*

Comparisons between two groups were made using Student's *t* tests or paired *t* tests when a repeated measure was used. Datasets containing more than two groups were analyzed using repeated-measures ANOVA with Tukey's multiple-comparison *post hoc* tests or two-way ANOVA as indicated. Single-phase decay curves were fitted to postsynaptic desensitization data, and values for time constants and plateaus were obtained using GraphPad Prism software. Postsynaptic currents induced by a GPCR agonist were only included in analyses if the amplitude was >10 pA. All data are shown as the mean ± SEM, and differences between groups were considered significant if $p < 0.05$.

**2.5 Results**

*Presynaptic, but not postsynaptic, MORs resist acute desensitization*

To examine the desensitization of presynaptic and postsynaptic MORs during exposure to ME, whole-cell voltage-clamp recordings were made from fluorescently labeled POMC neurons. Presynaptic and postsynaptic effects of ME were measured simultaneously. The MOR selective opioid agonist ME (30 µM) induced a postsynaptic outward current (42 ± 14 pA; $n$ = 6; Fig. 2.1*A*) that desensitized significantly within minutes of continued exposure ($p < 0.001$, one-way repeated-measures ANOVA; $\tau$ = 116 s; plateau, 46% of baseline; Fig. 2.1*B*). ME also caused a robust inhibition of the amplitude of eIPSCs (to 24 ± 3.5% of baseline; $n$ = 14; $p <$ 0.0001; Fig. 2.1*C*,*D*). Inhibition of eIPSC amplitude was maintained throughout the superfusion of ME (30 µM; $p$ = 0.27, one-way repeated-measures ANOVA; minutes 2 through 10; Fig. 2.1*C*,*D*). Together, these results indicate that postsynaptic, but not presynaptic, MORs regulating POMC neurons acutely desensitize when exposed to a maximal concentration of ME similar to previous results with the agonist DAMGO (Pennock and Hentges, 2011).

*Presynaptic resistance to desensitization is a property of multiple $G_{i/o}$-coupled receptors*

To determine whether differential presynaptic and postsynaptic desensitization may be a general property of $G_{i/o}$-coupled receptors, or rather a specific property of MORs, the ability of

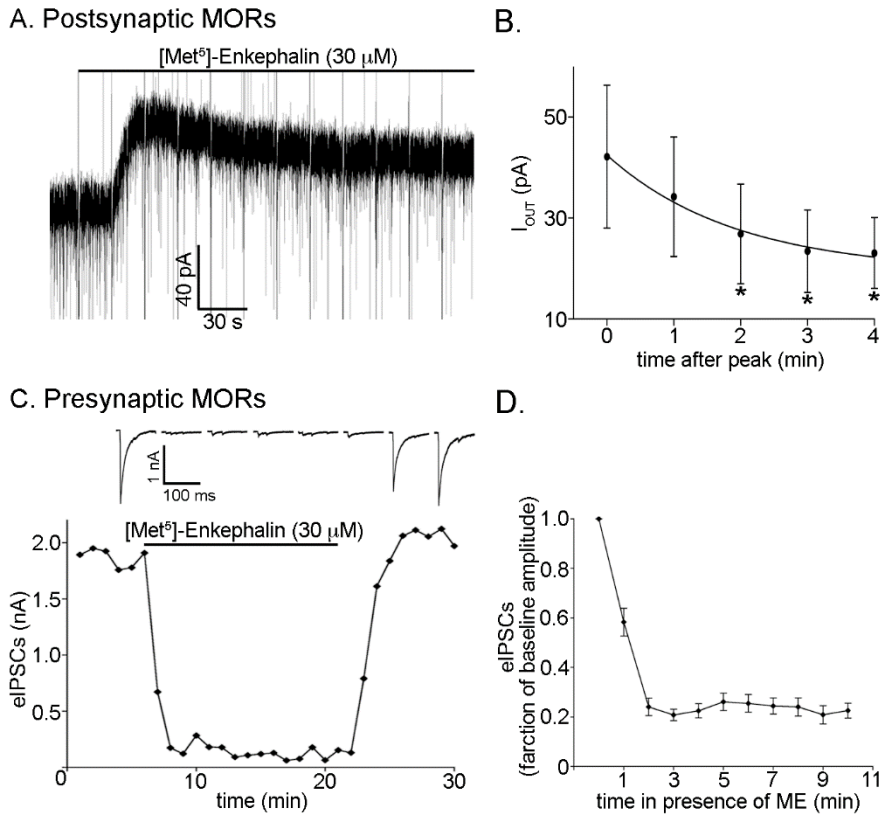


**Figure 2.1 Postsynaptic, but not presynaptic, MORs desensitize acutely. A)** Representative trace of an outward current induced by ME (30 μm), and the reduction in the amplitude of the current that occurs with prolonged ME exposure. Fast upward and downward deflections that occur at 20 s intervals represent stimulus artifacts that result from measuring presynaptic (eIPSC amplitude) and postsynaptic effects of MORs in a single recording. **B)** Compiled data from six recordings in which the amplitude of the outward current was measured at every minute for 4 min after the peak while under constant exposure to ME (30 μm). The curve fitted to the plot represents a single-phase exponential decay. The asterisks (*) represent points found to be significantly different from minute 0 using Tukey's multiple-comparison test. **C)** A representative plot of eIPSC amplitudes representing the effect of prolonged exposure to ME (30 μm) on eIPSC amplitude. Each point represents the average of three consecutive sweeps. Sample traces shown above the plot represent the average of three consecutive sweeps taken every third minute starting at the onset of agonist exposure. **D)** Compiled data representing the inhibition of eIPSC amplitude over the course of a 10 min exposure to ME (30 μm; *n* = 14). Error bars indicate SEM.

$GABA_B$, nociceptin, and κ-opioid receptors (KORs) to undergo or resist desensitization was examined. Superfusion of a maximal concentration of nociceptin (500 nM) induced a postsynaptic outward current (38 ± 4.8 pA peak) that declined within minutes to a plateau of 40% of baseline ($p < 0.0001$, one-way repeated-measures ANOVA; $\tau = 210$ s; $n = 6$; Fig. 2.2*A*). A maximal concentration of nociceptin (500 nM) also caused an inhibition of eIPSC amplitude

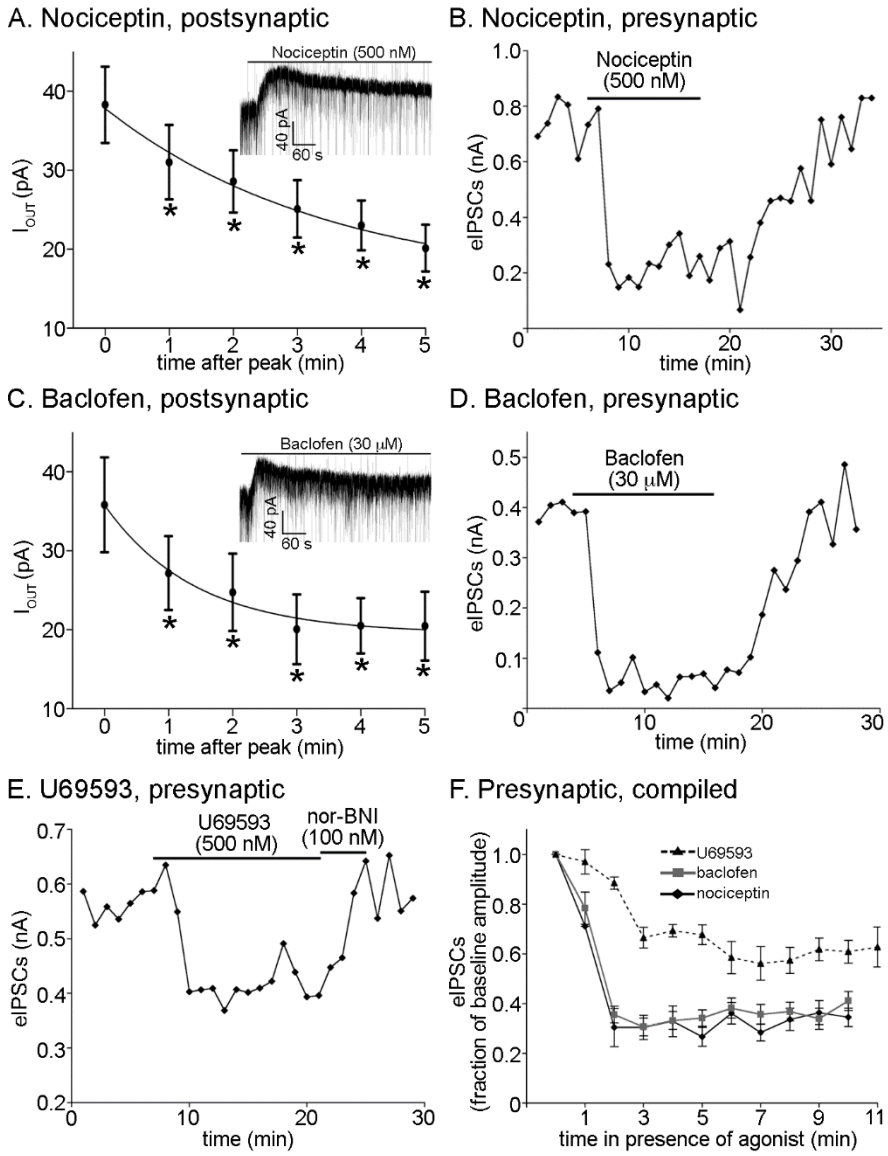


**Figure 2.2 Multiple $G_{i/o}$-coupled receptors presynaptic to POMC neurons resist acute desensitization. A)** Compiled data representing the amplitude of the postsynaptic current induced by nociceptin at every minute, beginning at the peak, for 5 min during constant exposure to the agonist (*n* = 6). The inset shows a sample trace of a postsynaptic current induced by nociceptin. A similar plot and inset is shown in **(C)** for recordings made in the presence of baclofen (*n* = 6). The asterisks represent points found to be significantly different from minute 0 using Tukey's multiple-comparison test. **B)** A representative plot showing the effect of prolonged exposure to a maximal concentration of nociceptin (500 nm) on eIPSC amplitude. Each point represents the average of three consecutive sweeps. Similar plots are shown for recordings made in the presence of baclofen **(*D*)** and U69593 **(*E*)**. **F)** Compiled data showing the inhibition of eIPSC amplitudes by U69593 (dashed line; *n* = 5), baclofen (gray line; *n* = 19), and nociceptin (black line; *n* = 7) during continued exposure to each agonist. Error bars indicate SEM.

(to 30 ± 7.7% of baseline; *n* = 7; Fig. 2.2*B*), but the inhibition of eIPSCs did not decline during the 10 min exposure to agonist (*p* = 0.47, one-way repeated-measures ANOVA; minutes 2

through 10; Fig. 2.2*B*,*F*). Similarly, the $GABA_BR$ agonist baclofen (30 μM, maximal concentration) induced an outward current (36 ± 6.0 pA; *n*= 6; Fig. 2.2*C*) that declined during exposure (plateau, 53% of baseline; $p < 0.0001$, one-way repeated-measure ANOVA; τ = 84 s; Fig. 2.2*C*). Baclofen also caused an inhibition of eIPSC amplitude (to 36 ± 3.4% of baseline; *n* = 19; Fig. 2.2*D*) that was maintained throughout the exposure ($p = 0.053$, one-way repeated-measures ANOVA; minutes 2 through 10; Fig. 2.2*D*,*F*).

Activation of the KOR inhibits neurotransmitter release onto POMC neurons but has no apparent postsynaptic effects (Pennock and Hentges, 2011)[2]. Superfusion of a maximal concentration of the KOR agonist U69593 (500 nM) resulted in inhibition of the amplitude of eIPSCs (to 66 ± 9.3% of baseline; *n* = 5; $p < 0.0001$; Fig. 2.2*E*,*F*). Similar to the MOR, $GABA_BR$, and nociceptin receptors, the KOR-mediated inhibition of eIPSC amplitude showed no desensitization during >10 min exposure to U69593 ($p = 0.071$, one-way repeated-measures ANOVA; minutes 3 through 13; *n* = 5; Fig. 2.2*F*). Nor-BNI (100 nM) was added upon the termination of U69593 superfusion to enhance wash/reversal (Fig. 2.2*E*). Together, these data show that resistance to acute desensitization may be a common characteristic of $G_{i/o}$-coupled receptors located on presynaptic terminals.

*Differential desensitization of presynaptic and postsynaptic $G_{i/o}$-coupled receptors in POMC neurons*

It is plausible that the cell type on which a receptor is located, not the subcellular compartment in which it is found, determines whether or not that receptor will be resistant to desensitization. To determine whether receptors located in presynaptic and postsynaptic compartments of a single cell type show differential acute desensitization, experiments were performed in which desensitization of presynaptic receptors within POMC neurons was studied

[2] An additional manuscript on this topic titled “Direct inhibition of hypothalamic proopiomelanocortin neurons by dynorphin A is mediated by the μ-opioid receptor” has since been published in *The Journal of Physiology*, and is located in Appendix II

using an optogenetic approach. A viral vector containing a floxed ChR2 sequence was injected into the arcuate nucleus of mice expressing Cre recombinase under the control of the POMC promoter. In slices prepared from these mice, brief pulses of blue light depolarized the POMC neurons resulting in the release of neurotransmitter onto unidentified neighboring neurons from which recordings were made. Superfusion of ME (30 μM) resulted in a robust inhibition of light-evoked IPSC amplitude (to 36 ± 10% of baseline; $n = 6$; $p < 0.0001$; 2.3*A*), which was reversed by the MOR antagonist CTAP (500 nM). Inhibition of the light-evoked IPSCs was maintained during the exposure to ME ($p = 0.14$, one-way repeated-measures ANOVA; minutes 3 through 10; $n = 6$; 2.3*B*), indicating that, unlike postsynaptic MORs in POMC neurons, MORs directly regulating transmitter release from POMC neurons do not undergo acute desensitization.

Baclofen (30 μM) also inhibited presynaptic release from POMC terminals as indicated by the reduction in light-evoked IPSC amplitude (to 27 ± 7.3% of baseline; $n = 6$; $p < 0.0001$; Fig. 2.3*C*). This inhibition was maintained during continued agonist exposure ($p = 0.051$, one-way repeated-measures ANOVA; minutes 2 through 10; $n = 6$; Fig. 2.3*D*). Thus, both μ-opioid and $GABA_B$ receptors undergo desensitization in the postsynaptic (Figs. 2.1, 2.2), but not presynaptic compartments (Fig. 2.3*A*–*D*).

Although KOR activation does not induce a GIRK-mediated current in POMC neurons (Pennock and Hentges, 2011), the KOR agonist U69593 (500 nM) significantly inhibited the amplitude of light-evoked IPSCs (to 45 ± 14% of baseline; $n = 4$;$p < 0.0001$; Fig. 2.3*E*). The inhibition of transmitter release was maintained during 14 min of continuous U69593 perfusion ($p = 0.94$, one-way repeated-measures ANOVA; minutes 4 through 14; $n = 4$; Fig. 2.3*F*). The inhibition of light-evoked IPSC amplitude was completely reversed by the KOR-selective antagonist nor-NBI (100 nM; Fig. 2.3*E*). Together, the data from the light-evoked release studies demonstrate that the ability of some receptors to resist desensitization is likely dependent on the location of the receptor within the neuron.

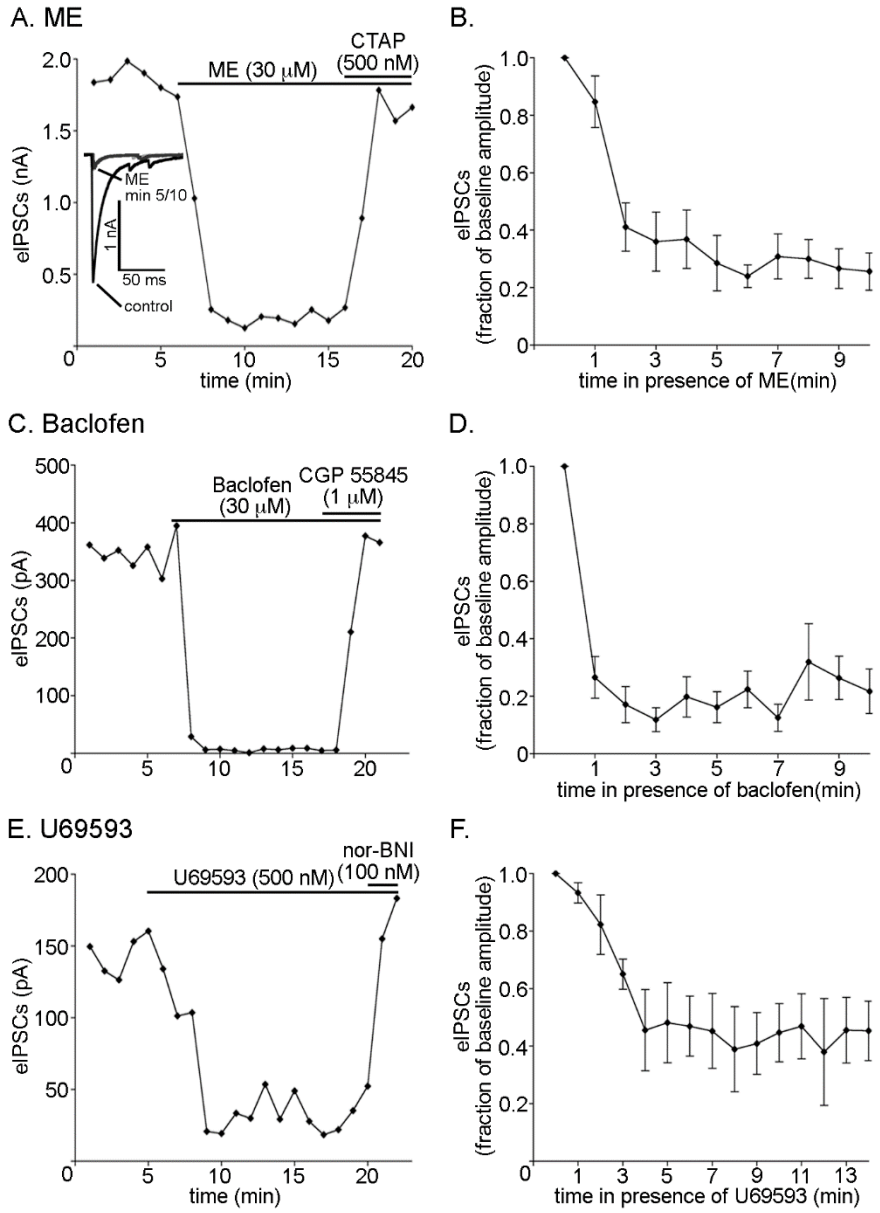


**Figure 2.3 $G_{i/o}$-coupled receptors on the axon terminals of POMC neurons resist desensitization. A)** A plot of light-evoked IPSCs recorded from a neuron within the arcuate nucleus innervated by a POMC neuron(s) expressing ChR2. Each point represents the average of three consecutive sweeps. Sample traces of light-evoked currents from minutes 0, 5, and 10 of ME superfusion are shown in the inset. Each trace is the average of three consecutive sweeps. Similar plots for recordings made in the presence of baclofen and U69593 are shown in **(C)** and **(E)**. Compiled data from recordings representing the average inhibition of light-evoked IPSC amplitude by ME ($n$ = 6), baclofen ($n$ = 6), and U69593 ($n$ = 4) are shown in **(B)**, **(D)**, and **(F)**, respectively. Error bars indicate SEM.

*Decreasing receptor number does not induce presynaptic desensitization*

To determine whether receptor reserve may account for the lack of desensitization observed for MORs, the irreversible MOR antagonist β-CNA was used to reduce receptor

reserve. β-CNA (50 nM) was superfused over the slice for 2 min following the washout of a brief application of ME (30 μM; Fig. 2.4*A*,*B*). The pulse of ME before β-CNA application was used to determine the maximal inhibition possible for ME before receptor reserve was reduced. After the superfusion of β-CNA and a washout period (>10 min), ME (30 μM) was again applied. β-CNA reduced the inhibition of eIPSC amplitude caused by ME to 60 ± 11% of its original value (83 ± 3.4% inhibition of eIPSC amplitude before β-CNA vs 49 ± 8.0% inhibition of eIPSC amplitude after β-CNA; $n = 3$; $p = 0.039$, paired $t$ test; Fig. 2.4*A*,*B*). Although β-CNA treatment reduced the inhibition of eIPSC amplitude by ME, there was still no decrease in ME-mediated inhibition (desensitization) over the course of the drug application ($p = 0.065$; $n = 3$; one-way repeated-measures ANOVA; minutes 2 through 11; Fig. 2.4*C*).

To estimate the size of the MOR receptor reserve presynaptic to POMC neurons, dose–response curves were constructed before and after β-CNA treatment and analyzed using Furchgott's method (Furchgott, 1966) (Fig. 2.4*D*,*E*). ME (30 μM) caused a much larger inhibition of eIPSC amplitude in control conditions compared with the inhibition after a 2 min exposure to β-CNA (79 ± 2.1% inhibition of eIPSC amplitude before β-CNA vs 42 ± 2.4% inhibition of eIPSC amplitude after β-CNA; $p < 0.0001$; $n = 36, 12$; Fig. 2.4*D*). Additionally, there was an approximately threefold shift in the $EC_{50}$ for ME after β-CNA (444 nM before β-CNA vs 1.336 μM after β-CNA). To estimate the fraction of the total number of presynaptic receptors inhibited by β-CNA, a double reciprocal plot of the agonist concentration needed to achieve a given inhibition of eIPSC amplitude before and after β-CNA treatment was constructed according to Furchgott's method (Eq. 1). This analysis revealed that the 44% decrease in the maximal inhibition of eIPSC amplitude by ME corresponded to a 59–68% reduction in the total number of presynaptic MORs (95% confidence interval for $q$ [0.3189, 0.4137]; Fig. 2.4*E*) and provided an estimated $K_A$ value near that of the $EC_{50}$ (95% confidence interval [0.693 μM, 1.315 μM]; Fig. 2.4*E*) under control conditions. These data suggest that presynaptic resistance to desensitization is not due to receptor reserve since removing enough receptors to potently blunt

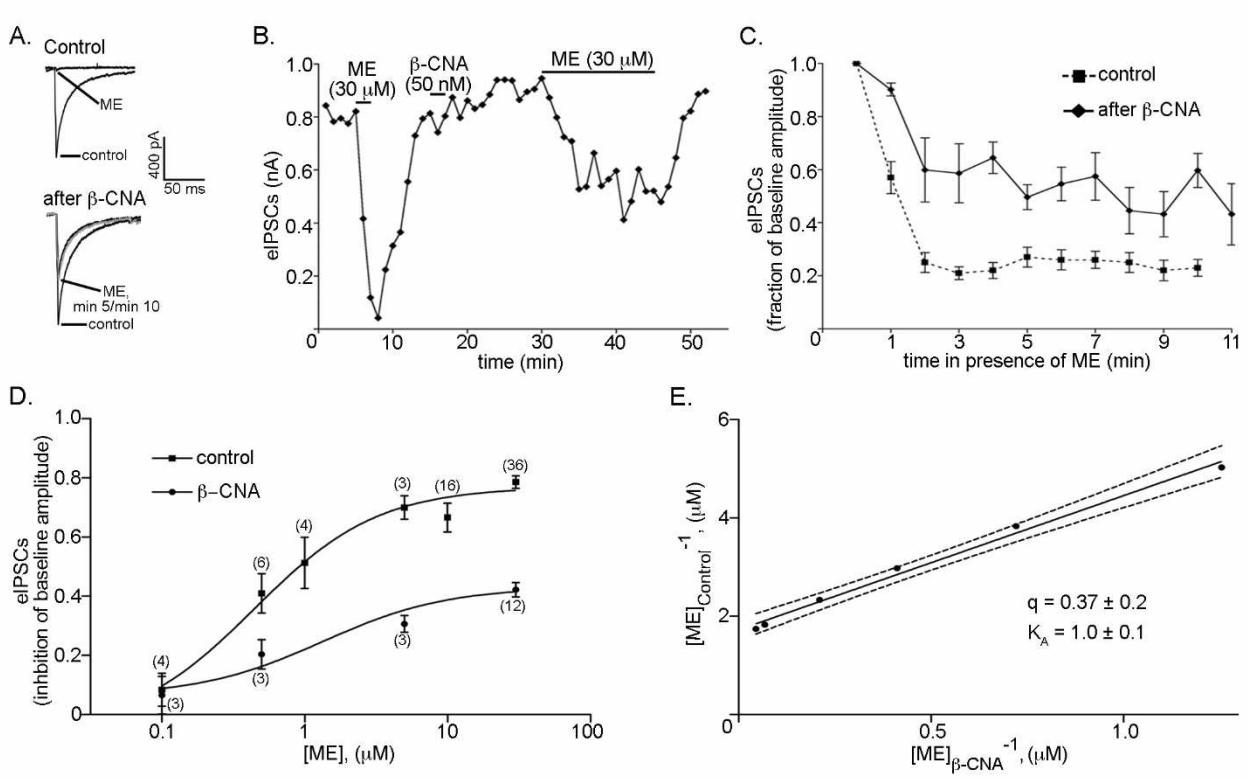


**Fig 2.4 Decreasing the number of functional receptors presynaptic to POMC neurons does not induce acute desensitization. A)** Sample traces indicating the inhibition of eIPSC amplitude in response to ME (30 μm) before (top traces) and after receptor knockdown with β-CNA (bottom traces). The sample traces in **(A)** represent the average of three consecutive sweeps taken under the conditions indicated. **B)** A plot of eIPSC amplitudes showing the effect of β-CNA on the inhibition of eIPSC amplitude by ME. Each point represents the average of three consecutive sweeps. **C)** Compiled data representing the effect of prolonged exposure to ME (30 μm) on eIPSC amplitude after treatment with β-CNA (solid line) (*n* = 3). **D)** Dose–response curves for ME-induced inhibition of eIPSC amplitude before (squares) and after (circles) treatment with β-CNA (50 nm; 2 min). The sample size for each point of the curve is shown in parentheses. **E)** A reciprocal plot of agonist concentrations that produced an equivalent inhibition of eIPSC amplitude before (vertical axis) and after (horizontal axis) β-CNA treatment was obtained using Furchgott's method (Eq. 1). Estimates of the fraction of receptors remaining after β-CNA treatment and the dissociation constant (in micromolar concentration) of ME at MORs presynaptic to POMC neurons are represented by *q* and $K_A$, respectively. The dashed lines represent the 95% confidence limits of the regression line. Error bars indicate SEM.

the maximal response did not cause presynaptic receptors to display desensitization.

Furthermore, the finding that removing ~50% of the surface receptors reduces the functional response by ~50% indicates that terminals regulating POMC neurons do not have a significant receptor reserve even under baseline conditions.

*Morphine does not cause desensitization of MORs in the presynaptic compartment*

MORs activated by morphine may desensitize through a mechanism distinct from those activated by ME or DAMGO in some systems (Johnson et al., 2006; Kelly et al., 2008). Thus, it

is possible that the environment of the presynaptic terminal may confer resistance to desensitization to a ME-activated MOR but not a morphine-activated receptor. To determine whether resistance to desensitization by presynaptic MORs may be agonist specific, eIPSC amplitude was measured during a 15 min superfusion of morphine (20 μM). Morphine caused a reduction in the amplitude of eIPSCs (to 52 ± 12% of baseline; $n = 4$; $p < 0.0001$) that was maintained throughout exposure to morphine ($p = 0.91$; $n = 4$; one-way repeated-measures ANOVA; minutes 3 through 13; Fig. 2.5*A*,*B*), indicating that presynaptic receptors do not undergo acute desensitization whether bound by full or partial agonists that likely confer different conformational states of the receptor.

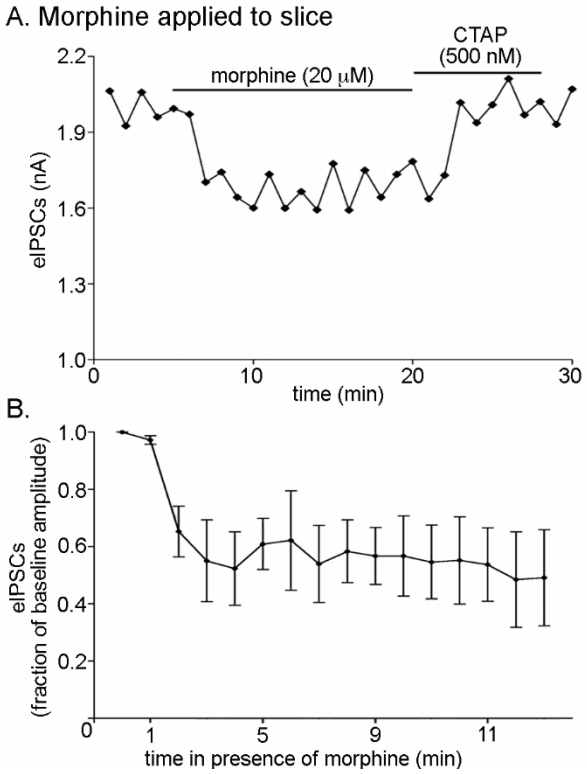


**Figure 2.5 Presynaptic MORs resist desensitization when activated with morphine. A)** Plot of eIPSC amplitudes during acute morphine exposure (20 μm). CTAP (500 nm) was added upon the cessation of morphine superfusion to speed wash time. Each point represents three consecutive sweeps. **B)** Compiled data representing the inhibition of eIPSC amplitude over 11 min of morphine (20 μm) superfusion ($n = 4$). Error bars indicate SEM.

Chronic morphine treatment (CMT) can reduce the efficacy of MOR agonists at presynaptic terminals (North and Vitek, 1980; Fyfe et al., 2010) and enhance the extent and rate of acute desensitization of postsynaptic MORs (Dang and Williams, 2005; Ingram et al., 2008). To determine whether CMT can modify presynaptic MOR signaling to enhance acute desensitization of these receptors in terminals presynaptic to POMC neurons, mice were treated with morphine (50 mg · $kg^{-1}$ · $d^{-1}$) or saline for 5–7 d using subcutaneous miniosmotic pumps before slice preparation. When slices were collected into morphine (1 μM) and maintained in morphine, application of naloxone (1 μM) caused a greater increase in the eIPSC amplitude (1.8 ± 0.13-fold increase in control; 4.4 ± 1.0-fold increase in CMT; $n$ = 4–6; $p$ = 0.01; Fig. 2.6*A*,*C*) and decrease in the paired-pulse ratio in cells from CMT mice compared with saline-treated mice (0.68 ± 0.04 in control; 0.51 ± 0.05 in CMT; $n$ = 4–6; $p$ = 0.01; Fig. 2.6*A*,*B*), indicating that morphine was effectively reaching the synapses and inhibiting transmitter release. Application of ME (30 μM) to slices from CMT mice that were collected and maintained in morphine-free aCSF resulted in a robust decrease in eIPSC amplitude (to 32 ± 3.2% of baseline; $n$ = 20; $p$ < 0.0001; Fig. 2.6*D*,*E*) that was maintained over the course of an 11 min exposure ($p$ = 0.32; $n$ = 5; one-way repeated-measures ANOVA; minutes 2 through 11) similar to that observed for tissue from untreated mice. Thus, presynaptic MORs resist desensitization after both acute and chronic exposure to morphine.

*A fraction of presynaptic $GABA_BRs$ acutely desensitize*

It is plausible that a property of the presynaptic environment could somehow prevent receptor desensitization. However, in 7 of 26 recordings made from POMC neurons in which baclofen (30 μM) was applied while recording eIPSC amplitude, the inhibition of eIPSC amplitude declined during baclofen application ($p$ < 0.0001, one-way repeated-measures ANOVA; minutes 2 through 10; $n$ = 7; Fig. 2.7*A*,*B*). Inhibition peaked by the second minute of agonist exposure, on average, and desensitized by ~50% by minute 10 (25 ± 6.9% of baseline at minute 2 vs 60 ± 7.4% of baseline at minute 10). In some recordings in which

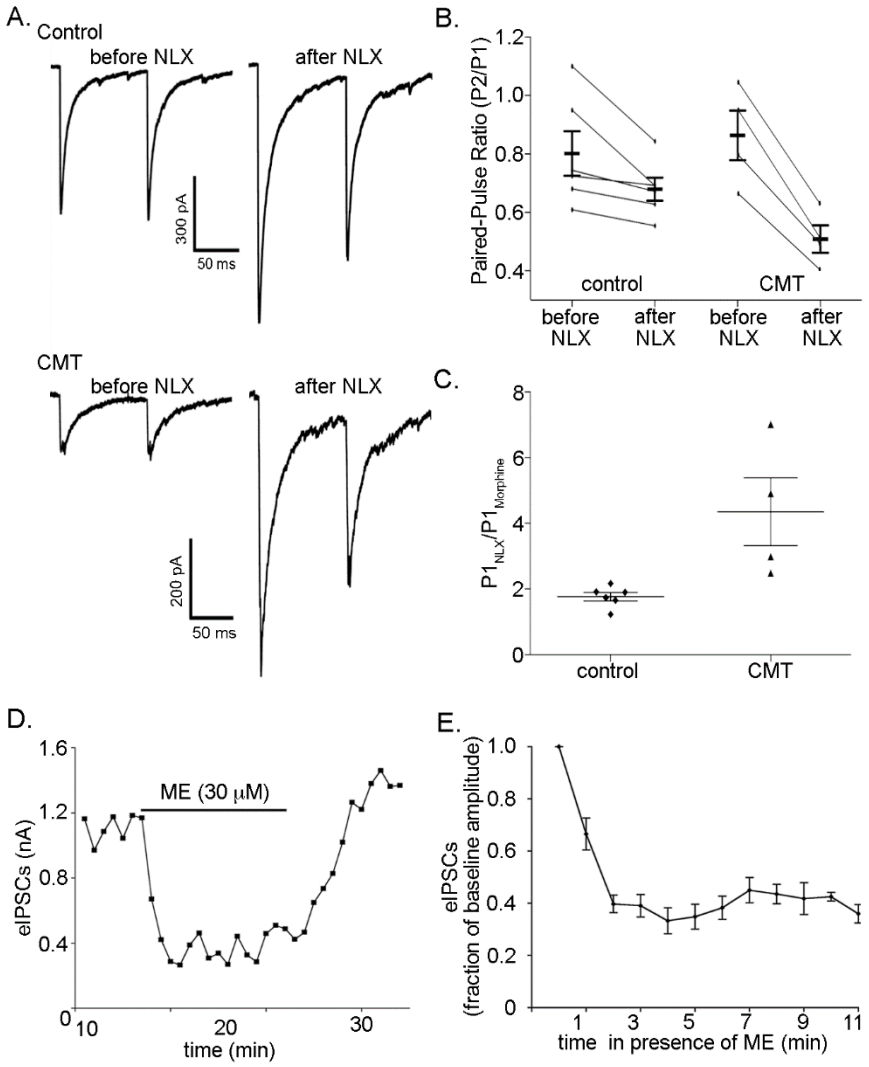


**Figure 2.6 Chronic morphine treatment does not enhance desensitization of presynaptic MORs. A)** Sample traces from recordings made in control and CMT (50 mg · $kg^{-1}$ · $d^{-1}$) mice that were prepared and maintained in 1 μm morphine until application of 1 μm naloxone (NLX) to induce precipitated withdrawal. IPSCs were evoked using paired stimuli 100 ms apart. **B)** NLX precipitated withdrawal decreased the paired-pulse ratio (P2/P1) to a greater extent in CMT mice than in controls. **C)** NLX also caused a larger increase in the amplitude of the IPSC evoked by the first of the paired stimuli (P1) in recordings made in slices prepared from CMT animals than in those prepared from controls. **D)** Plot of eIPSCs from a recording in which a slice prepared from an animal that had received CMT was exposed to ME (30 μm) for a prolonged period. Each point represents the average of three consecutive sweeps. **E)** Compiled data representing the effect of prolonged ME exposure on recordings made from slices prepared from mice that had received CMT (*n* = 5). Error bars indicate SEM.

$GABA_B$ receptor desensitization was observed, it was possible to subsequently perfuse a maximal concentration of ME for >10 min. In these recordings, only the $GABA_B$-mediated inhibition of eIPSC amplitude desensitized, whereas ME-mediated inhibition was maintained (data not shown). Additionally, $GABA_B$-mediated inhibition of miniature IPSC (mIPSC) frequency

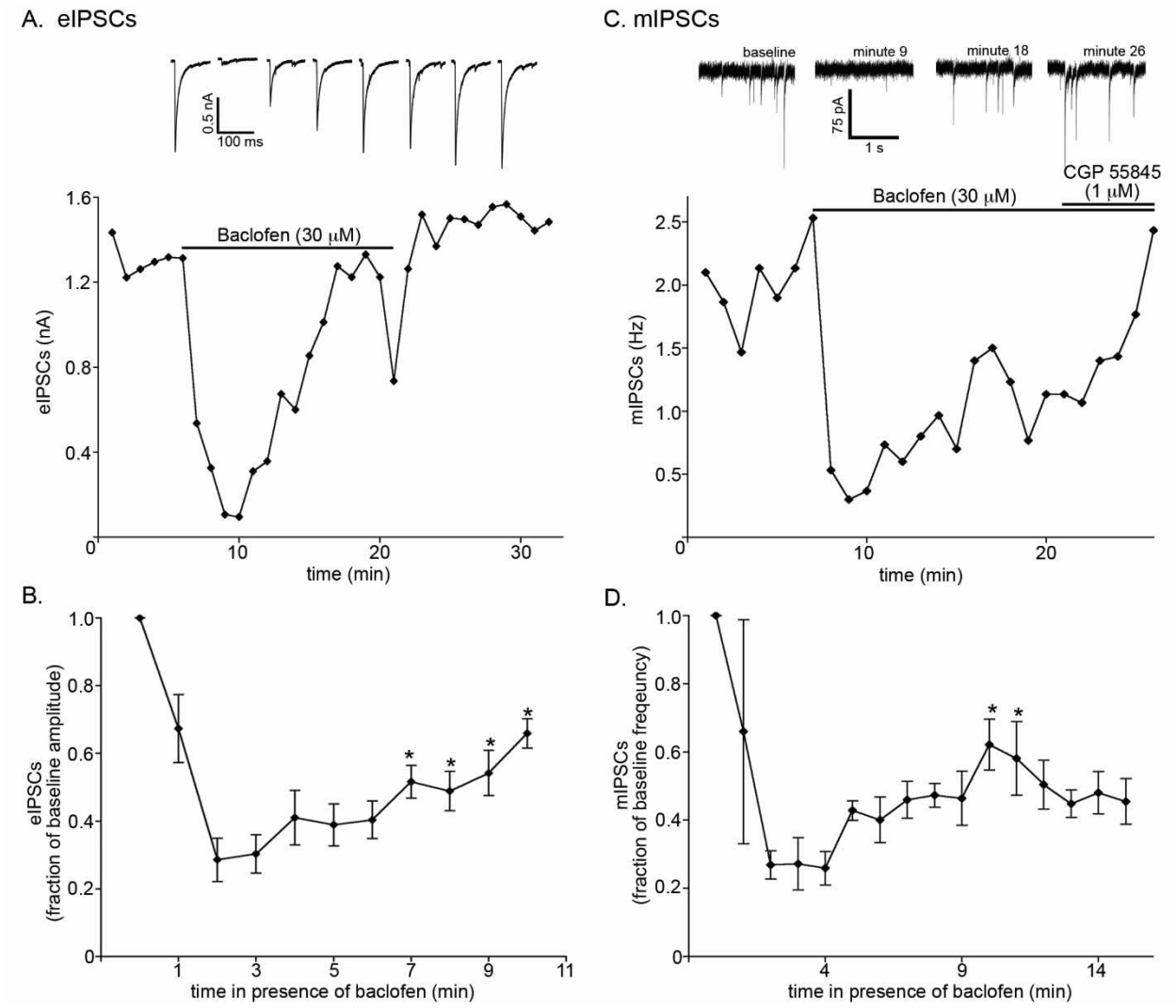


**Figure 2.7 A fraction of $GABA_B$Rs presynaptic to POMC neurons do desensitize**. **A)** A plot of eIPSC amplitudes from a recording in which the baclofen-induced inhibition of eIPSC amplitude declined robustly during application. Each point represents the average of three consecutive sweeps. The eIPSC traces shown above the plot are the average of three consecutive sweeps. Sample traces were taken at minutes 0 (baseline) and 4 (maximal inhibition) of baclofen superfusion and then every third minute thereafter. **B)** Compiled data showing the inhibition of eIPSC amplitude by baclofen at each minute during continuous exposure (*n* = 7). **C)** A plot of mIPSC frequency from a recording in which baclofen-induced inhibition of mIPSC frequency declined during baclofen application. Representative traces taken at various time points during a recording are shown above the plot. **D)** Compiled data showing the inhibition of mIPSC frequency by baclofen at each minute during continuous exposure (*n* = 3). The asterisks denote points that were found to be significantly different from the maximal inhibition caused by baclofen (minute 2) using Tukey's multiple-comparison test. Error bars indicate SEM.

was examined to determine whether ~25% of all inputs onto POMC neurons contain $GABA_B$ receptors that are able to undergo desensitization, or whether only ~25% of POMC neurons receive inputs with $GABA_B$ receptors that are able to undergo desensitization. Similar

to what was observed when using eIPSCs to measure $GABA_B$-mediated inhibition of neurotransmitter release, desensitization of $GABA_B$-mediated inhibition of mIPSC frequency was only observed in 25% of recordings made (3 of 12 recordings; Fig. 2.7*C*,*D*). This suggests that 25% of POMC neurons receive input from terminals on which $GABA_B$ receptors are able to undergo desensitization. If resistance or susceptibility of GPCRs to desensitization is due to a property of the terminals on which they are found, desensitization should have been observed in a similar fraction of recordings using agonists for the other receptors examined. Together, it appears that resistance to desensitization is likely conferred by a property of the receptor itself and not an intrinsic property of the terminals on which the receptors are found.

## 2.6 Discussion

The present data demonstrate that multiple $G_{i/o}$-coupled receptors located presynaptic to POMC neurons as well as on POMC terminals resist acute desensitization in response to agonist exposure, while the same receptors located on the somatodendritic region of POMC neurons robustly desensitize under identical conditions. MORs display resistance to desensitization in the presence of various agonists and after receptor number is drastically reduced. The current data together with previous reports of differential presynaptic and postsynaptic desensitization indicate that resistance to desensitization is a common and robust property of presynaptic $G_{i/o}$-coupled receptors. Comparative studies between presynaptic and postsynaptic receptor regulation could provide important insight into mechanisms underlying desensitization and could help explain complex physiologic responses to prolonged agonist exposure.

*Differential presynaptic and postsynaptic desensitization of multiple $G_{i/o}$-coupled receptors*

The ability of presynaptic, but not postsynaptic MORs to resist desensitization on POMC neurons is similar to the differential regulation of MORs previously reported in the periaqueductal gray (PAG) (Fyfe et al., 2010) and the locus ceruleus (Blanchet and Luscher, 2002). Thus, sustained presynaptic MOR signaling is not unique to terminals within the arcuate

nucleus. Differential desensitization of presynaptic and postsynaptic receptors also does not appear to be a phenomenon specific to the MOR since similar presynaptic resistance and postsynaptic susceptibility to desensitization was also found to occur for nociceptin and $GABA_B$ receptors regulating POMC neurons. κ-Opioid receptors, which were only found on presynaptic terminals, were also resistant to acute desensitization. Although KOR-induced postsynaptic currents were not observed in the present study, they likely possess the property of differential desensitization between presynaptic and postsynaptic receptors since KORs expressed in heterologous systems do display acute desensitization (Henry et al., 1995). $GABA_B$Rs and adenosine $A_1$ receptors ($A_1$Rs) located in the presynaptic but not postsynaptic compartment of cultured hippocampal neurons also resist acute agonist-induced desensitization (Wetherington and Lambert, 2002a, b), and a similar phenomenon has been described for $GABA_B$Rs regulating dopaminergic neurons in the VTA (Cruz et al., 2004). Thus, the ability to maintain signaling may be a general property of a variety of presynaptic receptors located throughout the brain.

*Presynaptic resistance to desensitization is independent of receptor reserve*

The apparent lack of acute desensitization observed for presynaptic MORs could simply reflect that there is a high receptor reserve presynaptically such that there is always a sufficient pool of non-desensitized receptors to mediate maximal inhibition of transmitter release. This does not appear to be the case, however, since reducing MOR receptor reserve with β-CNA did not unmask any acute presynaptic MOR desensitization, although inhibition of transmitter release was significantly reduced. These results are consistent with a previous study examining presynaptic MORs in the PAG that also resist desensitization after knockdown with β-CNA (Fyfe et al., 2010). The analysis of dose–response curves constructed under control conditions and after β-CNA exposure revealed that not only does resistance to desensitization by presynaptic MORs occur independent of receptor reserve, but that minimal MOR reserve is present on terminals presynaptic to POMC neurons to begin with.

CMT also can reduce receptor reserve (Christie et al., 1987) and enhance both the extent (Ingram et al., 2008) and rate (Dang and Williams, 2005) of MOR desensitization following a subsequent acute application of agonist. However, in the present study, CMT did not induce desensitization of presynaptic MORs nor reduce the presynaptic inhibition induced by μ receptor agonists as has been observed in other studies (North and Vitek, 1980; Fyfe et al., 2010). The presynaptic inhibition in the present study may be linked to the observation that terminals presynaptic to POMC neurons do not have a substantial receptor reserve even under basal conditions. Interestingly, $GABA_BRs$ and $A_1Rs$ located on the presynaptic terminals of hippocampal neurons also resist desensitization in a manner that is independent of receptor reserve (Wetherington and Lambert, 2002a, b). These similarities across multiple cell types and multiple systems imply that not only is resistance to desensitization a common property of presynaptic $G_{i/o}$-coupled receptors but that this resistance may occur through similar mechanisms. Furthermore, the fact that even prolonged exposure to agonist is not sufficient to reduce the presynaptic inhibition to a subsequent application of μ receptor agonist suggests that MORs in the presynaptic compartment resist desensitization by a specific mechanism rather than simply undergoing a postsynaptic-like desensitization in a slower manner.

*Contribution of presynaptic environment in the resistance to acute receptor desensitization*

MORs and $GABA_BRs$ undergo acute desensitization in the postsynaptic but not presynaptic compartment of POMC neurons as determined in the ChR2 experiments. This is consistent with previous studies showing that when hippocampal neurons form synapses onto themselves in culture, $GABA_B$ and adenosine $A_1$ receptors in the somatodendritic compartment undergo acute receptor-specific (not effector-dependent) desensitization, but those receptors located in the presynaptic terminal do not desensitize (Wetherington and Lambert, 2002a, b). Together, the data suggest that the cellular compartment in which a receptor resides may confer the tendency to undergo desensitization. However, the finding that presynaptic $GABA_BRs$ displayed acute desensitization in 10 of the 38 experiments (including both mIPSC and eIPSC

experiments) in the present study indicates that compartmentalization alone may not be sufficient to confer resistance to desensitization. Furthermore, $GABA_BRs$ in CA3 neurons of the neonatal rat hippocampus located on the postsynaptic membrane or in presynaptic glutamatergic terminals do not desensitize with acute agonist exposure, although $GABA_BRs$ receptors located on GABAergic terminals desensitize readily (Tosetti et al., 2004). Resistance to desensitization also occurs in postsynaptic $GABA_BRs$ located on GABAergic, but not dopaminergic, neurons of the VTA (Cruz et al., 2004). These discrepancies between the patterns of presynaptic and postsynaptic desensitization of $GABA_BRs$ and the other receptors examined suggests that, although there is a strong correlation between compartmentalization and desensitization, compartmentalization alone is not sufficient to explain differential desensitization between presynaptic and postsynaptic $G_{i/o}$-coupled receptors.

Such discrepancies between the $GABA_BR$ and the other receptors examined may be the result of variable expression of the $GABA_bR1_{a/b}$ splice variants. Although these splice variants are preferentially targeted to either terminal regions ($R1_a$) or somatodendritic regions ($R1_b$) of neurons, studies using $R1_a$ and $R1_b$ knock-outs suggest that either variant can be expressed at GABAergic terminals and postsynaptic membranes (Vigot et al., 2006). Variable expression patterns of the $GABA_BR1$ splice variants may explain the discrepancies between the patterns of presynaptic and postsynaptic desensitization of the $GABA_BR$ and other receptors that have been examined. Determining whether and why a certain $GABA_BR$ heterodimer composition is resistant to desensitization may provide insight into the mechanism by which other receptors resist acute desensitization.

*Implications of differential presynaptic and postsynaptic receptor desensitization*

The physiologic consequences of differential presynaptic and postsynaptic desensitization for specific receptors remain to be determined, but the data suggest that acute and chronic agonist exposure can differently affect cellular activity as postsynaptic responses may desensitize while presynaptic actions are maintained. In POMC neurons, for example, the

initial action of MOR agonists is direct postsynaptic inhibition, whereas the prolonged effect of MOR activation would likely be disinhibition since the majority of inputs to POMC neurons are GABAergic (Pinto et al., 2004; Hentges et al., 2009). Similar differential desensitization of $GABA_BRs$ located on GABAergic terminals and postsynaptic $GABA_BRs$ in the neonatal hippocampus has been suggested to underlie the induction of epileptiform discharges in this region (Vardya et al., 2010). The lack of presynaptic MOR desensitization may explain the observation that synaptic transmission increases upon withdrawal of MOR agonist in multiple brain regions (Bonci and Williams, 1997; Hack et al., 2003; Bie and Pan, 2005), consistent with a continued action at presynaptic MORs until removal of drug. This increase in synaptic transmission after the cessation of chronic opioid exposure has been implicated in withdrawal symptoms such as hyperalgesia (Bie and Pan, 2005; Bie et al., 2005; Heinl et al., 2011). Thus, the differential presynaptic and postsynaptic desensitization of certain GPCRs may have significant functional consequences.

*Conclusions*

Whereas the postsynaptic actions of MORs and other $G_{i/o}$-coupled receptors decline within minutes of agonist exposure, these same receptors on the presynaptic terminals of POMC neurons and presynaptic to POMC neurons continue to inhibit transmitter release. Thus, during continued agonist exposure, postsynaptic responses will likely decline while presynaptic actions will be maintained. The mechanisms limiting presynaptic receptor desensitization remain to be determined, but the data here suggest that compartment-specific modification of the receptor may underlie the resistance.

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# Chapter 3: Desensitization-Resistant MORs and $GABA_BRs$ and Desensitization-Susceptible $GABA_BRs$ Inhibit GABA Release Onto POMC Neurons Through Similar Mechanisms

## 3.1 Overview

In the previous chapter, evidence was presented that presynaptic MORs are regulated in a manner that is distinct from their postsynaptic counterparts. We also found that desensitization-resistant and –susceptible $G\alpha_{i/o}$-coupled GPCRs exist on axon terminals presynaptic to POMC neurons. This finding demonstrated that while resistance to desensitization is a common property of these receptors, it is not ubiquitous. This finding also opened the door for comparative studies examining the coupling of desensitization-resistant and –susceptible receptors, which will be described in this chapter.

We hypothesize that resistance to desensitization is due to receptor level differences in MORs, and other GPCRs, located in axon terminals versus receptors located on the soma and dendrites of neurons. However, it is possible that resistance to desensitization is conferred by a particular receptor-effector coupling. If this were the case then we would expect desensitization-resistant and desensitization-susceptible GPCRs located on axon terminals to be differentially coupled. In this chapter, the receptor-effector coupling of MORs on axon terminals presynaptic to POMC neurons is compared to that of $GABA_BRs$ that are also located on the same population terminals. While MORs located presynaptic to POMC neurons have been shown to be completely resistant to desensitization, inhibition induced by $GABA_BRs$ exhibits acute desensitization in approximately one quarter of experiments. If resistance to desensitization is conferred by receptor-effector coupling then we would expect differential coupling between desensitization-resistant MORs and $GABA_BRs$ versus the population of $GABA_BRs$ that are susceptible to desensitization. If all of these receptors couple to the same set of effectors it will provide more evidence that resistance to desensitization is due to receptor-level properties.

The findings presented in this chapter support the hypothesis that resistance to desensitization is due to receptor-level properties as both desensitization-resistant and desensitization-susceptible receptors located on axon terminals presynaptic to POMC neurons were found to be similarly coupled. In addition to this finding, we found that presynaptic MORs and $GABA_BRs$ inhibit spontaneous and evoked release, and that inhibition of each type of release occurs through a different mechanism. Desensitization of $GABA_BR$-mediated inhibition occurred when measuring either type of release, and resistance to desensitization by the MOR was maintained when measuring either type of release, providing more evidence that desensitization, or lack thereof, occurs at the level of the receptor.

The following chapter was published May 1, 2016 in *Journal of Neurophysiology* Volume 115 no. 5 under the title "Desensitization-resistant and –sensitive GPCR-mediated Inhibition of GABA Release Occurs by $Ca^{2+}$-dependent and –independent Mechanisms at a Hypothalamic Synapse", and has been reproduced with the permission of the American Physiological Society.[3] I designed and carried out the experiments described in this manuscript with guidance from Shane T. Hentges. I also drafted the manuscript with the aid of Shane T. Hentges.

**3.2 Summary**

Whereas the activation of $G\alpha_{i/o}$-coupled receptors commonly results in postsynaptic responses that show acute desensitization, the presynaptic inhibition of transmitter release caused by many $G\alpha_{i/o}$-coupled receptors is maintained during agonist exposure. However, an exception has been noted where $GABA_B$ receptor ($GABA_BR$)-mediated inhibition of inhibitory postsynaptic currents (IPSCs) recorded in mouse proopiomelanocortin (POMC) neurons exhibit acute desensitization in ~25% of experiments. To determine whether differential effector coupling confers sensitivity to desensitization, voltage-clamp recordings were made from POMC neurons to compare the mechanism by which μ-opioid receptors (MORs) and $GABA_BRs$ inhibit

[3] See Appendix III for permissions

transmitter release. Neither MOR- nor $GABA_BR$-mediated inhibition of release relied on the activation of presynaptic $K^+$ channels. Both receptors maintained the ability to inhibit release in the absence of external $Ca^{2+}$ or in the presence of ionomycin-induced $Ca^{2+}$ influx, indicating that inhibition of release can occur through a $Ca^{2+}$-independent mechanism. Replacing $Ca^{2+}$ with $Sr^{2+}$ to disrupt G-protein-mediated inhibition of release occurring directly at the release machinery did not alter MOR- or $GABA_B$-mediated inhibition of IPSCs, suggesting that reductions in evoked release can occur through the inhibition of $Ca^{2+}$ channels. Additionally, both receptors inhibited evoked IPSCs in the presence of selective blockers of N- or P/Q-type $Ca^{2+}$ channels. Altogether, the results show that MORs and $GABA_BRs$ can inhibit transmitter release through the inhibition of calcium influx and by direct actions at the release machinery. Furthermore, since both the desensitizing and nondesensitizing presynaptic receptors are similarly coupled, differential effector coupling is unlikely responsible for differential desensitization of the inhibition of release.

### 3.3 Introduction

Proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus have been the focus of many recent studies due to their important role in the regulation of energy balance (Mercer et al., 2013). Studies examining the regulation of POMC neuron activity have revealed that the release of GABA onto POMC neurons is inhibited by multiple $G\alpha_{i/o}$-coupled receptors, including the μ-opioid receptor (MOR) and $GABA_B$ receptor ($GABA_BR$) (Pennock et al., 2012). Electrophysiological recordings demonstrate that at the majority of POMC neurons, a sustained (≥10 min) application of a maximal concentration of an agonist for the MOR or the $GABA_BR$ induces robust of inhibition of GABA release onto POMC neurons that does not diminish over the course of the application (Pennock et al., 2012). This sustained inhibition of transmitter release may indicate that presynaptic MORs and $GABA_BRs$ are relatively resistant to acute desensitization. This is in contrast to postsynaptic MORs and $GABA_BRs$ located on POMC neurons, both of which exhibit robust desensitization during a similar application of an

agonist (Pennock et al., 2012). Resistance to acute desensitization of inhibitory G-protein-coupled receptors (GPCRs) mediating presynaptic inhibition is a common property of these receptors that has been described at a variety of other synapses (Blanchet and Luscher, 2002; Wetherington and Lambert, 2002a, b; Cruz et al., 2004; Fyfe et al., 2010), however, desensitization of $GABA_BR$-mediated inhibition of neurotransmitter release has been reported (Tosetti et al., 2004; Pennock et al., 2012). At present, it is not clear what determines whether the inhibition of release by presynaptic $G\alpha_{i/o}$-coupled receptors is resistant or susceptible to acute desensitization.

A previous study by our group suggests that resistance or susceptibility to desensitization by presynaptic $G\alpha_{i/o}$-coupled GPCRs may occur in a receptor-autonomous fashion (Pennock et al., 2012). However, another possible explanation for differential desensitization of MOR- and $GABA_BR$-mediated inhibition of GABA release is that some $GABA_BRs$ couple to downstream effectors that desensitize rather than desensitization occurring at the receptors themselves. Both the MOR and $GABA_BR$ are known to inhibit release through multiple distinct pathways, including activation of voltage-dependent $K^+$ channels (Vaughan et al., 1997; Zhu and Pan, 2005), inhibition of $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (Hori et al., 1992; Dittman and Regehr, 1996; Takahashi et al., 1998), and inhibition of release downstream of $Ca^{2+}$ entry through actions directly at the vesicular release machinery (Capogna et al., 1993, 1996; Dittman and Regehr, 1996).

In the present study, MOR- and $GABA_BR$-mediated inhibition of inhibitory postsynaptic currents (IPSCs) in POMC neurons was measured in recording conditions that disrupt *1*) activation of voltage-dependent $K^+$ channels, *2*) $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels, and *3*) GPCR-mediated inhibition of GABA release occurring at the release machinery. The data demonstrate that neither the MOR- nor $GABA_BR$-mediated inhibition of release requires the activation of voltage-dependent $K^+$ channels. Additionally, both receptors appear to inhibit GABA release through inhibiting $Ca^{2+}$ entry into presynaptic terminals as well

as by inhibiting the release machinery downstream of $Ca^{2+}$ influx. Desensitization of $GABA_BR$-mediated inhibition of GABA release was detected under conditions that disrupted $Ca^{2+}$ influx and conditions that disrupt GPCR-mediated inhibition of the release machinery, indicating that differential effector coupling is unlikely to confer susceptibility or resistance to GPCR-mediated inhibition of release.

### 3.4 Materials and Methods

*Animals*

Mice expressing enhanced green fluorescent protein (eGFP) (Cowley et al., 2001) or Discosomared (DsRed) (Hentges et al., 2009) driven by the POMC promoter were backcrossed for >11 generations on the C57BL/6 background. Animals were housed at a controlled temperature (22–24℃) on a constant 12:12 -h light-dark cycle. Standard rodent chow and tap water were provided ad libitum. Transgenic mice were identified using standard PCR genotyping. Brain slices were prepared from male and female mice between 6 and 12 wk of age. All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee and met the United States Public Health Service guidelines.

*Brain-slice preparation*

POMC-eGFP or POMC-DsRed mice were deeply anesthetized using isoflurane followed by rapid removal of the brain and transfer of the tissue into ice-cold artificial cerebral spinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 $NaH_2PO_4$, 21.4 $NaHCO_3$, and 11 glucose. aCSF solutions were saturated with a 95% $O_2$-5% $CO_2$ mixture. Sagittal brain slices (240 mm) were prepared using a VT1200 S vibratome (Leica). Brain slices were transferred into warm (37℃) aCSF containing MK -801 (15 μM). Brain slices were allowed at least 45 min of rest before being transferred to the recording chamber.

*Electrophysiology*

After placement in the recording chamber, brain slices were continuously superfused with warm (37℃), 95% $O_2$-5% $CO_2$-saturated aCSF. When indicated, $CaCl_2$ in the external

solution was replaced with either 2.4 mM $SrCl_2$ or 2.4 mM $MgCl_2$ (total [$MgCl_2$] = 3.6 mM). Recording pipettes were filled with an internal solution containing (in mM): 57.5 CsCl, 57.5 $CsSO_3CH_3$, 20 NaCl, 1.5 $MgCl_2$, 5 HEPES ($K^+$ salt), 0.1 EGTA, 2 Mg-ATP, 0.5 Na-GTP, and 10 phosphocreatine, pH 7.3. Recording pipettes had a tip resistance between 1.5 and 2.5 MΩ when filled with internal solution. POMC neurons in the arcuate nucleus were identified by the presence of either eGFP or DsRed fluorescence in brain slices taken from POMC-eGFP or POMC-DsRed mice, respectively. A ≥1-GΩ seal was obtained on neurons before negative pressure was applied to rupture the cell membrane. Cells were held at a potential of −60 mV, and no series resistance compensation was applied.

Inhibitory postsynaptic potentials were isolated by recording in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM). Miniature IPSCs (mIPSCs) were collected during 15-s sweeps taken every 15 s. Events were detected using rise time kinetics. Events with a rise time of <100 μs were rejected. mIPSCs were recorded in the presence of tetrodotoxin (TTX; 300 nM). To elicit evoked IPSCs (eIPSCs), current was delivered through a bipolar stimulating electrode placed in the middorsal arcuate nucleus. When using a $CaCl_2$-based external recording solution, paired 0.5-ms stimuli (100 ms between pulses) were delivered every 20 s. When using a $SrCl_2$-based external recording solution, a single 0.5-ms stimulus was applied every 20 s. Asynchronous events evoked by $Sr^{2+}$ were collected in the 10 s following the single stimulus using detection parameters identical to those used to detect mIPSCs. Trains of stimuli were evoked by applying 30 0.5-ms stimuli, each spaced 100 ms apart. Injected currents were 10–100 μA in amplitude.

All recordings were made using an Axoclamp MultiClamp 700B or Axopatch 200B amplifier. AxoGraph X software (AxoGraph) was used to collect data. Recordings were collected at 10 kHz and digitally filtered at 1 kHz. Recordings were discarded if the series resistance exceeded 20 MΩ or changed significantly during the course of the recording.

Baclofen- or [D-Ala$^2$, N-Me-Phe$^4$, Gly$^5$-ol]-enkephalin acetate (DAMGO)-induced inhibition of IPSC frequency or amplitude was considered to have desensitized if two conditions were met: *1*) the inhibition of IPSC frequency or amplitude was reduced by ≥15% over the course of 7 min of drug application; and *2*) the inhibition was reversed and IPSC frequency or amplitude did not exceed that measured before drug application. Baclofen- or DAMGO-induced inhibition was considered resistant to desensitization if the inhibition was reversed but did not exhibit at least a 15% reduction over the course of the drug application.

*Drugs*

Stock solutions of DAMGO (Sigma-Aldrich), R(+)-baclofen hydrochloride (baclofen; Sigma-Aldrich), D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ (CTAP; Tocris Bioscience), ω-conotoxin GVIA (Alomone Labs), ω-agatoxin IVA (Alomone Labs), and tetrodotoxin citrate (TTX; Tocris Bioscience) were prepared in distilled water (at least 1,000× final concentration). Stock solution of ionomycin (Sigma-Aldrich), (2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride (CGP 55845; Sigma-Aldrich), 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX; Sigma-Aldrich), and (+)-MK-801 hydrogen maleate (MK-801; Sigma-Aldrich) were prepared in DMSO (at least 10,000× final concentration). Tetraethylammonium chloride (TEA; Sigma-Aldrich), 4-aminopyridine (4-AP; Sigma-Aldrich), and barium chloride ($Ba^+$; Sigma-Aldrich) were added directly to aCSF at the final concentration used.

*Statistics*

Data were compared using the paired Student's *t*-test, repeated-measures ANOVA followed by Tukey multiple-comparison test, one-way ANOVA, or a two-way ANOVA followed by the Bonferroni multiple-comparison test as indicated. Time constants and plateaus of the decay of eIPSC amplitudes evoked by a train of stimuli were obtained by fitting compiled and normalized data to a single-phase decaying exponential. All data were analyzed using

GraphPad Prism. Data are shown as means ± SE, and differences between groups were considered significant if $p < 0.05$.

**3.5 Results**

*Activation of $K^+$ channels is not required for MOR- or $GABA_BR$-mediated inhibition of GABA release onto POMC neurons*

The MOR can inhibit release of neurotransmitter from presynaptic terminals via the activation of voltage-dependent $K^+$ channels (Vaughan et al., 1997; Zhu and Pan, 2005). If MOR- or $GABA_BR$-mediated inhibition of GABA release onto POMC neurons requires the activation of voltage-dependent $K^+$ channels, then the presence of the voltage-dependent $K^+$ channel inhibitor TEA or 4-AP should occlude inhibition by that receptor. However, in the presence of TEA (10 mM), DAMGO still inhibited mIPSC frequency [0.13 ± 0.03 of control; $n$ = 5; $p < 0.001$, $t$ = 29.80, degrees of freedom (df) = 4, paired $t$-test; Fig. 3.1B; nonnormalized, 8.1 ± 1.2 to 1.2 ± 0.4 Hz; $p < 0.001$, $t$ = 9.04, df = 4, paired $t$-test] and eIPSC amplitude (0.33 ± 0.11 of control; $n$ = 6; $p = 0.004$, $t$ = 6.26, df = 3, paired $t$-test; Fig. 3.1B; nonnormalized, 503 ± 106 to 144 ± 33 pA; $p = 0.03$, $t$ = 2.95, df = 3, paired $t$-test). Similarly, baclofen inhibited both mIPSC frequency (0.34 ± 0.07 of control; $n$ = 6; $p < 0.001$, $t$ = 9.68, df = 5, paired $t$-test; Fig. 3.1B; nonnormalized, 6.4 ± 2.0 to 2.3 ± 0.8 Hz; $p = 0.01$, $t$ = 3.09, df = 5, paired $t$-test) and eIPSC amplitude (0.33 ± 0.12 of control; $n$ = 6; $p < 0.001$, $t$ = 6.26, df = 5, paired $t$-test; Fig. 3.1, A and B; nonnormalized, 763 ± 87 to 239 ± 92 pA; $p = 0.005$, $t$ = 4.10, df = 5, paired $t$-test) in the presence of TEA.

Application of 4-AP (100 μM) increased the frequency of IPSCs (2.2 ± 0.4-fold increase; $n$ = 28; $p = 0.002$, $t$ = 3.20, df = 27, paired $t$-test; Fig. 3.1A) in a manner reversible by TTX (2.4 ± 0.6 in 4-AP to 0.85 ± 0.06 in TTX relative to baseline; $n$ = 18; $p = 0.002$, $F = 7.43$, df = 50, repeated-measures ANOVA; IPSC frequency in TTX was not significantly different from control using Tukey multiple-comparison test). In the absence of TTX, the frequency of IPSCs measured in the presence of 4-AP was decreased by both DAMGO (0.28 ± 0.06 of control; $n$ =

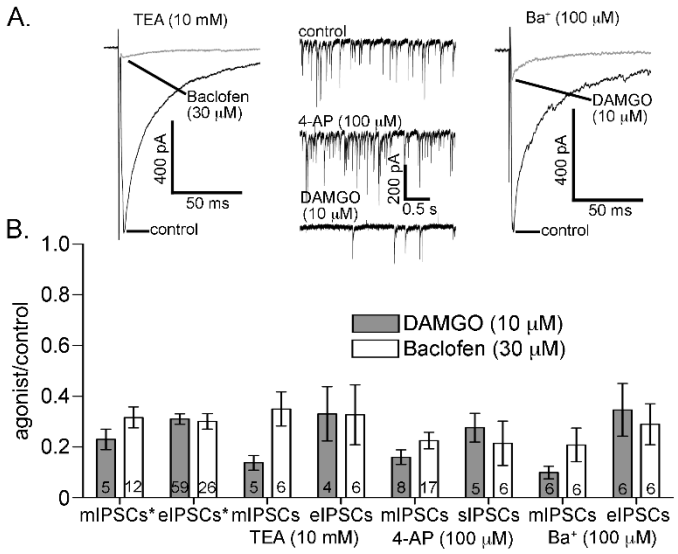


**Figure 3.1 MOR- and $GABA_BR$-mediated inhibition of release does not require the activation of $K^+$ channels. A)** Sample traces demonstrating either DAMGO- or baclofen-induced inhibition of eIPSC amplitude or spontaneous IPSC (sIPSC) frequency in the presence of the $K^+$-channel blockers TEA (10 mM), 4-AP (100 μM), or $Ba^+$ (100 μM). **B)** Compiled and normalized data demonstrating the magnitude of DAMGO- and baclofen-induced inhibition of mIPSC frequency and eIPSC amplitude in the presence of TEA, 4-AP, and $Ba^+$. The shown values for DAMGO- and baclofen-induced inhibition of mIPSC frequency and eIPSC amplitude in the absence of $K^+$-channel blockers (marked with an asterisk) were previously published in Pennock and Hentges (2011; DAMGO) and Pennock et al. (2012; baclofen). Numbers displayed within the bars of the graph represent the sample size for that experiment.

5; $p < 0.001$, $t = 12.75$, df = 4, paired *t*-test; Fig. 3.1, A and B; nonnormalized, 13.8 ± 6.5 to 3.0 ± 0.9 Hz; $p = 0.07$, $t = 1.89$, df = 4, paired *t*-test) and baclofen (0.21 ± 0.09 of control; $n = 6$; $p < 0.001$, $t = 8.98$, df = 5, paired *t*-test;Fig. 3.1B; nonnormalized, 16.6 ± 6.8 to 6.0 ± 4.8 Hz; $p = 0.003$, $t = 4.67$, df = 5, paired *t*-test). The frequency of mIPSCs measured in the presence of 4-AP and TTX was also inhibited by both DAMGO (0.16 ± 0.03 of control; $n = 8$; $p < 0.001$, $t = 28.96$, df = 7, paired *t*-test; Fig. 3.1B; nonnormalized, 14.9 ± 2.9 to 2.6 ± 0.7 Hz; $p < 0.001$, $t = 5.38$, df = 7, paired *t*-test) and baclofen (0.23 ± 0.03 of control; $n = 17$; $p < 0.001$, $t = 23.74$, df = 16, paired *t*-test; Fig. 3.1B; nonnormalized, 8.1 ± 1.6 to 2.2 ± 0.6 Hz; $p = 0.003$, $t = 5.58$, df = 16, paired *t*-test).

It has also been suggested that G-protein-coupled inwardly rectifying $K^+$ channels (GIRKs) may mediate inhibition of release by presynaptic inhibitory GPCRs (Ladera et al., 2008; Michaeli and Yaka, 2010). To determine whether GIRK activation plays a role in either MOR- or $GABA_BR$-mediated inhibition of GABA release onto POMC neurons, DAMGO- and baclofen-

induced inhibition of mIPSC frequency and eIPSC amplitude was measured in the presence of the GIRK blocker $Ba^+$ (100 μM). In the presence of $Ba^+$, DAMGO inhibited mIPSC frequency (0.09 ± 0.02 of control; $n = 6$; $p < 0.001$, $t = 36.67$, df = 5, paired *t*-test; Fig. 3.1B; nonnormalized, 7.9 ± 3.1 to 0.6 ± 0.3 Hz; $p = 0.025$, $t = 2.55$, df = 5, paired *t*-test) and eIPSC amplitude (0.35 ± 0.10 of control; $n = 6$; $p < 0.001$, $t = 6.30$, df = 5, paired *t*-test; Fig. 3.1, A and B; nonnormalized, 544 ± 100 to 198 ± 71 pA; $p = 0.005$, $t = 4.03$, df = 5, paired *t*-test). Similarly, baclofen-induced inhibition of mIPSC frequency still occurred in the presence of $Ba^+$ (0.21 ± 0.07 of control; $n = 6$; $p < 0.001$, $t = 11.94$, df = 5, paired *t*-test; Fig. 3.1B; nonnormalized, 7.1 ± 3.3 to 2.2 ± 1.4 Hz; $p = 0.014$, $t = 3.09$, df = 5, paired *t*-test), as did inhibition of eIPSC amplitude (0.29 ± 0.08 of control; $n = 6$; $p < 0.001$, $t = 8.79$, df = 5, paired *t*-test; Fig. 3.1B; nonnormalized, 513 ± 85 to 174 ± 64 pA; $p < 0.001$, $t = 7.51$, df = 5, paired *t*-test).

DAMGO- and baclofen-induced inhibition of mIPSC frequency and eIPSC amplitude measured from POMC neurons in the absence of $K^+$-channel blockers has been previously characterized (Pennock and Hentges, 2011; Pennock et al., 2012) (Fig. 3.1B). These previously published results were compared to DAMGO- and baclofen-induced inhibition of mIPSC frequency and eIPSC amplitude in the presence of TEA, 4-AP, and $Ba^+$ to determine whether the presence of $K^+$-channel blockers attenuates MOR- or GABABR-mediated inhibition of GABA release (Fig. 3.1B). The presence of $K^+$-channel blockers did not significantly affect the magnitude of DAMGO-induced inhibition of mIPSC frequency ($p = 0.06$, $F = 2.84$, df = 23, 1-way ANOVA) or eIPSC amplitude ($p = 0.90$, $F = 0.19$, df = 70, 1-way ANOVA) or baclofen-induced inhibition of mIPSC frequency ($p = 0.15$, $F = 1.91$, df = 40, 1-way ANOVA) or eIPSC amplitude ($p = 0.74$, $F = 0.42$, df = 43, 1-way ANOVA). Together, these results suggest that DAMGO- and baclofen-induced inhibition of GABA release onto POMC neurons does not rely on the activation of presynaptic voltage-dependent $K^+$ channels or GIRKs.

*Inhibition of $Ca^{2+}$ influx is not required for MOR- or $GABA_BR$-mediated inhibition of GABA release*

To determine whether MORs or $GABA_BRs$ inhibit GABA release onto POMC neurons by decreasing $Ca^{2+}$ influx into presynaptic terminals, recordings were made in $Ca^{2+}$-free external solution. Switching from a $Ca^{2+}$-containing to a $Ca^{2+}$-free external recording solution eliminated eIPSCs measured from POMC neurons (406 ± 80 to 2 ± 2 pA; $n = 3$; $p = 0.02$, $t = 4.88$, df = 2, paired *t*-test; data not shown), indicating that $Ca^{2+}$ was successfully removed from the extracellular space, and thus $Ca^{2+}$ influx was eliminated. Unlike eIPSC amplitude, mIPSC frequency was maintained in the absence of external $Ca^{2+}$ (8.77 ± 2.0 Hz in standard aCSF vs. 8.9 ± 2.0 Hz in $Ca^{2+}$-free aCSF; $n = 20$; $p = 0.65$, $t = 0.46$, df = 19, paired *t*-test; Fig. 3.2, A and E,top traces, and B and F). In the absence of external $Ca^{2+}$, robust inhibition of mIPSC frequency was induced by both DAMGO (6.0 ± 1.5 to 0.9 ± 0.2 Hz; $n = 10$; $p = 0.004$, $t = 3.80$, df = 9, paired *t*-test; Fig. 3.2, A–C) and baclofen (11.7 ± 3.0 to 4.6 ± 1.7 Hz; $n = 13$; $p < 0.001$, $t = 5.22$, df = 12, paired *t*-test; Fig. 3.2, E–G). Consistent with previous results (Pennock et al. 2012), DAMGO-induced inhibition of mIPSC frequency was resistant to acute desensitization ($p = 0.23$, $F = 1.24$, df = 153; $n = 7$; repeated-measures ANOVA; Fig. 3.2D). Also consistent with previous results, baclofen-induced inhibition of mIPSC frequency could be either resistant ($p = 0.05$, $F = 1.59$, df = 181; $n = 7$ of 10 recordings; repeated-measures ANOVA; Fig. 3.2D) or susceptible ($p < 0.001$, $F = 2.61$, df = 77; $n = 3$ of 10 recordings; repeated-measures ANOVA; Fig. 3.2H) to acute desensitization. The amount of desensitization that occurred in each recording is represented as a scatterplot in Fig. 3.2I. Altogether, the above results indicate that MOR and $GABA_BR$ can inhibit transmitter release by a mechanism other than preventing $Ca^{2+}$ influx into GABAergic terminals.

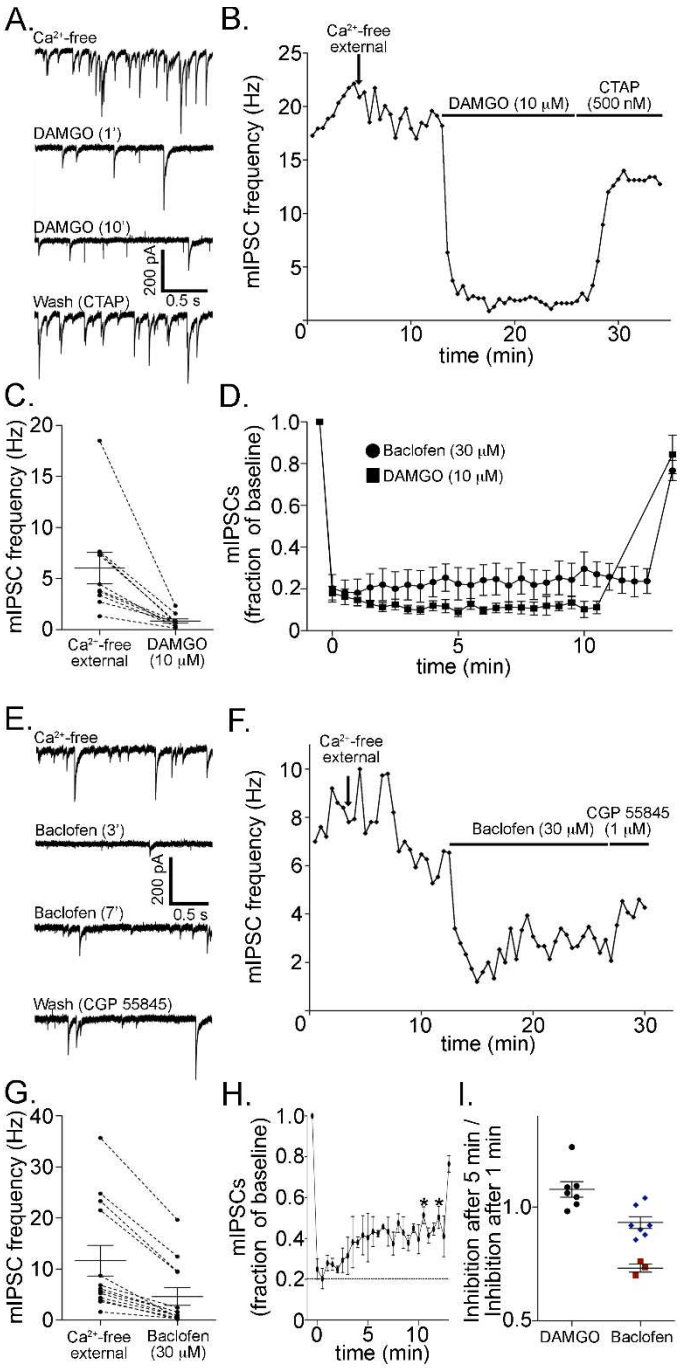


**Figure 3.2 MOR- and $GABA_BR$-mediated inhibition of mIPSC frequency in the absence of external $Ca^{2+}$. A)** Sample traces demonstrating DAMGO-induced reduction in mIPSC frequency measured from POMC neurons when recording in a $Ca^{2+}$-free external recording solution and reversal on application of the MOR-selective antagonist CTAP (500 nM). **B)** A plot of the recording from which the traces in **(A)** were taken. **C)** compiled data from the experiments represented in **(A)** and **(B)**. **D)** Compiled data from recordings in which DAMGO (■)- or baclofen (●)-induced inhibition of mIPSC frequency exhibited resistance to acute desensitization. The inhibition was reversed by the addition of antagonist. **E)** Sample traces demonstrating baclofen-induced reduction in mIPSC frequency measured from POMC neurons when recording in a $Ca^{2+}$-free recording solution and reversal with the application of the $GABA_BR$ antagonist CGP 55845. Acute desensitization of the baclofen-induced inhibition is shown in the trace taken from the 7th minute of the baclofen application. *(continued on next page)*

(Figure 3.2 continued) **F)** A plot of the recording from which the traces in **(E)** were taken. **G)** compiled data from the experiments represented in **(E)** and **(F)**. **H)** Compiled data from recordings in which baclofen-induced inhibition of mIPSC frequency exhibited acute desensitization. *I*: scatterplot demonstrating the inhibition of mIPSC frequency by DAMGO (●) or baclofen (♦ for nondesensitizing recordings; ■ for desensitizing recordings) after 5 min of continuous drug application divided by the inhibition after 1 min. Points marked with an asterisk were found to be significantly different from peak inhibition using Tukey multiple-comparison test.

*Unregulated $Ca^{2+}$ influx into GABAergic terminals presynaptic to POMC neurons does not occlude MOR- or $GABA_BR$-mediated inhibition of GABA release*

To explore further the possibility that inhibition of $Ca^{2+}$ influx into GABAergic terminals is not necessary for MOR- and $GABA_BR$-induced inhibition of release, agonist actions were examined under the condition of unregulated $Ca^{2+}$ influx. To achieve unregulated $Ca^{2+}$ influx into terminals presynaptic to POMC neurons, slices were exposed to the $Ca^{2+}$ ionophore ionomycin. A brief (2–5 min) application of ionomycin (1 μM) resulted in a robust increase in IPSC frequency measured from POMC neurons in the presence of TTX (11.59 ± 1.6 to 23.58 ± 2.0 Hz; $n = 26$; $p < 0.001$, $t = 7.99$, df = 25, paired t-test; average 2.8 ± 0.4-fold increase; Fig. 3.3, A and B). After reaching a peak, the ionomycin-induced increase in mIPSC frequency is not maintained over time (Fig. 3.3A). To lessen both the continued increase in mIPSC frequency that occurs in the high concentration of ionomycin and the decline in mIPSC frequency that occurs when application is stopped, a reduced concentration of ionomycin (100 nM) was continuously superfused over the slice after the initial brief, high-concentration ionomycin application (Fig. 3.4, A and E). Ionomycin had no effect in $Ca^{2+}$-free external recording solution (11.32 ± 4.5 to 10.0 ± 3.8 Hz; $n = 6$; $p = 0.12$, $t = 1.85$, df = 5, paired *t*-test; Fig. 3.3, C and D), confirming the $Ca^{2+}$-dependent nature of the ionomycin-induced increase in IPSC frequency.

Under this condition of unregulated $Ca^{2+}$ influx, a maximal concentration of DAMGO (10 μM) still inhibited IPSC frequency (21.6 ± 2.6 to 8.6 ± 1.7 Hz; $n = 13$; $p < 0.001$, $t = 7.15$, df = 12, paired *t*-test; Fig. 3.4, A–C). A concentration of DAMGO that produced half-maximal inhibition under control conditions (100 nM) produced similar inhibition of IPSC frequency in the presence of ionomycin (56 ± 11% of the inhibition produced by 10 μM DAMGO; Fig. 3.4D), implying that

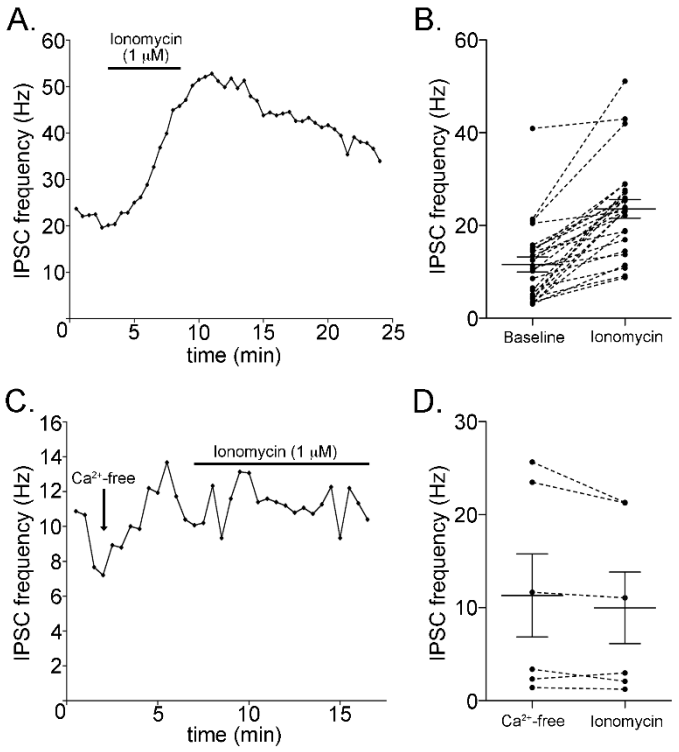


**Fig. 3.3 Ionomycin induces a $Ca^{2+}$-dependent increase in IPSC frequency measured from POMC neurons**. **A)** Plot of IPSC frequency measured from POMC neurons demonstrating an ionomycin-induced increase in IPSC frequency. **B)** Compiled data for ionomycin-induced increases in IPSC frequency measured from POMC neurons. **C)** Plot of IPSC frequency demonstrating a lack of an ionomycin-induced increase in IPSC frequency when recording in a $Ca^{2+}$-free external recording solution. **D)** Compiled data demonstrating the effect of ionomycin on IPSC frequency when recording in a $Ca^{2+}$-free external recording solution. All recordings were made in the presence of TTX (300 nM).

receptor function was not compromised during these experiments. A maximal concentration of baclofen (30 μM) also induced robust inhibition of IPSC frequency in the presence of ionomycin (24.0 ± 2.3 to 13.7 ± 1.8 Hz; $n = 13$; $p < 0.001$, $t = 7.74$, df = 12, paired $t$-test; Fig. 3.4, E–G). Baclofen (2 μM), which is near the $EC_{50}$ for baclofen under control conditions, produced an approximately half-maximal inhibition of IPSC frequency in the presence of ionomycin-induced $Ca^{2+}$ influx (51 ± 18% of the inhibition produced by 30 μM baclofen; Fig. 3.4H), demonstrating that $GABA_BR$ function is also not compromised by this experimental paradigm. The observation that MOR- and $GABA_BR$-induced inhibition of release is maintained when $Ca^{2+}$ influx cannot be prevented further indicates that these receptors can prevent transmitter release via a mechanism other than reduced $Ca^{2+}$ influx.

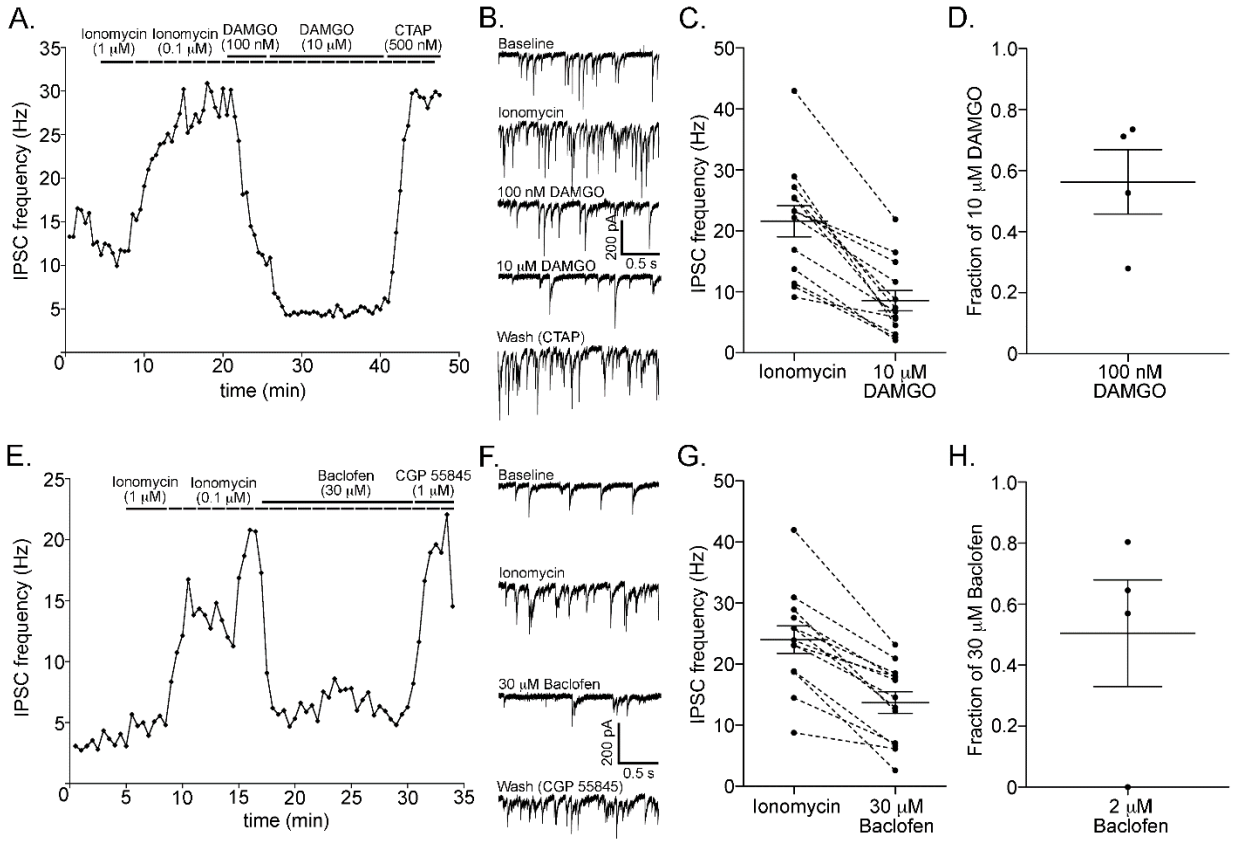


**Fig. 3.4 MOR- and $GABA_BR$-mediated inhibition of IPSC frequency is maintained in the presence of ionomycin-induced $Ca^{2+}$influx. A)** A plot of a recording demonstrating a stable ionomycin-induced increase in IPSC frequency measured from POMC neurons that was inhibited by a submaximal (100 nM) and maximal (10 μM) concentration of DAMGO. **B)** Sample traces taken from the recording plotted in *A*. **C)** Compiled data for DAMGO-induced inhibition of IPSC frequency in the presence of ionomycin. **D)** A submaximal concentration of DAMGO (100 nM) that is near the $EC_{50}$ of DAMGO still produces an approximately half-maximal inhibition in the presence of ionomycin. **E)** A plot of a recording demonstrating baclofen-induced inhibition of the ionomycin-induced increase in IPSC frequency. *F*: sample traces from the recording plotted in **(E)**. **G)** Compiled data for baclofen-induced inhibition of IPSC frequency in the presence of ionomycin. **H)** A concentration of baclofen (2 μM) that is approximately half-maximal under normal recording conditions is also approximately half-maximal in the presence of ionomycin. All recordings were made in the presence of TTX (300 nM).

*$Sr^{2+}$-evoked GABA release onto POMC neurons is inhibited by the MOR and $GABA_BR$*

Whereas the previous two experiments indicate that the MOR and $GABA_BR$ do not require inhibition of $Ca^{2+}$ influx to reduce transmitter release, the experimental paradigms used do not provide information about evoked, synchronous release. To determine whether inhibition of synchronous, $Ca^{2+}$-dependent release is dependent on the inhibition of $Ca^{2+}$ influx, $Ca^{2+}$ in the external recording solution was replaced with $Sr^{2+}$. Like $Ca^{2+}$, $Sr^{2+}$ influx into presynaptic terminals via voltage-dependent $Ca^{2+}$ channels results in the release of neurotransmitter from

vesicles into the synapse (Miledi, 1966; Dodge et al., 1969; Goda and Stevens, 1994; Ohno-Shosaku et al., 1994). However, unlike $Ca^{2+}$, $Sr^{2+}$ does not cause synaptotagmin to compete with Gβγ at the SNARE complex (Shin et al., 2003; Bhalla et al., 2005). Therefore, replacing $Ca^{2+}$ with $Sr^{2+}$ provides a means to determine whether GPCR-mediated inhibition of evoked release is due to reduced influx through $Ca^{2+}$ channels since competitive interactions at the synaptotagmin/SNARE complex are occluded when release is evoked with $Sr^{2+}$ (Hamid et al., 2014).

Switching from a $Ca^{2+}$-based to $Sr^{2+}$-based external recording solution while evoking GABA release from terminals presynaptic to POMC neurons decreased the amplitude of eIPSCs measured from POMC neurons (1.48 ± 0.27 to 0.37 ± 0.07 nA; $n = 7$; $p = 0.002$, paired *t*-test; Fig. 3.5, A and B) but caused an increase in the number of IPSCs measured in the 10 s following stimulation that was reversed when the stimulation ceased ($Ca^{2+}$ external, 9.7 ± 4.1 Hz; $Sr^{2+}$ external, 14.7 ± 6.4 Hz; $Sr^{2+}$ external with no stimulus, 10.8 ± 4.8 Hz; $n = 7$; $p = 0.05$, $F = 3.75$, $df = 20$, repeated-measures ANOVA; Fig. 3.5, A–D). $Sr^{2+}$ did not increase mIPSC frequency, as was indicated by recordings made in the presence of TTX (8.8 ± 1.3 to 8.4 ± 1.2 Hz; $n = 11$; $p = 0.38$, $t = 0.93$, $df = 10$, paired *t*-test; Fig. 3.5, E and F). These results confirm that the observed increase in transmitter release in the 10 s after simulation in the presence of $Sr^{2+}$ is due to action potential propagation and subsequent $Sr^{2+}$ influx. These findings are consistent with previous studies demonstrating decreased eIPSC amplitude and increased delayed release in the presence of $Sr^{2+}$ (Morishita and Alger, 1997; Behrends and ten Bruggencate, 1998; Rumpel and Behrends, 1999).

In the presence of a $Sr^{2+}$-based external recording solution, DAMGO inhibited the amplitude of eIPSCs measured from POMC neurons (213 ± 46 to 49 ± 13 pA; $n = 13$; $p = 0.002$, $t = 4.07$, $df = 12$, paired *t*-test; Fig. 3.6, A and D) and decreased the frequency of delayed IPSCs measured in the 10 s after an electrical stimulus (10.7 ± 2.1 to 1.6 ± 0.5 Hz; $n = 13$; $p <$

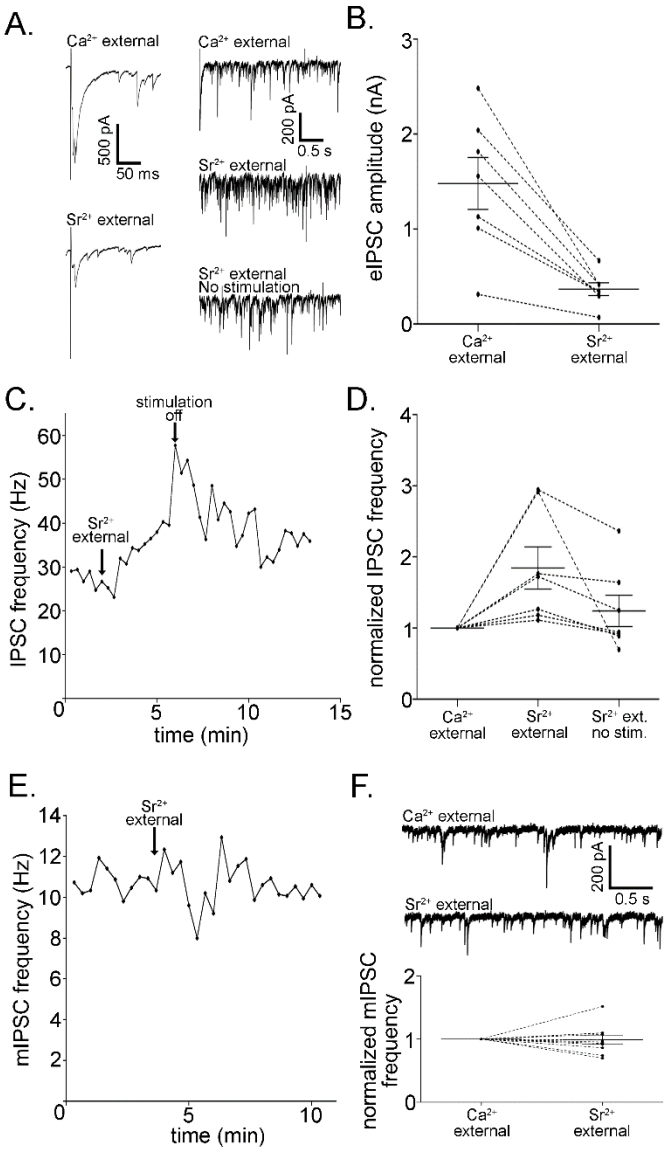


**Figure 3.5 Substituting $Sr^{2+}$ for $Ca^{2+}$ in the external recording solution results in a decrease in eIPSC amplitude and an action-potential-dependent increase in delayed IPSCs. A)** Sample traces demonstrating decreased eIPSC amplitude and increased IPSC frequency in the presence of $Sr^{2+}$. **B)** Compiled data showing a decrease in eIPSC amplitude in $Sr^{2+}$-based external recording solution. **C)** Sample plot of a recording demonstrating that switching from a $Ca^{2+}$-based to a $Sr^{2+}$-based external recording solution while evoking neurotransmitter release (single stimulus every 20 s) results in an increase in delayed IPSCs occurring after the stimulus that dissipates after the stimulus is no longer applied ($Sr^{2+}$ext. no stim.). **D)** Compiled data for the experiment represented in **(C)**. **E)** Sample plot of a recording made in the absence of electrical stimulation and in the presence of TTX (300 nM). Replacing $Ca^{2+}$ with $Sr^{2+}$ had no effect on IPSC frequency under these conditions. **F)** Sample traces and compiled data demonstrating a lack of $Sr^{2+}$-induced increase in IPSC frequency in the absence of electrical stimulation and in the presence of TTX.

0.001, *t* = 5.49, df = 12, paired *t*-test; Fig. 3.6, A–C). The DAMGO-induced inhibition of both the frequency of delayed IPSCs and the immediate evoked IPSCs was maintained during prolonged application (delayed IPSCs: *n* = 6; p = 0.44, F = 1.02, df = 119, repeated-measures ANOVA; eIPSCs: *n* = 6, p = 0.75, F = 0.76, df = 119, repeated-measures ANOVA; Fig. 3.6, E and F). Baclofen also inhibited eIPSC amplitude (198 ± 30 to 42 ± 9 pA; *n* = 31; p < 0.001, *t* = 6.88, df = 30, paired *t*-test; Fig. 3.6, G and J) and delayed IPSC frequency (19.7 ± 2.5 to 7.5 ± 1.8 Hz; *n* = 31; p < 0.001, *t* = 11.22, df = 30, paired *t*-test; Fig. 3.6, G–I) measured from POMC neurons while using a $Sr^{2+}$-based external solution. Consistent with previous results, the baclofen-induced inhibition of delayed IPSCs exhibited acute desensitization in a fraction of recordings (n = 5 of 25 recordings; P < 0.001, F = 9.79, df = 109, repeated-measures ANOVA; Fig. 3.6, K and L). However, baclofen-induced inhibition of eIPSCs in the same recordings did not exhibit statistically significant desensitization (n = 5; P = 0.11, F = 1.47, df = 109, repeated-measures ANOVA; Fig. 3.6, M and N), but this may be due to the high amount of variability in the amplitude of $Sr^{2+}$-evoked IPSCs. Recordings of baclofen-induced inhibition of delayed events that did not exhibit clear desensitization also showed a statistically significant change in inhibition over the course of a prolonged baclofen exposure (delayed IPSCs: *n* = 20; p < 0.001, F = 2.35, df = 459, repeated-measures ANOVA; Fig. 3.6, E and L). This may be the result of including recordings that were near, but did not meet, the criteria to be included in the group of recordings that were considered to have desensitized. Baclofen-induced inhibition of synchronous release from these same recordings exhibited no desensitization (eIPSCs: *n* = 20; p = 0.36, F = 1.09, df = 459, repeated-measures ANOVA; Fig. 3.6, F and N). Altogether, the recordings made in the presence of $Sr^{2+}$ suggest that both MOR and $GABA_BRs$ can reduce transmitter release by inhibiting $Ca^{2+}$ channels since $Sr^{2+}$ likely occludes direct actions at vesicular release machinery.

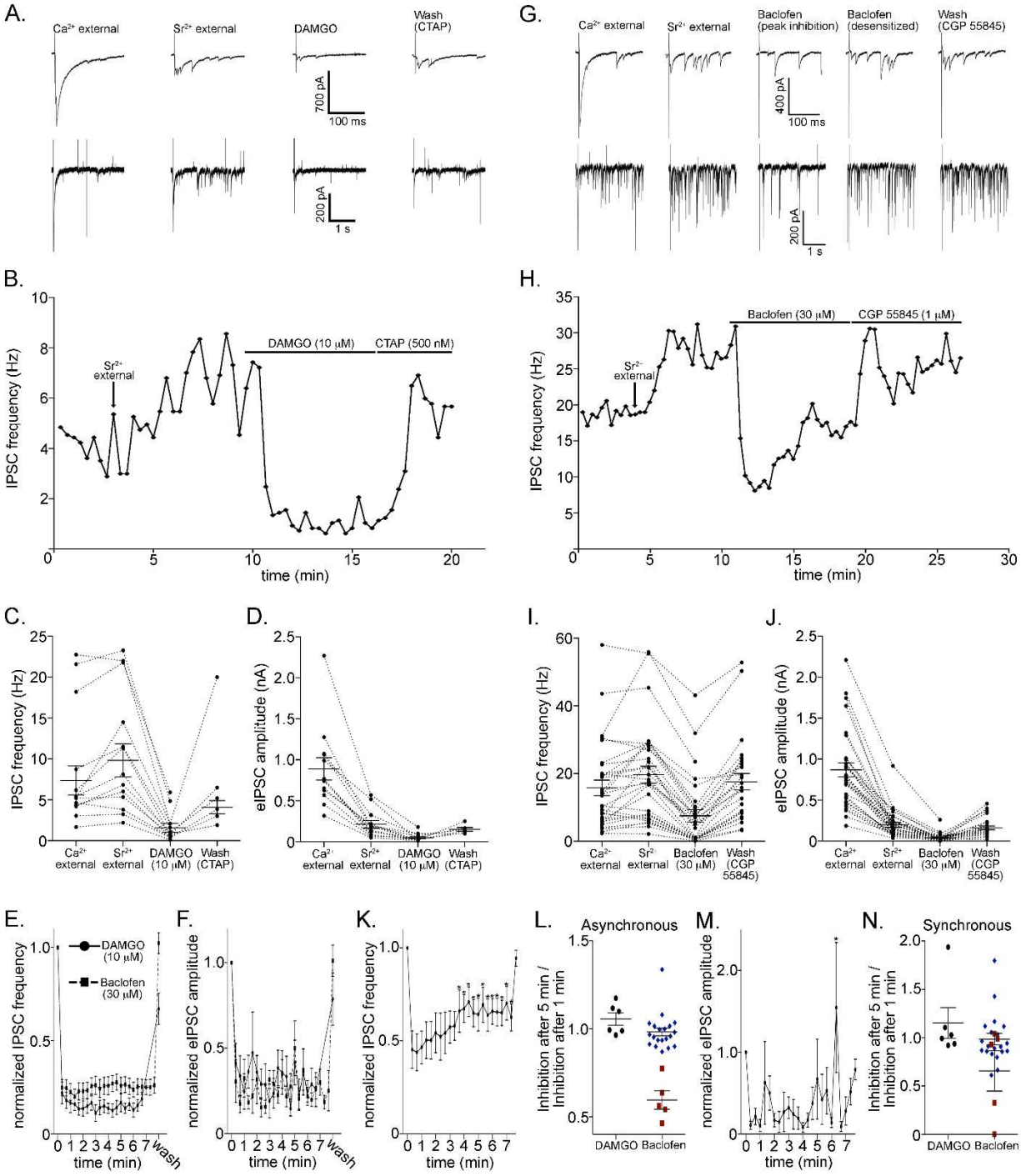


**Figure 3.6 $Sr^{2+}$-evoked IPSCs are inhibited by both MOR and $GABA_BR$ agonists. A)** Sample traces demonstrating decreased eIPSC amplitude and increased frequency of delayed IPSCs while evoking neurotransmitter release in the presence of $Sr^{2+}$-based external recording solution as well as subsequent inhibition of both immediate and delayed $Sr^{2+}$-evoked IPSCs by DAMGO. The MOR antagonist CTAP was used to reverse the DAMGO-induced inhibition. **B)** Plot of IPSC frequency over time taken from the same recording as the sample traces in **(A)**. **C,D)** Compiled data for the experiments represented in **(A)** and **(B)**. **E,F)** Compiled data demonstrating resistance to desensitization of DAMGO (●)- and baclofen (■)-induced (*continued on next page*)

(Figure 3.6 continued) inhibition of delayed IPSCs and eIPSCs recorded in the presence of $Sr^{2+}$. **G)** Sample traces demonstrating the decrease in eIPSC amplitude and IPSC frequency observed when substituting $Sr^{2+}$ for $Ca^{2+}$ in the external recording solution as well as inhibition of $Sr^{2+}$-evoked events by baclofen and subsequent desensitization of the baclofen-induced inhibition. CGP 55845 was used to reverse the baclofen-induced inhibition. **H)** Plot of IPSC frequency from the recording shown as sample traces in **(G)**. **I,J)** Compiled data for the experiments represented in **(G)** and **(H)**. **(K)** Compiled data for recordings in which baclofen-induced inhibition of the frequency of delayed IPSCs exhibited acute desensitization. **(L)** Scatterplot demonstrating the inhibition of IPSC frequency by DAMGO (●) or baclofen (⧫ for nondesensitizing recordings; ■ for desensitizing recordings) after 5 min of continuous drug application divided by the inhibition after 1 min. **(M)** Normalized eIPSC amplitudes from the same recordings used to produce the graph in **(K)**. **(N)** Scatterplot demonstrating the inhibition of eIPSC amplitude by DAMGO (●) or baclofen (⧫ for nondesensitizing recordings; ■ for desensitizing recordings) after 5 min of continuous drug application divided by the inhibition after 1 min. Recordings were placed in the same group (nondesensitizing or desensitizing) that they were placed into in **(L)**. Points marked with an asterisk were found to be significantly different from peak inhibition using Tukey multiple-comparison test.

*The MOR and $GABA_BR$ inhibit N- and P/Q-type voltage-dependent $Ca^{2+}$-channel-dependent evoked GABA release onto POMC neurons*

Inhibition of $Sr^{2+}$-evoked GABA release onto POMC neurons by the MOR and $GABA_BR$ implies that both receptors can inhibit release via the inhibition of $Ca^{2+}$ influx, likely through the inhibition of presynaptic voltage-dependent $Ca^{2+}$ channels (VDCCs). However, the receptors may differentially couple to different types of VDCCs within presynaptic terminals. To determine whether the MOR or $GABA_BR$ preferentially couple to different types of VDCCs, the inhibition of GABA release by each receptor was measured in the presence of a blocker of N- or P/Q-type VDCCs. If the MOR or $GABA_BR$ selectively couples to either N- or P/Q-type VDCCs, then we expect inhibition of GABA release by that receptor to be reduced in the presence of a blocker of the VDCC it couples to.

To ensure that $Ca^{2+}$ influx through N- and P/Q-type VDCCs is sufficient to account for evoked GABA release onto POMC neurons, eIPSCs were evoked using a train of 30 stimuli (each separated by 100 ms) in the absence of VDCC blockers, after application of the N-type VDCC blocker ω-conotoxin GVIA (5 μM), and after the application of the P/Q-type VDCC blocker ω-agatoxin IVA (100 nM) subsequent to ω-conotoxin GVIA application. IPSCs evoked using a train of stimuli had a peak current of 1,061 ± 171 pA after the first stimulus. Amplitudes of subsequent IPSCs in the train decreased ($\tau$ = 72 ms; 1-phase exponential decay) until

reaching a steady-state amplitude that was ~47% (503 ± 24 pA) of the first IPSC in the train (*n* = 5; Fig. 3.7, A and B). Application of the N-type VDCC blocker ω-conotoxin GVIA (5 μM) reduced the amplitude of all IPSCs evoked by the stimulus train [amplitude of 1st eIPSC = 605 ± 44 pA; plateau = 322 ± 19 pA; τ = 118 ms; 1-phase exponential decay; *n* = 5; p = 0.27, F = 1.38, df numerator (dfn) = 1, df denominator (dfd) = 8, 2-way repeated-measures ANOVA; Fig. 3.7, A and B]. Subsequent application of the P/Q-type VDCC blocker ω-agatoxin IVA (100 nM) further reduced the amplitude of IPSCs evoked by the stimulus train (amplitude of 1st eIPSC = 51 ± 13 pA; *n* = 5; p = 0.02, F = 8.34, dfn = 1, dfd = 8, 2-way repeated-measures ANOVA; Fig. 3.7, A and B). After blocking both N- and P/Q-type VDCCs, the amplitude of eIPSCs during the stimulus train no longer exhibited exponential decay (Fig. 3.7B) and had an average amplitude of 49 ± 3 pA (*n* = 150, average of all 30 eIPSC amplitudes during the stimulus train from 5 cells). To ensure that the relatively small currents being measured after applying both blockers were not simply due to measurement of a stimulus artifact, DAMGO or baclofen was applied to the slice after ω-conotoxin GVIA and ω-agatoxin IVA. The residual currents measured after applying both blockers were further inhibited by both DAMGO (16 ± 1 pA; *n* = 60; 2 cells; Fig. 3.7A) and baclofen (9 ± 1 pA; *n* = 60; 2 cells; data not shown), demonstrating that these remaining currents are unlikely to be due to a measurement of a stimulus artifact. It is unclear whether this residual current after applying both blockers is due to incomplete blockade of N- and P/Q-type channels or from $Ca^{2+}$ influx through a third type of VDCC. Because of the high frequency of spontaneous events measured from POMC neurons, it is also likely that some of the residual current measured is due to spontaneous events occurring near in time to stimuli applied during the trains.

Application of ω-agatoxin IVA (100 nM) alone significantly reduced the amplitude of IPSCs evoked during a 30-stimulus train (amplitude of 1st IPSC: 688 ± 90 to 372 ± 47 pA; plateau: 297 ± 10 to 165 ± 5 pA, 1-phase exponential decay; *n* = 14; p = 0.009, F = 8.01, dfn = 1, dfd = 26, 2-way repeated-measures ANOVA; Fig. 3.8, A–C). IPSCs evoked by the train were

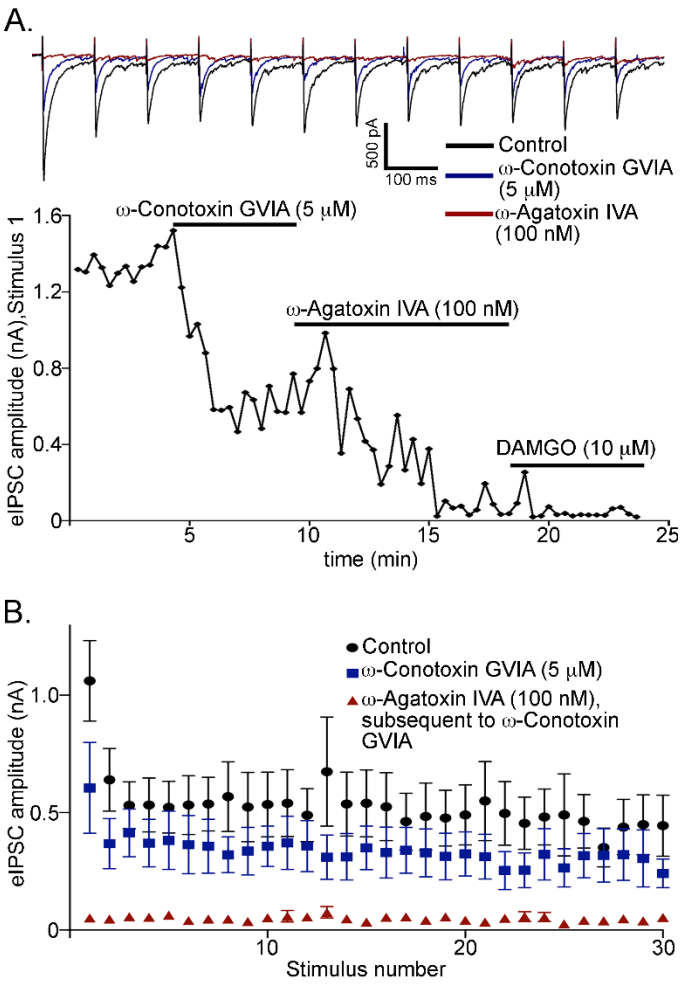


**Figure 3.7 Evoked GABA release onto POMC neurons is mediated by presynaptic N- and P/Q-type voltage-dependent $Ca^{2+}$channels. (A)** Sample traces and plot demonstrating the inhibition of IPSCs evoked using a train of stimuli (100 ms between stimuli) by application of the N-type voltage-dependent $Ca^{2+}$-channel (VDCC) blocker ω-conotoxin GVIA (5 μM) followed by application of the P/Q-type VDCC blocker ω-agatoxin IVA (100 nM). The sample traces show the 1st 12 eIPSCs evoked by a 30-stimulus train and are the average of 6 sweeps each recorded 20 s apart. The sample plot demonstrates the amplitude of the eIPSC evoked by the 1st stimulus of the train for each sweep taken during the recording. **(B)** Compiled data representing the average amplitude of eIPSCs evoked using a train of 30 stimuli before and after the application of the VDCC blockers ω-conotoxin GVIA and ω-agatoxin IVA.

further inhibited by subsequent application of either the MOR agonist DAMGO ($n$ = 6; p = 0.03, F = 6.89, dfn = 1, dfd = 10, 2-way repeated-measures ANOVA; Fig. 3.8B) or the $GABA_BR$ agonist baclofen ($n$ = 8; p < 0.001, F = 27.83, dfn = 1, dfd = 14, 2-way repeated-measures ANOVA; Fig. 3.8, A and C), indicating that neither the MOR nor $GABA_BR$ inhibits evoked GABA release onto POMC neurons by selective inhibition of P/Q-type VDCCs. Similarly, application of ω-conotoxin GVIA (5 μM) reduced the amplitude of IPSCs evoked during the stimulus train

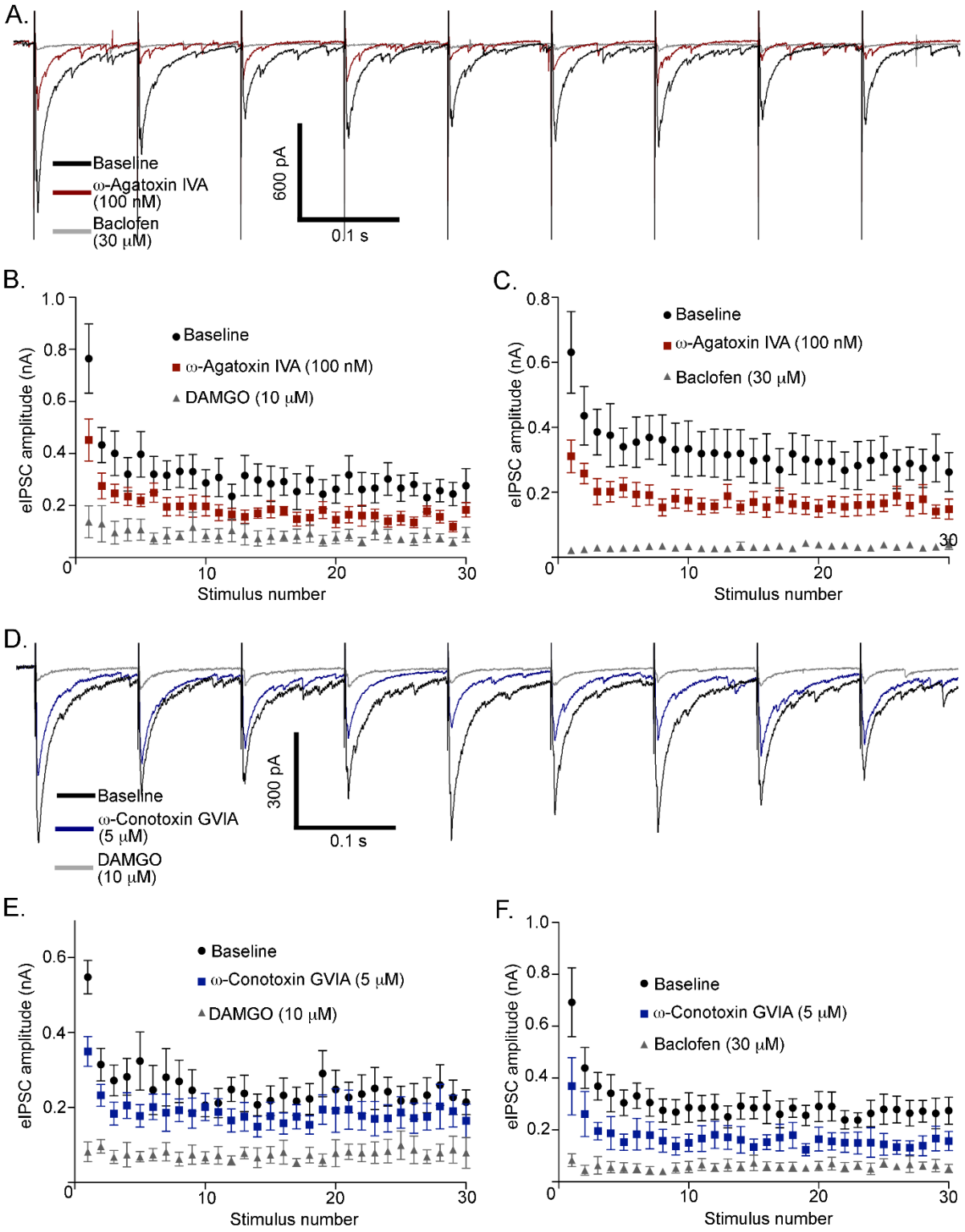


**Figure 3.8 The MOR and $GABA_BR$ mediate inhibition of N- and P/Q-type VDCC-dependent GABA release onto POMC neurons. (A)** Sample traces showing the 1st 9 eIPSCs evoked by a 30-stimulus train (100 ms between stimuli) before and after application of the P/Q-type voltage-dependent $Ca^{2+}$-channel (VDCC) blocker ω-agatoxin IVA (100 nM) as well as inhibition of the remaining current by the $GABA_BR$ agonist baclofen (30 μM). Sample traces are the average (*continued on next page*)

(*Figure 3.8 continued*) of 6 sweeps each recorded 20 s apart. **B,C)** Compiled data demonstrating the inhibition of trains of 30 eIPSCs by the P/Q-type VDCC blocker ω-agatoxin IVA and subsequent application of DAMGO or baclofen, respectively. **(D)** Sample traces showing the 1st 9 eIPSCs evoked by a 30-stimulus train (100 ms between stimuli) before and after application of the N-type VDCC blocker ω-conotoxin GVIA (5 μM) as well as inhibition of the remaining current by the MOR agonist DAMGO (10 μM). **E,F)** Compiled data demonstrating inhibition of trains of 30 eIPSCs by the N-type VDCC blocker ω-conotoxin GVIA and subsequent application of DAMGO or baclofen, respectively.

(amplitude of 1st IPSC: 743 ± 84 to 425 ± 71 pA; plateau: 325 ± 10 to 207 ± 8 pA, 1-phase exponential decay; $n$ = 19; p = 0.04, F = 4.66, dfn = 1, dfd = 36, 2-way repeated-measures ANOVA; Fig. 3.8, D–F). Again, IPSCs evoked by the train of stimuli after blocker application were further inhibited by subsequent application of either DAMGO ($n$ = 6; p = 0.02, F = 7.68, dfn = 1, dfd = 10, 2-way repeated-measures ANOVA; Fig. 3.8, D and E) or baclofen ($n$ = 8; p = 0.04, F = 5.41, dfn = 1, dfd = 14, 2-way repeated-measures ANOVA; Fig. 3.8F), indicating that neither the MOR nor $GABA_BR$ inhibits GABA release onto POMC neurons by selectively inhibiting N-type VDCCs. Together, the finding that DAMGO and baclofen induce robust inhibition of GABA release when either N- or P/Q-type VDCCs are blocked indicates that neither receptor couples selectively to one type of VDCC in presynaptic terminals.

### 3.6 Discussion

The present study was designed to determine the coupling of MORs and $GABA_BRs$ in terminals presynaptic to POMC neurons to address whether differential coupling may underlie sensitivity or resistance to desensitization of the inhibition of transmitter release. Both MOR and $GABA_BR$ agonists inhibited GABA release onto POMC neurons in the absence of external $Ca^{2+}$ as well as in the presence of ionomycin-induced $Ca^{2+}$ influx into terminals, suggesting a $Ca^{2+}$-independent mechanism of MOR- and $GABA_BR$-mediated inhibition of release. MOR- and $GABA_BR$-mediated inhibition of GABA release was also maintained when $Ca^{2+}$ in the external recording solution was replaced with $Sr^{2+}$, which is consistent with a $Ca^{2+}$-dependent mechanism of inhibition. Both receptors inhibited GABA release evoked by trains of stimuli in a manner that is consistent with inhibition of release via inhibition of voltage-dependent $Ca^{2+}$ channels. Together, these data demonstrate that MOR- and $GABA_BR$-mediated inhibition

of GABA release onto POMC neurons likely occurs through both $Ca^{2+}$-independent and -dependent mechanisms.

GPCR-mediated activation of voltage-dependent $K^+$ channels (VDKCs) (Vaughan et al., 1997; Zhu and Pan, 2005) and activation of G-protein-coupled inwardly rectifying $K^+$ channels (GIRKs) on presynaptic terminals have been described as a mechanism by which inhibition of release can occur (Ladera et al., 2008; Michaeli and Yaka, 2010). However, neither the VDKC blockers 4-AP or TEA nor the GIRK blocker $Ba^+$ had any effect on MOR- or $GABA_BR$-mediated inhibition of GABA release from terminals presynaptic to POMC neurons (Fig. 3.1). Thus it is unlikely that MORs or $GABA_BRs$ rely on the activation of $K^+$ channels to inhibit release from these terminals.

Both MORs and $GABA_BRs$ inhibit evoked and spontaneous GABA release from terminals presynaptic to POMC neurons (Pennock et al., 2012), despite the fact that spontaneous GABA release from these terminals occurs in a $Ca^{2+}$-independent fashion, whereas evoked release does require $Ca^{2+}$. This alone may have hinted at the dual mechanisms of inhibition of release by MORs and $GABA_BRs$. For example, both $GABA_BRs$ and adenosine $A_1$ receptors inhibit glutamate release evoked from granule cells to Purkinje cells in the rat cerebellum through modulation of voltage-dependent $Ca^{2+}$ channels. However, only $GABA_BRs$ inhibit mIPSCs. This suggests that $GABA_BRs$ in that particular synapse also inhibit release through a second mechanism that occurs downstream of $Ca^{2+}$ entry (Dittman and Regehr, 1996). Similar to $GABA_BRs$ that modulate glutamate release in the granule cell to Purkinje cell synapse in the cerebellum, MORs and $GABA_BRs$ presynaptic to POMC neurons may utilize two distinct pathways to inhibit evoked and miniature GABA release onto POMC neurons.

Previous studies have demonstrated that the majority of spontaneous IPSCs measured from POMC neurons are unaffected by application of TTX (Pinto et al., 2004; Hentges et al., 2005; Pennock and Hentges, 2011). Furthermore, in the present study, it is shown that mIPSCs

are largely unaffected when $Ca^{2+}$ is removed from the external recording solution (Fig. 3.2). Together, these findings suggest that spontaneous GABA release onto POMC neurons occurs in a manner that is largely independent of $Ca^{2+}$ influx. Therefore, inhibition of this $Ca^{2+}$-independent release induced by DAMGO and baclofen (Fig. 3.2) cannot be due to inhibition of $Ca^{2+}$ influx and may occur through a mechanism that is not dependent on modulation of presynaptic $Ca^{2+}$.

Unregulated $Ca^{2+}$ influx induced at terminals presynaptic to POMC neurons by the $Ca^{2+}$ ionophore ionomycin also failed to occlude either MOR- or $GABA_BR$-mediated inhibition of GABA release onto POMC neurons (Fig. 3.4). This finding suggests that not only is inhibition of $Ca^{2+}$ influx not required to inhibit GABA release, but also that regulation of internal $Ca^{2+}$ concentrations is unlikely to be important in inhibiting nonevoked release from these terminals. It is worth noting that the magnitude of the inhibition of IPSC frequency by DAMGO or baclofen in the presence of ionomycin is decreased compared to experiments made under normal recording conditions [~50% of baseline in the present study vs. 70–80% of baseline (Pennock and Hentges, 2011; Pennock et al., 2012)]. Previous experiments have shown that there is a small receptor reserve for MORs presynaptic to POMC neurons (Pennock et al., 2012). If $GABA_BRs$ on the same terminals have a similarly small receptor reserve, one explanation for the reduced magnitude of inhibition is simply that the terminals contain an insufficient amount of receptors to inhibit the increased release induced by ionomycin. However, the extent of receptor reserve for $GABA_BRs$ on terminals presynaptic to POMC neurons is unknown.

Another potential explanation for the reduced magnitude of MOR- and $GABA_BR$-mediated inhibition of GABA release is that only vesicles that would normally be involved in $Ca^{2+}$-independent release are being inhibited. There is strong evidence that evoked and spontaneous release arise from distinct pools of vesicles in central mammalian GABA (Mathew and Hablitz, 2008; Chung et al., 2010) and glutamate (Sara et al., 2005; Atasoy et al., 2008;

Sara et al., 2011) terminals as well as in the cholinergic terminals of the *Drosophila* neuromuscular junction (Koenig and Ikeda, 1999; Melom et al., 2013). If this is the case in GABAergic terminals presynaptic to POMC neurons, the MOR and $GABA_BR$ may use a $Ca^{2+}$-dependent mechanism to inhibit $Ca^{2+}$-dependent release while inhibiting $Ca^{2+}$-independent release through a $Ca^{2+}$-independent mechanism. Ionomycin-induced $Ca^{2+}$ influx would then only occlude inhibition of release occurring at sites that are normally involved in evoked GABA release, whereas $Ca^{2+}$-independent inhibition of sites involved in spontaneous release would still occur. Distinct release sites for spontaneous and evoked GABA release onto POMC neurons may also explain why δ-opioid receptors presynaptic to POMC neurons inhibit spontaneous, but not evoked, release (Pennock and Hentges, 2011). This has also been suggested as an explanation as to how group II metabotropic glutamate receptors selectively inhibit spontaneous GABA release at the interneuron-Purkinje cell synapse in the rat cerebellum (Glitsch, 2006). However, this explanation is only relevant in GABAergic terminals presynaptic to POMC neurons if MOR- and $GABA_BR$-mediated inhibition of evoked release occurs in a $Ca^{2+}$-dependent manner as was shown in the present study.

To determine whether MOR- and $GABA_BR$-mediated inhibition of evoked release occurs through a $Ca^{2+}$-dependent mechanism, inhibition induced by both receptors was measured after $Ca^{2+}$ was replaced with $Sr^{2+}$ in the external recording solution. Voltage-dependent $Ca^{2+}$ channels have a high conductance for $Sr^{2+}$ (Xu-Friedman and Regehr, 1999; Babai et al., 2014), and $Sr^{2+}$ influx results in neurotransmitter release by binding and activating $Ca^{2+}$ sensors on the vesicular release machinery (Babai et al., 2014). $Sr^{2+}$-evoked release has also been shown to occlude $Ca^{2+}$-independent inhibition of evoked glutamate release by serotonin $5\text{-}HT_{1B}$ receptors ($5\text{-}HT_{1B}$Rs) in CA1-subiculum synapses in the rat hippocampus (Hamid et al., 2014). $5\text{-}HT_{1B}$Rs inhibit release via a competitive interaction between Gβγ and $Ca^{2+}$-synaptotagmin at the SNARE complex (Blackmer et al., 2001; Blackmer et al., 2005; Gerachshenko et al., 2005; Yoon et al., 2007; Wells et al., 2012; Hamid et al., 2014). However, this competitive interaction may be

absent between Gβγ and $Sr^{2+}$-synaptotagmin (Hamid et al., 2014). If MOR- or $GABA_BR$-mediated inhibition of evoked GABA release onto POMC neurons occurs only through direct actions on release machinery, then there should be no effect of agonists on release that is evoked with $Sr^{2+}$ in lieu of $Ca^{2+}$. However, the present results show that both MOR- and $GABA_BR$-mediated inhibition of evoked GABA release was maintained when release was evoked using $Sr^{2+}$ (Fig. 3.6), indicating that direct actions on the release machinery are not necessary.

$Sr^{2+}$-evoked GABA release onto POMC neurons was characterized by a decrease in the amplitude of the eIPSC measured immediately after electrical stimulation as well as an increase in delayed, asynchronous IPSCs following stimulation (Fig. 3.5). This is consistent with previous studies that characterized $Sr^{2+}$-evoked release in glutamatergic (Goda and Stevens, 1994; AbdulGhani et al., 1996; Xu-Friedman and Regehr, 1999) and GABAergic (Morishita and Alger, 1997; Behrends and ten Bruggencate, 1998; Rumpel and Behrends, 1999) synapses. Both the initial synchronous eIPSC as well as delayed IPSCs occurring in the 10 s after an electrical stimulus were robustly inhibited by DAMGO and baclofen. Because of the high basal frequency of spontaneous IPSCs measured from POMC neurons before the $Sr^{2+}$-evoked increase in IPSC frequency, it is not possible to discern which IPSCs are actually the result of increased $Sr^{2+}$ in the presynaptic terminal. However, the magnitude of DAMGO- and baclofen-induced inhibition was often great enough that it could not be accounted for simply by assuming that all inhibition was occurring at the sites responsible for basal spontaneous release. These data suggest that both the MOR and $GABA_BR$ are preventing the influx of $Sr^{2+}$ into presynaptic terminals, which most likely occurs through inhibition of voltage-dependent $Ca^{2+}$ channels. A second possible explanation is that inhibition of $Sr^{2+}$-evoked release is occurring through a $Ca^{2+}$-independent mechanism that is distinct from the one previously reported for $5\text{-}HT_{1B}Rs$ and that is not occluded by $Sr^{2+}$-synaptotagmin.

When release was evoked using $Sr^{2+}$, desensitization of baclofen-induced inhibition of asynchronous GABA release occurring in the 10 s following stimulation was robust in 5/25 recordings (Fig. 3.6, *K* and *L*). However, baclofen-induced inhibition of synchronous GABA release in these same recordings did not demonstrate clear desensitization in all cases (Fig. 3.6, *M* and *N*). Although baclofen-induced inhibition of GABA release is sufficient to account for both spontaneous IPSCs and $Sr^{2+}$-evoked asynchronous IPSCs, we cannot determine whether desensitization of that inhibition is occurring in synapses that are being stimulated electrically. The origins of inputs that are electrically stimulated in this study are unknown and likely have varying degrees of overlap with the inputs responsible for the spontaneous events measured from POMC neurons. This might explain why a given recording may exhibit desensitization of the inhibition of IPSC frequency but not exhibit desensitization of the inhibition of eIPSC amplitude. This could also occur in the reverse direction where inhibition of $Sr^{2+}$-evoked IPSC amplitude desensitizes but desensitization of the inhibition of IPSC frequency is not detected.

Although experiments using $Sr^{2+}$ to evoke GABA release suggest that both the MOR and $GABA_BR$ inhibit $Ca^{2+}$ influx into terminals, it is possible that the receptors couple to different types of voltage-dependent $Ca^{2+}$ channels found within presynaptic terminals. N- and P/Q-type, along with R-type, VDCCs are often responsible for providing the $Ca^{2+}$ influx needed for synchronous neurotransmitter release (Meir et al., 1999), and all can be modulated by G proteins (Dolphin, 2003). Applying selective blockers for N- and P/Q-type VDCCs inhibited the amplitude of IPSCs evoked by a train of 30 stimuli by slightly more than 95% (Fig. 3.7), indicating that the vast majority of $Ca^{2+}$ influx needed to evoke GABA release onto POMC neurons occurs through these types of presynaptic VDCCs. Both DAMGO and baclofen robustly inhibited eIPSC amplitude when either N-type or P/Q-type VDCCs were blocked pharmacologically (Fig. 3.8). This indicates that although both receptors are likely inhibiting $Ca^{2+}$ influx into presynaptic terminals, neither the MOR nor $GABA_BR$ is selectively inhibiting one type of VDCC or the other.

Inhibition of release by inhibitory GPCRs located on presynaptic terminals has frequently been reported to exhibit resistance to acute desensitization (Blanchet and Luscher, 2002; Wetherington and Lambert, 2002a, b; Cruz et al., 2004; Fyfe et al., 2010; Pennock et al., 2012). The $GABA_BR$ has proven to be an exception to this in some brain regions (Tosetti et al., 2004; Pennock et al., 2012). A possible explanation for the desensitization of $GABA_BR$-mediated inhibition of release is that the receptor is differentially coupled from receptors that resist desensitization. If this is the case, it may be that inactivation of the downstream effector, and not desensitization of the $GABA_BR$ itself, is responsible for the observed loss in $GABA_BR$-mediated inhibition during a sustained exposure to agonist. However, both MOR- and $GABA_BR$-mediated inhibition was sustained under all conditions tested in the present study, and desensitization of $GABA_BR$-mediated inhibition was observed when measuring both $Ca^{2+}$-dependent and -independent release. Together, these findings provide further evidence that resistance or susceptibility to desensitization of inhibition of release by presynaptic inhibitory GPCRs occurs upstream of effectors either at the receptors themselves or possibly the G proteins they couple to.

MORs and $GABA_BRs$ located presynaptic to POMC neurons were able to inhibit GABA release in the absence of external $Ca^{2+}$ as well as in the presence of unregulated $Ca^{2+}$ influx, suggesting a $Ca^{2+}$-independent mechanism of inhibition. However, activation of both receptors also resulted in inhibition of GABA release evoked with $Sr^{2+}$, which is known to disrupt a mechanism of $Ca^{2+}$-independent inhibition of release (Hamid et al., 2014). This suggests that both receptors use $Ca^{2+}$-independent and -dependent mechanisms to inhibit GABA release onto POMC neurons. Strong coupling to two separate effector systems may explain how both receptors inhibit both $Ca^{2+}$-independent spontaneous release as well as $Ca^{2+}$-dependent evoked release. However, differential effector coupling cannot explain differential desensitization of the inhibition of release by presynaptic MORs and $GABA_BRs$ since both receptors appear to be coupled to similar downstream effectors.

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## Chapter 4: Single Particle Tracking of the Mu Opioid Receptor in AtT20 Cells

### 4.1 Overview

The previous two chapters established that desensitization-resistant MORs are regulated in a manner that diverges from that of postsynaptic MORs, and that resistance to desensitization cannot be accounted for by particular receptor-effector coupling. While these experiments provide useful information on the physiology of presynaptic MORs, they ultimately served to rule out possible explanations for resistance to desensitization. It is likely that resistance to desensitization by presynaptic MORs is conferred by some basic physical property of the receptors that differs from postsynaptic receptors. Whether this is due to differential covalent modification of pre- and postsynaptic MORs, or due to the presence of protein-protein interaction between the receptor and some other molecule, is unknown. However, whatever the mechanism underlying resistance to desensitization might be, it seems clear that standard electrophysiological and pharmacological techniques alone are insufficient to determine its nature.

If differential covalent modification or protein-protein interactions are responsible for stabilizing presynaptic MORs in a way that prevents desensitization it is reasonable to expect that the mobility of presynaptic MORs in the plasma membrane would be altered relative to postsynaptic receptors. Measuring the mobility of pre- and postsynaptic MORs could determine whether acute physical differences exist between these two populations of receptors. However, there is not currently an established technique used to make such measurements for the MOR. In this chapter, a cell line (AtT20 cells) expressing MORs that are modified to allow attachment of a fluorophore are used to establish the validity of single-particle tracking (SPT) as a measure of agonist-induced changes in MOR mobility. This particular cell line was chosen due to its frequent use in studies of MOR pharmacology and physiology, and because MORs expressed

in AtT20 cells have receptor-effector coupling that is similar to MORs located on the somato-dendritic region of neurons.

MOR mobility was measured under basal conditions (no MOR agonist present) or after ten minutes of exposure to the MOR agonist DAMGO. We found that under basal conditions MORs were split into a mobile and an immobile population, and that DAMGO exposure results in a higher fraction of receptors on the plasma membrane being found in the immobile state. These findings demonstrate that SPT is a viable method for measuring agonist-induced changes in the diffusion of MORs within the plasma membrane. The establishment of this technique will allow for future studies focusing on differential mobility of MORs in located in different neuronal compartments, which will in turn provide insight into the physical differences that may exist between these receptors. These experiments have also paved the way for future studies to determine the physical basis of the mobile and immobile population of MORs. It is possible that differential mobility of MORs on the plasma membrane is related to differential recruitment of downstream signaling cascades.

The work presented in the following chapter is currently being prepared for publication as a manuscript. I designed and carried out the experiments described in this chapter, and received critical assistance from Diego Krapf in the analysis and interpretation of the data. I drafted the chapter in its current form with input from Diego Krapf and Shane T. Hentges.

**4.2 Summary**

Mu opioid receptor (MOR) function, and that of other G-protein coupled receptors, is commonly studied via the activation of a particular downstream effector of the receptor, e.g. activation of an ion channel conductance or inhibition of cAMP production. Measuring agonist-induced changes in MOR mobility using single-particle tracking can provide an alternative measure for agonist-induced changes of the MOR that possesses high temporal and spatial resolution, and does not rely on measuring outputs provided by effectors as a proxy for receptor activation. In the present study, single-particle tracking of quantum dot labeled FLAG-tagged

MORs was carried out in AtT20 cells. Under basal conditions, FLAG-MORs were found to exist in mobile and immobile states. After the cells were treated with the MOR agonist DAMGO both mobile and immobile receptor populations were still present, albeit with a significantly high fraction of immobile trajectories. These data demonstrate that activation of FLAG-MORs with DAMGO results in robust changes in mobility of the receptor. While the physical basis of the mobile and immobile population of receptors is still unknown, we speculate that the mobile and immobile populations of FLAG-MORs recruit distinct downstream signaling pathways. The characterization of agonist-induced changes in FLAG-MOR mobility carried out in the present study provides a framework for future studies of MOR signaling using single-molecule imaging techniques.

**4.3 Introduction**

The mu opioid receptor (MOR) is responsible for the analgesic effects of exogenously administered opioids (e.g. morphine, codeine, fentanyl), as well as the actions of several endogenous opioids (Williams et al., 2001). Activation of the MOR results in the inhibition of adenylyl cyclase, inhibition of voltage-dependent $Ca^{2+}$-channels (VDCCs), activation of G-protein coupled inwardly rectifying $K^+$ channels (GIRKs), and the activation of several downstream signaling cascades that alter cellular function through transcriptional regulation (Williams et al., 2001; Williams et al., 2013). Most functional studies of the MOR have focused on the output produced by the inhibition or activation of these effectors.

Measuring the outputs provided by downstream effectors of the MOR (e.g. VDCCs, GIRKs, adenylyl cyclase) has provided a useful proxy for MOR activity. For example, electrophysiological studies measuring MOR-mediated activation or inhibition of ion channels provide a real-time, ensemble measure of MOR activation and desensitization in a given cell (Dang and Christie, 2012). Electrophysiological experiments can also determine the functional consequences of MOR activation on the cellular or circuit level via measurements of actions potential firing.

Biochemical and fluorescence imaging studies have provided evidence that compartmentalization of the receptor in the plasma membrane is likely critical to the functional output of the receptor. MORs exist in cholesterol rich domains on the plasma membrane, and colocalize with putative markers of signaling microdomains (Zhao et al., 2006; Levitt et al., 2009). Depletion of cholesterol also results in attenuation of both acute (Gaibelet et al., 2008; Levitt et al., 2009) and prolonged MOR signaling (Zhao et al., 2006; Levitt et al., 2009). Additionally, activation of the MOR is also known to result in a redistribution of the receptors within the plasma membrane, and this redistribution of receptors is likely critical to their function (Gaibelet et al., 2008; Zheng et al., 2008; Halls et al., 2016).

Studies of the MOR mobility in live cells using fluorescence recovery after photobleaching (FRAP) have shown that MORs exist in mobile and immobile MORs exist on the plasma membrane of SH-SY5Y cells (Sauliere et al., 2006), and that activation of the receptors alter their mobility (Sauliere-Nzeh et al., 2010). Specifically, application of the full MOR agonist DAMGO resulted in approximately half of the receptors becoming confined in relatively small domains and half becoming freely diffusible, while application of the partial agonist morphine resulted in a uniform reduction in MOR mobility (Sauliere-Nzeh et al., 2010). These studies provide compelling evidence that the compartmentalization and redistribution of receptors observed in biochemical studies of the MOR correspond to alterations in mobility of the receptor that are detectable using a real-time, ensemble measure of MOR diffusion.

Single-particle tracking of MORs expressed in normal rat kidney (NRK) cells has also been performed, albeit with conflicting results. One study reported that most receptors undergo rapid diffusion within microdomains that themselves diffuse, while a smaller fraction of receptors under slow, directed diffusion (Daumas et al., 2003). A second study reported rapid “hop” diffusion of the receptor between double nested compartments on the NRK cell membrane (Suzuki et al., 2005). However, neither study explored agonist-induced changes in diffusion of the receptor using this measure.

Using single-particle tracking to study agonist-induced changes in MOR mobility will be a useful complement to previous studies in several ways. Single-particle tracking will allow for the detection of subpopulations of MORs based on their mobility, thus unmasking heterogeneity within the total population of MORs that is difficult to detect with ensemble measurements. It can also potentially provide a bridge between real-time electrophysiological experiments and biochemistry, which provides a static picture of a receptor's interactions at a given time point. Extensive work has been performed characterizing the protein-protein and lipid-protein interactions that are critical to MOR activation and function (Lopez and Salome, 2009), but the real-time dynamics of these interactions is still not clear. Probing agonist-induced changes in MOR mobility with single-particle tracking, and determining what subpopulations of MORs exist based on this measure, will open the door for studies examining the dynamics of these interactions.

In the present study, we performed single-particle tracking of modified MORs at physiological temperature (37℃) both under basal conditions and in the presence of DAMGO, a full agonist of the MOR. We employed an MOR construct that possesses an N-terminal FLAG epitope (FLAG-MOR) conjugated to a quantum dot via an anti-FLAG antibody. Experiments were performed in AtT20 cells that stably express FLAG-MOR (Borgland et al., 2003).

Analysis of trajectories of Qdot 565-conjugated FLAG-MORs reveals that activation of FLAG-MORs with DAMGO results in a reduction in the mobility of the receptor. Further quantitative diffusion analysis shows that receptors can be found in a mobile or immobile state under basal conditions, with most trajectories exhibiting relatively high mobility. Activation of the receptor with 10 μM DAMGO results in changes in the diffusion of the receptor. In particular, DAMGO treatment causes a reduction in the number of mobile trajectories and an increase in the number of immobile trajectories. These changes in the diffusive state of the receptor are also observed as an overall reduction in the ensemble-averaged effective diffusion coefficient. Together these findings explain the reduction in the average MSD of trajectories measured in

the presence of DAMGO. These findings show that activation of the FLAG-MOR does not results in a uniform decrease in the mobility of the receptors, but instead causes an increased fraction of FLAG-MORs to be found in an immobile state.

### 4.4 Materials and Methods

*Cell Culture*

AtT20 cells stably expressing the FLAG-MOR (provided by Dr. MacDonald Chrisite, University of Sydney) were maintained at 37℃/5% $CO_2$ in Dulbecco's Modified Eagle Medium (DMEM; Gibco by Life Technologies) supplemented with 5% fetal bovine serum (ATCC), Glutamax (1:100, final concentration of 2mM L-alanyl-L-glutamine), and penicillin/streptomycin (Gibco by Life Technologies). Once cells reached confluence they exposed to 0.25% trypsin-EDTA (Gibco by Life Technologies) and re-plated at lower density. Cells were maintained for no more than 12 passages beyond their original plating.

*Imaging*

To prepare for imaging, AtT20 cells were be diluted and plated on MatTec dishes containing the same DMEM solution as described above. On the fourth day after plating these cells were labeled and imaged. Labeling was performed immediately before imaging. For labeling, cells were rinsed multiple times with a saline solution containing (in mM): 35 KCl, 120 NaCl, 1 $CaCl_2$, 25 HEPES, 10mM glucose, and pH 7.4 (adjusted using NaOH). After thoroughly washing to remove DMEM, the plates were filled with saline containing 1% (w/v) bovine serum albumin (BSA; Sigma) and incubated for 10 minutes at 37°C. The cells were then incubated for 5 minutes at 37℃ in the presence of a biotinylated anti -FLAG antibody (1:1000 dilution; final concentration 1 µg/mL; BioM2 Anti-FLAG; Sigma). After multiple rinses with saline (still containing 1% BSA) to remove unbound antibody, the cells were then incubated for 8 minutes at 37℃ in the presence of streptavidin coated quantum dots (1:10000, final concentration of 100 pM; Streptavidin Qdot 565; Life Technologies; lot # 1750483). The cells were rinsed multiple

time with saline lacking BSA to remove unbound SA Qdot 565, as well as BSA, from the culture dish.

Imaging was carried on a spinning disk microscope equipped with a temperature controlled. The full MOR agonist DAMGO (10 μM; Sigma) was added to half of the culture dishes before placing them in the chamber. All culture dishes were kept in the chamber at 37℃ for 10 minutes before images were acquired. This was done to allow desensitization of the FLAG-MOR to reach a steady state before imaging in DAMGO treated dishes, and to ensure that non-treated dishes were imaged at the same time after labeling as treated dishes. Qdot 565 was excited using a 488 nm laser, and acquired at 565 nm. Videos were acquired at a rate of 20 frames/s and were 5000 frames in length.

*Image Processing and Analysis*

Images were background subtracted using a rolling-ball algorithm in ImageJ software. A Gaussian kernel filter was then applied to the images using a standard deviation of 0.8 pixels. After processing, fluorescent particles were detected using the u-track algorithm (Jaqaman et al., 2008) in MATLAB. Each 5000-frame video was broken into five separate 1000-frame segments for analysis. Trajectories less than 25 frames in length were excluded from further analysis. Trajectories were then converted to text files and analyzed using custom codes in LabView.

*Statistical Analysis*

Data sets were compared using an unpaired Student's or Welch's *t*-test as indicated, and *p* values of less than 0.05 were considered significant. GraphPad Prism (version 7.01) was used to perform statistical tests as well as to obtain descriptive statistics. Compiled data are shown as the mean ± SEM, or as histograms.

## 4.5 Results

*Activation of FLAG-MORs with DAMGO reduces the average MSD of the receptors*

Qdot 565-labeled FLAG-MORs located on the basal surface of AtT20 cells were imaged using spinning disk confocal microscopy in either the absence or presence of the full MOR agonist DAMGO (n=7 videos for each condition). Signaling of MORs expressed in these cells has been extensively characterized, and it is known that MORs expressed in AtT20 couple to endogenously expressed GIRKs (Celver et al., 2004; Yousuf et al., 2015), P/Q-type VDCCs (Borgland et al., 2003), adenylyl cyclase (Thompson et al., 2016), and G-protein coupled receptor kinases (Celver et al., 2004; Dang and Christie, 2012). Thus, the use of AtT20 cells stably expressing FLAG-MORs provides an experimental system with relatively consistent expression of the receptor, known receptor-effector coupling pathways, and is a cell type extensively used in function studies of the MOR.

After locating a field of view with a sufficient number of labeled FLAG-MORs (most cells in a given dish did not have labeled FLAG-MOR), videos of 5000 frames were obtained at a frame rate of 20 frames/s (50 ms exposure). A differential interference contrast (DIC) image of AtT20 cells and a fluorescent image of FLAG-MORs labeled with quantum dots are shown in Fig. 4.1 A and B, respectively. Similar images are shown for DAMGO treated cells in Fig. 4.1 D,E. The labeling shown in these images is typical for the experiments carried out during this study. Trajectories of labeled FLAG-MOR obtained in the absence or presence of 10 µM DAMGO were obtained using the u-track algorithm (Jaqaman et al., 2008). Trajectories obtained from the cells shown in the previous panels are shown in Fig. 4.1 C and F.

The most common method for obtaining information from trajectories obtained via single-particle tracking is based on the mean square displacement (MSD). For an individual trajectory the MSD is obtained by averaging over the time series,

$$\overline{\delta^2(t_{lag})} = \frac{1}{T - t_{lag}} \int_0^{T-t_{lag}} \left| \boldsymbol{r}\left(t + t_{lag}\right) - \boldsymbol{r}(t) \right|^2 dt,$$

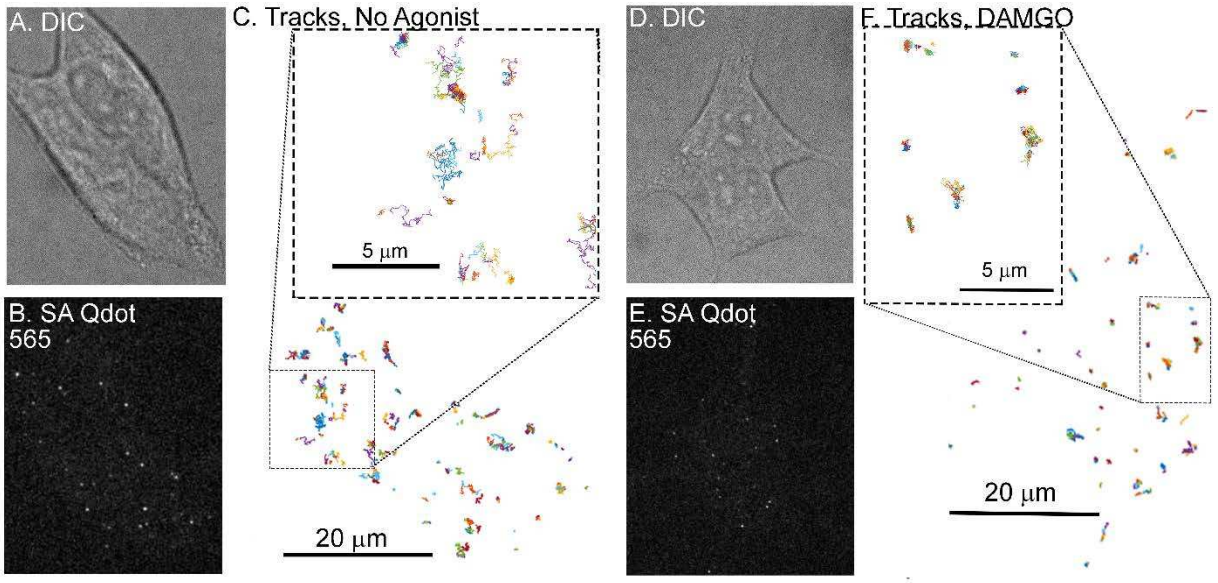


**Figure 4.1 Single-particle tracking of FLAG-MORs in AtT20 cells**. **A)** A DIC of AtT20 cells and **(B)** a fluorescent image of SA Qdot 565 conjugated FLAG-MORs in the same cells. **C)** Trajectories of SA Qdot 565-conjugated FLAG-MORs imaged in the absence of DAMGO from the cells shown in **(A)** and **(B)**. All of the trajectories obtained from a single 1000 frame video segment are shown. The inset shows a zoomed image of trajectories from the area indicated by the dashed box. Both mobile and immobile trajectories can be seen in the inset. **D)** A DIC image of AtT20 cells that were treated with DAMGO for ten minutes prior to imaging and **(E)** a fluorescent image of SA Qdot 565-conjugated FLAG-MORs in the same cells. Imaging was performed in the presence of DAMGO. **G)** Trajectories of SA Qdot 565 conjugated FLAG-MORs imaged from cells shown in **(D)** and **(E)**. All of the trajectories obtained from a single 1000 frame video segment are shown. The inset shows a zoomed image of trajectories from the area indicated by the dashed box.

where $\overline{\delta^2(t_{lag})}$ is the time-averaged MSD, $\boldsymbol{r}(t)$ is the two-dimensional position of the particle at time $t$, $t_{lag}$ is the lag time (the time between successive points when calculating the MSD), and $T$ is the duration of the trajectory. For normal diffusion processes (i.e. Brownian motion), the MSD scales linearly in lag time, $\overline{\delta^2(t_{lag})} = 4Dt_{lag}$. This relationship allows for the obtainment of the diffusion coefficient (*D*) from the MSD. However, measurements obtained in live cells often exhibit anomalous diffusion, which manifests as a deviation from this simple law (Barkai et al., 2012; Hofling and Franosch, 2013; Metzler et al., 2014; Krapf, 2015). This results in non-linear scaling of the MSD,

$$\overline{\delta^2(t_{lag})} = Kt_{lag}^{\alpha},$$

where $\alpha$ is the anomalous exponent and $K$ is the generalized diffusion coefficient, which has units of $cm^2/s^{\alpha}$. Processes with $0 < \alpha < 1$ are considered subdiffusive, and those with $\alpha > 1$ are considered superdiffusive.

Fig. 4.2 A and B show the MSD, in logarithmic scale, of 64 randomly-chosen trajectories obtained from cells under basal conditions or cells treated with DAMGO, respectively. The logarithm of the MSD can be expressed as, $\log(MSD) = \log(K) + \alpha\log(t_{lag})$, and thus the anomalous exponent $\alpha$ can be obtained from the slope of the MSD plot and the generalized diffusion coefficient $K$ can be obtained by the *y*-intercept (i.e., extrapolate to $t_{lag}$=1s). The dotted lines in Figure 4.2 A, B represent a linear MSD (i.e., α=1). The MSD of most trajectories possess a slope that is less than that of these lines, which is indicative of subdiffusion (α<1).

The large spread of anomalous exponent $\alpha$ and generalized diffusion coefficient $K$ values suggest a marked heterogeneity in the diffusion behaviors of FLAG-MORs. Fig. 4.2C shows a histogram for values of the anomalous exponent $\alpha$ obtained from FLAG-MORs under basal conditions. Two populations are clear visible, one with a relatively narrow distribution centered at α=0.06 and the other with a broader distribution centered at α=0.7. After ten minutes in the presence of DAMGO these two populations are still present, but treatment with DAMGO results in a higher proportion of receptors being located in the low α population (Fig. 4.2D). To quantify this shift, the fraction of trajectories with α<0.2 and α>0.4 were calculated. The average fraction of receptors with α<0.2 increased from 0.23±0.01 under basal conditions to 0.33±0.3 after ten minutes in the presence of DAMGO (n=7 experiments per condition; *p*=0.007; Welch's *t*-test; Fig. 4.2E). Likewise, the fraction of receptors with α>0.4 decreased from 0.61±0.01 under basal conditions to 0.47±0.03 after DAMGO (*p*=0.003; Welch's *t*-test; Fig. 4.2E).

In order to compare our data to ensemble measurements, such as FRAP, an ensemble average of the time-averaged MSD of FLAG-MORs was generated for trajectories obtained in

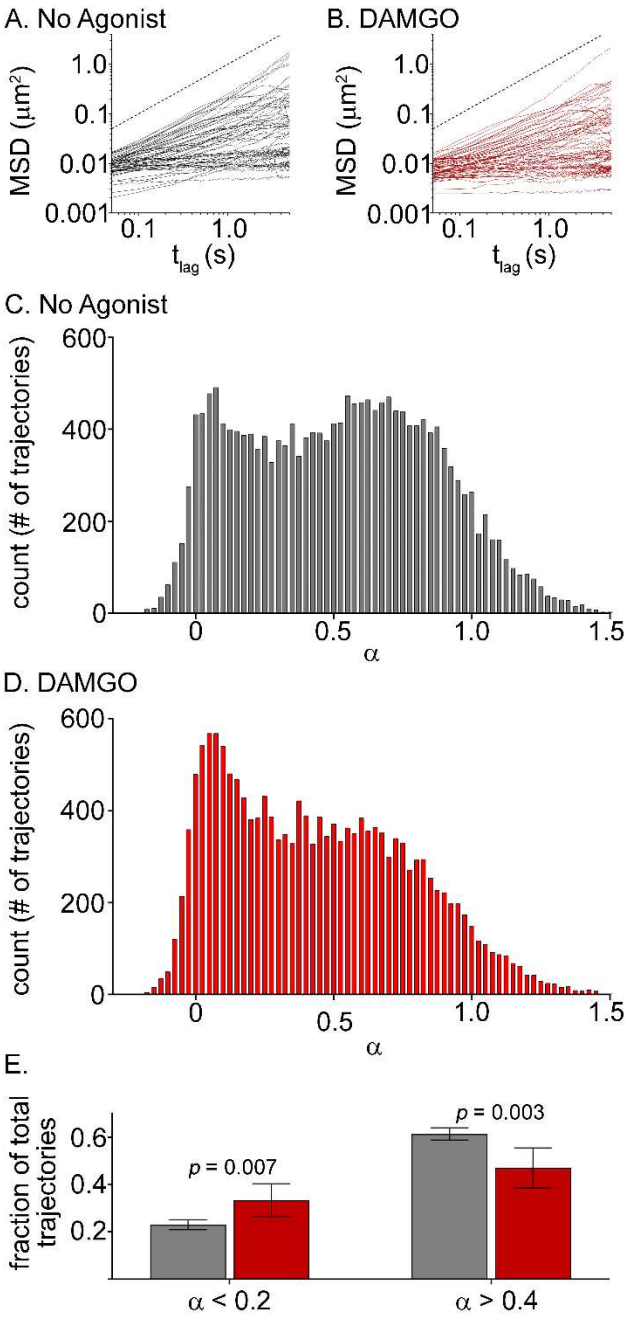


**Figure 4.2 Activation of the FLAG-MOR with DAMGO results in a higher fraction of immobile receptors (A,B)** 64 randomly selected MSD plots from trajectories at least 200 frames in length are shown from experiments in which cells did not receive DAMGO treatment (black; **A**) and from experiments from where cells were treated with DAMGO (red; **B**). The dashed lines in both plots represent the slope of MSD plots where α=1. These plots are shown to demonstrate the qualitative similarity between MSD plots of trajectories from both sets of experiments. **(C)** A histogram plotting the distribution of α values for trajectories obtained in the absence of DAMGO. The plot exhibits a clear bimodal distribution with peaks near α=0.0 and α=0.7. *(Continued on next page)*

*(Figure 4.2 continued)* **(D)** The histogram of α values from trajectories obtained in the presence of DAMGO exhibit the same bimodal distribution, but an increase in the size of the peak at α=0.08 and a decrease in the size of the peak at α=0.7. **(E)** The average fraction of FLAG-MOR trajectories where α<0.2 and α>0.4 is shown as a bar graph for experiments where cells were not treated with DAMGO (grey) or were treated with DAMGO (red). The are shown as the mean ± SEM. *P*-values were acquired using Welch's *t*-test.

the presence or absence of DAMGO (Fig. 4.3). The ensemble-averaged time-averaged MSD, $MSD = Kt_{lag}^{\alpha}$, exhibits anomalous diffusion behavior in both the absence and presence of 10 μM DAMGO. The average MSD of FLAG-MORs was reduced in the presence of DAMGO, and linear regression for $t_{lag} \leq 500$ms indicates that the average diffusion coefficient of FLAG-MORs is also reduced from $K$=0.11 μm$^2$/s$^{0.71}$ under basal conditions to $K$=0.08 μm$^2$/s$^{0.65}$ in the presence of DAMGO. The anomalous exponent $\alpha$ of the averaged MSD also decreases from α=0.71 under basal conditions to α=0.65 in the presence of DAMGO. Thus, the effects of ten minutes in the presence of DAMGO on FLAG-MOR mobility also manifest as a reduction in mobility with ensemble measurements of MOR diffusion.

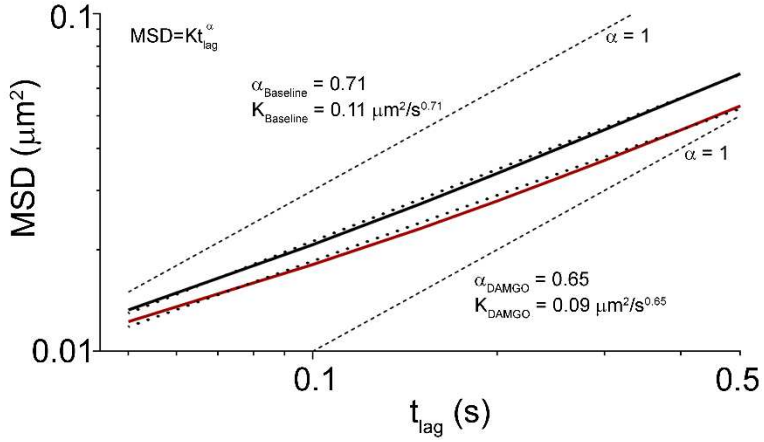


**Figure 4.3 Ten minutes in the presence of DAMGO causes a reduction in the average MSD of FLAG-MORs**. A log-log plot of the average mean square displacement (MSD) of all trajectories obtained in from cells that were not treated with DAMGO (black line) or that were obtained from cells that were treated with DAMGO for ten minutes before imaging, as well as imaged in the presence of DAMGO (red line). The dashed lines represent the slope of the MSD of particles that undergo normal diffusion (α=1). The first ten data points from each data set (up to $t_{lag}$=500 ms) were fit using linear regression in the log scale. The linear fit of these data is shown as the dotted line overlaying each plot. This fit was then used to calculate values for α and K, which are shown on the graph as $K_{Baseline}$ and $\alpha_{Baseline}$ for experiments performed in the absence of DAMGO, and $K_{DAMGO}$ and $\alpha_{DAMGO}$ for experiments performed in the presence of DAMGO.

*DAMGO induces minimal changes in the diffusion of receptors within the mobile and immobile population of receptors*

To determine if DAMGO results in changes in the diffusion of receptors located within the mobile (α>0.4) or immobile (α<0.2) population of receptors we compared the generalized diffusion coefficients (K) of FLAG-MORs from both populations under basal conditions or after ten minutes in the presence of 10 μM DAMGO. DAMGO induced a reduction in the average value for K consistent with the values calculated in Fig. 4.2 (0.114± 0.001 $\mu m^2/s^\alpha$ to 0.087±0.001 $\mu m^2/s^\alpha$; n=18456 with no agonist, n=16320 with DAMGO; p<0.0001; Welch's *t*-test), as well as a shift to a smaller median value (0.0613 $\mu m^2/s^\alpha$ to 0.0382 $\mu m^2/s^\alpha$). Histograms showing the distribution of the values for K for the entire population of FLAG-MORs in the absence or presence of DAMGO demonstrate a clear increase in the proportion of FLAG-MOR trajectories with low values for K, as well as a decrease in proportion of trajectories with higher K values (Fig. 4.4A). Plotting each trajectory's K as a function of α demonstrates a strong correlation between these two values (No agonist: r=0.93; p<0.0001; DAMGO: r=0.91; p<0.0001; Spearman's correlation; Fig. 4.4B). Performing linear regression on the log transformed values of K as a function of α demonstrates that the relationship between K and α is nearly identical in the absence or presence of DAMGO (No agonist: slope=1.438±0.004; DAMGO: slope=1.458±0.005; Fig. 4.4B). These findings are consistent with the DAMGO-induced increase in the proportion of immobile FLAG-MORs shown in Figure 4.2.

Ten minutes in the presence of DAMGO did cause a small decrease in the average value of K for trajectories where α>0.4 (0.172±0.002 $\mu m^2/s^\alpha$ to 0.153±0.002 $\mu m^2/s^\alpha$; n=11233 with no agonist, n=8238 with DAMGO; p<0.0001; unpaired Student's *t*-test), as well as the median value for K (0.120 $\mu m^2/s^\alpha$ to 0.101 $\mu m^2/s^\alpha$). The histogram of K values from trajectories where α>0.4 shows a clear decrease in the number of trajectories with relatively high values for K (K>0.05 $\mu m^2/s^\alpha$; Fig. 4.4C). Consistent with this, we found that experiments performed in the presence of DAMGO had a significantly smaller fraction of trajectories with K>0.05 $\mu m^2/s^\alpha$

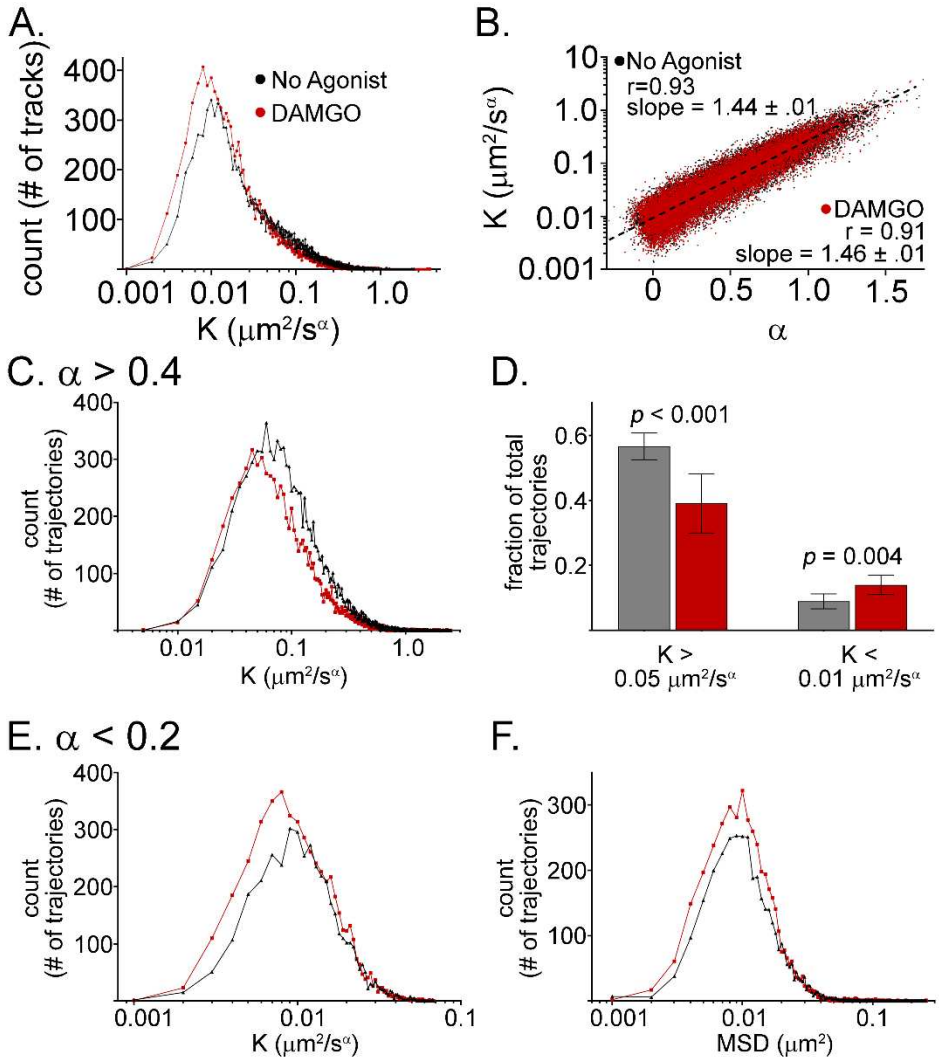


**Figure 4.4. DAMGO treatment results in an increased fraction of trajectories with low generalized diffusion coefficients (A)** A histogram showing the distribution of generalized diffusion coefficients (K) in experiments in which cells were not treated with DAMGO (black) and experiments where cells did receive DAMGO treatment (red). **(B)** A scatter plot with the K value of each trajectory plotted against the α value for that same trajectory. Analysis of the plots demonstrated a strong correlation between values for α and K that was unchanged by DAMGO (Spearman's correlation), and linear regression of log(K) as a function of α demonstrated the slope of this relationship was also unchanged by DAMGO. **(C)** A histogram showing the distribution of K values from trajectories where α>0.4. Experiments that did (red) or did not (black) receive DAMGO treatment are shown. This histogram demonstrates that the number of trajectories with relatively high values of K (K>0.05 $\mu m^2/s^\alpha$) are decreased after treatment with DAMGO. **(D)** This was quantified by calculating the average fraction of trajectories where K>0.05 $\mu m^2/s^\alpha$ for experiments that did (red) or did not (grey) receive DAMGO treatment (*p*-value acquired using Welch's *t*-test). **(E)** A histogram similar to the one shown in *C* showing the distribution of K values from trajectories where α<0.02. An increase in the number of trajectories with relatively low values for K (K<0.01 $\mu m^2/s^\alpha$) can be seen in trajectories from experiments that received DAMGO treatment. This effect is quantified in *D*. **(F)** A histogram of steady-state MSD values (MSD at $t_{lag}$=1.25 s) from trajectories where α<0.02. The number of values included in this plot is decreased versus *E* because not all trajectories were long enough to obtain this value. Treatment of cells with DAMGO did not cause any change in the distribution of these values.

(0.57±0.02 with no agonist to 0.39±0.03 with DAMGO; n=7 experiments for each condition; p<0.001; unpaired Student's *t*-test; Fig. 4.4D).

For trajectories with α<0.2, DAMGO also caused a slight decrease in the average value of K (0.0143±0.0001 $\mu m^2/s^\alpha$ to 0.0135±0.0001 $\mu m^2/s^\alpha$; n=4292 with no agonist, n=5077 with DAMGO; p<0.0001; unpaired *t*-test), as well as the median value of K (0.0123 $\mu m^2/s^\alpha$ to 0.0116 $\mu m^2/s^\alpha$). In contrast to the distribution of K values for trajectories with α>0.4, the histogram for K values from trajectories with α<0.2 shows an increase in the number relatively low K values (K<0.01 $\mu m^2/s^\alpha$; Fig. 4.4E). Consistent with this, the fraction of trajectories where K<0.01 $\mu m^2/s^\alpha$ increased significantly in the presence of DAMGO (0.09±0.01 with no agonist to 0.14±0.01 with DAMGO; n=7 experiments for each condition; p=0.004; unpaired Student's *t*-test; Fig. 4.4D). Finally, the steady state MSD of trajectories with α<0.2 was unaltered by DAMGO, demonstrating the radius that the immobile population of receptors is confined within is unchanged by activation of the receptor (Fig. 4.4F). Together, these data demonstrate that while DAMGO causes a robust shift of FLAG-MORs from a more mobile to a less mobile state, receptors found within the mobile and immobile populations have similar properties whether in the absence or presence of DAMGO.

**4.6 Discussion**

The present study was undertaken to characterize changes in FLAG-MOR diffusion induced by the full MOR agonist DAMGO using single particle tracking. We found that ten minutes in the presence of DAMGO resulted in an apparent overall reduction in the mobility of FLAG-MORs, as indicated by a reduced average MSD of FLAG-MORs in the presence of DAMGO. Analysis of the anomalous diffusion exponents (α) for trajectories obtained in the absence and presence of DAMGO demonstrated an increased immobile, and decreased mobile, fraction of receptors after ten minutes in the presence of a maximal concentration of DAMGO. DAMGO caused an increase in the number of immobile trajectories (α<0.2) with low generalized diffusion coefficient values (K<0.01 $\mu m^2/s^\alpha$), but their steady-state MSD values

remained unchanged. Ten minutes in the presence of DAMGO also decreased the number of mobile trajectories (α>0.4) with relatively high values for K (K>0.05 $\mu m^2/s^\alpha$). While DAMGO increased the fraction of trajectories with low values for α and K, it did not alter the positive correlation between a trajectory's α and K values. Together, these findings demonstrate that FLAG-MORs can be in a mobile or immobile state under basal conditions, and that activation of the receptor with DAMGO results in an increase in the proportion of immobile receptors. This study demonstrates the viability of single-particle tracking as a direct measure of agonist-induced changes of the MOR that possesses the resolution to detect subpopulation of MORs based on their mobility.

FLAG-MORs expressed in AtT20 cells exhibited a clear bimodal distribution based on their anomalous diffusion exponent (α) under basal conditions, with a population of receptors centered around an α value of 0.08 and another centered around an α value of 0.7. This can be interpreted as a mobile (population with higher α values) and immobile (population with low α values) population of receptors present on the plasma membrane of these cells. Mobile and immobile populations of MORs have also been reported in previous live-cell imaging studies using SPT (Suzuki et al., 2005) and FRAP (Sauliere et al., 2006). The physical basis of these two population of receptors is not currently understood, but it may be that a receptor's mobility is indicative of the downstream signaling pathways it is acting through.

Ten minutes in the presence of a maximal concentration (10 μM) of the full MOR agonist DAMGO resulted in an increased fraction of immobile, and decreased fraction of mobile, FLAG-MOR trajectories. Ten minutes is sufficient time for desensitization of the MOR to reach a steady-state and for internalization have reached near maximum in AtT20 cells, HEK 293 cells, and neurons (Alvarez et al., 2002; Borgland et al., 2003; Arttamangkul et al., 2006). However even after acute desensitization has reached a maximum, MOR signaling persists (Borgland et al., 2003). Therefore, the FLAG-MORs remaining on the surface of the AtT20 cells after ten

minutes in the presence of DAMGO in these experiments are likely maintaining the steady-state signaling that remains after acute desensitization has reached a maximum.

A similar DAMGO-induced increase in the immobile population of MORs was observed in SH-SY5Y cells using FRAP (Sauliere-Nzeh et al., 2010). In that study, this increase in immobile receptors was attributed to trapping of the receptors in clathrin-coated pits because the increase was prevented by incubating the cells with 500 mM sucrose. If this interpretation is correct, it may suggest that the mobile population of receptors is responsible for maintaining signaling after desensitization has occurred. However, a more recent study of FLAG-MORs expressed in HEK 293 cells showed no increase in colocalization of the receptor with clathrin after ten minutes in the presence of DAMGO (Halls et al., 2016). This study also demonstrated that specific agonists induce differential redistribution of the receptor within the membrane, and that this differential redistribution plays a role in the particular downstream signaling molecules they recruit (Halls et al., 2016). Therefore, it may be that both the mobile and immobile populations of FLAG-MORs play distinct roles in signaling by the receptor.

Biochemical studies have suggested that the location of the MOR either inside or outside of putative lipid raft domains may influence its signaling. One study demonstrated colocalization of MORs with adenylyl cyclase caveolin-1, which is a marker of putative lipid rafts (Zhao et al., 2006). A second study by the same group found that translocation into and out of these putative domains was agonist dependent, and that receptors located within the putative rafts were responsible for G-protein dependent signaling while receptors located outside of the putative rafts were responsible for β-arrestin dependent signaling (Zheng et al., 2008). It is possible that mobile and immobile receptors could correspond to receptors outside or inside of these putative raft domains, respectively. However, it is worth noting that two other studies did not find MORs localized in the Triton X-100 insoluble membrane fractions thought to contain lipid rafts either before or after treatment with and agonist (Sauliere-Nzeh et al., 2010; Halls et al., 2016), so the relationship between MOR signaling and lipid rafts remains controversial.

Precoupling of GPCRs to G-protein, and potentially forming precoupled GPCR-G-protein-effector complexes, may be an important factor in receptor mobility. Evidence for GPCR-G-protein precoupling has been presented for multiple receptor types (Tian et al., 1994; Gales et al., 2005; Nobles et al., 2005). In addition to precoupling, interactions between GPCRs and G-protein increase after activation of the receptor with an agonist (Gales et al., 2005). There is also evidence that G-protein-coupled inwardly rectifying $K^+$ channels (GIRKs) precouple to G-proteins (Riven et al., 2006), as well as evidence that P/Q-type voltage-dependent $Ca^{2+}$ channels can exist in a preformed complex with G-proteins and $GABA_B$ receptors on presynaptic terminals (Laviv et al., 2011). Precoupled receptor-G-protein-effector complexes could provide an interesting explanation for the immobile population of inactive FLAG-MORs, and can potentially be addressed in future studies examining the colocalization of immobile receptors with ion channels and other effectors known to couple to the MOR.

The use of biased agonists may also shed light on signaling that is mediated by the mobile and immobile population of receptors. Biased agonism, also referred to as functional selectivity, refers to the ability of agonist to preferentially recruit certain pathways that a receptor couples to. This results in agonists with different efficacies for different types of signaling, e.g. G-protein-mediated or β-arrestin-mediated signaling in the case of the MOR (Kelly, 2013). The MOR has a large number of endogenous and exogenous agonists whose signaling biases and efficacies for G-protein- and β-arrestin-mediated signaling have been extensively characterized (McPherson et al., 2010; Thompson et al., 2015, 2016). This will allow for the design of experiments that determine if heavily G-protein or β-arrestin biased MOR agonists differentially modulate the receptors mobility.

*Conclusions*

The present study demonstrated that agonist-induced changes in FLAG-MOR diffusion can be readily detected using single-particle tracking. Under basal conditions the population of FLAG-MORs is split between receptors in a mobile or immobile state. Ten minutes in the

presence of the MOR agonist DAMGO results in an increased fraction of receptors being found in the immobile state. It is possible that the two distinct mobility states of FLAG-MOR in the presence of DAMGO represent the recruitment of distinct signaling pathways by the receptor. However, future studies examining the colocalization of mobile and immobile receptors with known downstream effectors of the MOR, as well as studies using heavily G-protein or β-arrestin biased agonists to activate the receptor, will be needed to parse out the specific role of mobile and immobile MORs. This study also provides a basis for future studies that wish to use direct, agonist-induced changes of MOR mobility to complement common techniques such as electrophysiology and biochemistry.

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# Chapter 5: Conclusions

Significant effort has gone into understanding the desensitization of postsynaptic MORs during prolonged or repeated activation of the receptors, while little attention has been paid to the naturally occurring desensitization-resistant population of MORs located on axon terminals. Desensitization of postsynaptic MORs has proven to be a complicated process that cannot be eliminated by the knockdown of any one signaling cascade (Williams et al., 2013). Thus, the presence of desensitization-resistant presynaptic receptors provides a compelling target for studies examining the mechanisms underlying desensitization.

Understanding the differences in regulation of pre- and postsynaptic MORs can provide important insights into the signaling pathways required for MOR desensitization to occur, and can provide a better understanding of how the opioid receptor system functions as a whole. The studies presented in the previous chapters have provided compelling evidence that presynaptic MORs indeed undergo regulation that is unique from that of postsynaptic receptors and provide strategies for further studying these differences. A better understanding of the function and regulation of presynaptic MORs, and other GPCRs located on axon terminals, can potentially provide insight for future therapeutic strategies and drug development, as well as a more sophisticated understanding of how these receptors modulate the flow of information through chemical synapses.

## 5.1 Regulation of Presynaptic MORs is Distinct from That of Postsynaptic MORs

One explanation for the apparent resistance to desensitization by presynaptic MORs was that they are not resistant to desensitization at all, but that we are not detecting desensitization under normal recording conditions. Postsynaptic MORs, or MORs expressed in cell lines, also appear resistant to acute desensitization under the certain conditions, but desensitization of these receptors can be readily unmasked with the correct manipulations. One mechanism which could cause apparent resistance to acute desensitization would be if

terminals presynaptic to POMC neurons (as well as the PAG (Fyfe et al., 2010), LC (Blanchet and Luscher, 2002), and VTA (Lowe and Bailey, 2015)), have a high MOR reserve. If more receptors are present than are needed to maximally activate the effector being measured, then losing even a large number of functional receptors due to desensitization may not result in an acute decrease in signal (Connor et al., 2004). In this particular scenario, that would mean that even if significant desensitization of presynaptic MORs occurs, there would be no decrease in the inhibition of neurotransmitter release when a maximal concentration of an agonist is continuously applied. However, we found that removing enough functional receptor to reduce the maximal inhibition of GABA release by presynaptic MORs did not unmask acute desensitization of the receptor (Fig. 2.4). This finding demonstrates that the apparent resistance to desensitization by presynaptic MORs is not merely due to a large enough density of receptors on presynaptic terminals to maintain maximal inhibition of GABA release despite attrition from desensitization. This finding is consistent with similar work performed in the PAG (Fyfe et al., 2010) and VTA (Lowe and Bailey, 2015), which provides evidence that the phenomenon we are studying here is a general property of presynaptic MORs.

Additionally, not only did we find that receptor reserve was not responsible for the ability of presynaptic MORs to maintain signaling in the presence of a maximal concentration of agonist, it was also discovered that terminals presynaptic to POMC neurons do not even possess a reserve of MORs. Removal of approximately 63% of functional receptors from these terminals, as calculated by Furchgott's method (Furchgott and Bursztyn, 1967), only resulted in an approximately 50% decrease in the maximal inhibition of GABA release by presynaptic MORs. The measured acute desensitization of postsynaptic MORs often results from up to a 90% loss of functional receptors on the plasma membrane (Connor et al., 2004). This finding would suggest that MORs located on axon terminals may remain stable at the plasma membrane during prolonged signaling, unlike postsynaptic MORs that are internalized after

desensitization (Williams et al., 2013). However, future studies examining the trafficking of presynaptic MORs will be necessary to determine whether this is the case.

Another potential explanation for the apparent resistance to desensitization by presynaptic MORs is that desensitization occurs in a manner similar to that of postsynaptic MORs, but at a rate or magnitude that makes it difficult to measure using electrophysiology. If this were the case, one would expect manipulations known to increase the rate and extent of postsynaptic MORs to have a similar effect on presynaptic receptors. In an attempt to potentiate desensitization of presynaptic MORs we treated mice chronically with the opiate morphine (50 mg/kg per day for 5-7 days), which has been shown to enhance desensitization of postsynaptic MORs (Christie et al., 1987; Dang and Williams, 2004; Bagley et al., 2005; Dang and Williams, 2005). However, even after chronic morphine treatment MORs on terminals presynaptic to POMC neurons did not exhibit acute desensitization (Fig. 2.6). This is consistent with a study performed in the PAG (Fyfe et al., 2010), which again demonstrates that the phenomenon being studied here is not merely a peculiarity of the synapses we have chosen to perform our studies in.

**5.2 Resistance to Desensitization Persists When MORs Are Activated With Morphine**

Activation of GPCRs can lead to the recruitment of multiple downstream signaling pathways, and use of different agonists for a given GPCR often results in preferential recruitment of one of these pathways. This phenomenon is known as biased agonism or functional selectivity (Urban et al., 2007). Differential activation of G-protein-dependent versus β-arrestin-dependent signaling pathways is often used to describe the bias of opioid agonists (Kelly, 2013), but there is also evidence that agonist bias can alter the pathways through which the MOR desensitizes (Kelly et al., 2008). In particular, there are several studies that suggest the MOR desensitizes through a GRK- or PKC-dependent mechanism when activated by DAMGO or morphine, respectively (Bailey et al., 2004; Johnson et al., 2006; Bailey et al., 2009). We thought it was possible that axon terminals lack the biochemical machinery mediating

desensitization of the ME or DAMGO activated receptor, while containing the necessary machinery to desensitize the morphine activated receptor.

To test this hypothesis, inhibition of GABA release onto POMC neurons was measured in the presence of morphine. Like ME- (Fig. 2.1) and DAMGO- induced inhibition (Pennock and Hentges, 2011), morphine-induced inhibition of GABA release did not exhibit acute desensitization (Fig. 2.5). This finding suggests that presynaptic MORs are resistant to desensitization in general, and that the observed resistance to desensitization was most likely not due to our choice of agonist. More specifically, there does not appear to be any bias towards PKC-mediated desensitization in terminals presynaptic to POMC neurons.

A more recent study has reported that morphine-induced inhibition of GABA release in the VTA is desensitized under conditions where PKC activity is increased pharmacologically (Lowe and Bailey, 2015). In this study, morphine-induced, but not DAMGO-induced, inhibition of GABA release from terminals in the VTA was attenuated by the preincubation of slices with the phorbol ester PMA. This was interpreted as acute desensitization occurring during the relatively long (>5 minutes) wash-in of morphine, thus the “maximal” inhibition observed was in fact the steady-state signaling of the receptor after desensitization had reached completion. Although this finding was reported as selective desensitization of the morphine activated MOR, it is important to note that acute desensitization of the receptor (i.e. a decrease in maximum response over the course of several minutes of continuous exposure to morphine; Fig. 1.2) was not reported. Attenuation of presynaptic MOR signaling via phosphorylation by PKC is an intriguing finding because it demonstrates that the same biochemical pathways that are considered important for desensitization of postsynaptic MORs are present in axon terminals, but that these pathways are not recruited by presynaptic MORs under normal conditions.

However, Phorbol esters such as PMA are also known to enhance neurotransmitter release from axon terminals through PKC-dependent and PKC-independent mechanisms (Brose and Rosenmund, 2002; Silinsky and Searl, 2003). Therefore, a potential alternative

explanation for the decrease in morphine-induced inhibition of GABA release is that off-target effects of PMA are attenuating MOR-mediated inhibition of release in a manner independent of MOR phosphorylation. In fact, there is evidence that effects of phorbol esters on postsynaptic morphine-induced MOR signaling may also be PKC-independent (Arttamangkul et al., 2015). While PMA clearly and selectively attenuates morphine-induced inhibition of GABA release, more studies are needed to determine definitively the role of PKC in this effect and to more generally determine the role of kinase-mediated signaling in the function of presynaptic MORs.

### 5.3 Resistance to Acute Desensitization is a Common Property of $G\alpha_{i/o}$-coupled GPCRs Located on Axon Terminals

Resistance to desensitization by presynaptic, but not postsynaptic, MORs has been observed in the LC (Blanchet and Luscher, 2002), PAG (Fyfe et al., 2010), VTA (Lowe and Bailey, 2015), and the arcuate nucleus (Pennock and Hentges, 2011). This demonstrates that resistance to desensitization is likely a common property of presynaptic MORs. Resistance to desensitization by presynaptic MORs has also been found to occur independent of receptor reserve (Fyfe et al., 2010; Lowe and Bailey, 2015) and cannot be induced by CMT (Fyfe et al., 2010) in all brain regions examined. This may indicate that resistance to desensitization in these different brain regions is conferred by a shared mechanism. Interestingly, it is possible that this common mechanism is shared by not only presynaptic MORs located in different brain regions, but also other $G\alpha_{i/o}$-coupled receptors located on axon terminals.

Prior to our studies, resistance to desensitization similar to that exhibited by presynaptic MORs was observed for $GABA_BRs$ in the VTA and cultured hippocampal neurons (Wetherington and Lambert, 2002b; Cruz et al., 2004), as well as for $A_1$ adenosine receptors in cultured hippocampal neurons (Wetherington and Lambert, 2002a). These findings, along with those from studies of presynaptic MORs, demonstrate that resistance to desensitization is a common property of multiple $G\alpha_{io}$-coupled GPCRs when they are located on axon terminals in

different brain regions. We wanted to determine if resistance to desensitization was a common property of multiple $G\alpha_{i/o}$-coupled GPCRs located on the same population of terminals.

Like the MOR, nociceptin receptors, kappa opioid receptors (KOR), and $GABA_BRs$ inhibit GABA release onto POMC neurons. Also like the MOR, all three of these receptors exhibit resistance to acute desensitization when they are located on terminals presynaptic to POMC neurons (Fig. 2.2). In addition to this, MORs, KORs, and $GABA_BRs$ located on POMC terminals also resist acute desensitization (Fig. 2.3). This demonstrates that multiple $G\alpha_{i/o}$-coupled receptors located on the same population of terminals exhibit resistance to acute desensitization, and it is demonstrated in two separate populations of terminals. Additionally, the compartment specific nature of desensitization is also common to multiple $G\alpha_{i/o}$-coupled receptors, as postsynaptic MORs, nociception receptors, and $GABA_BRs$ located on the somato-dendritic region of POMC neurons all robustly desensitize (Fig. 2.1, 2.2).

It is now known that MORs located on GABAergic terminals in the VTA resist desensitization (Lowe and Bailey, 2015), which had already been demonstrated for $GABA_BRs$ on the same terminals (Cruz et al., 2004). Additionally, $GABA_BRs$ located on GABAergic and glutamatergic terminals in the PAG were shown to resist desensitization (Liu et al., 2013), which is consistent with MORs in the PAG (Fyfe et al., 2010). Our findings, along with those of other groups, consistently point to the commonality of resistance to desensitization among presynaptic $G\alpha_{i/o}$-coupled GPCRs. It has also been demonstrated that resistance to desensitization by presynaptic $GABA_BRs$ and $A_1$ adenosine receptors in cultured hippocampal neurons is not conferred by a high receptor reserve (Wetherington and Lambert, 2002b, a). This is consistent with multiple studies, including our own (Fig. 2.4), examining resistance to desensitization by the MOR (Fyfe et al., 2010; Lowe and Bailey, 2015), and may point to a shared mechanism conferring resistance to desensitization across different types of $G\alpha_{i/o}$-coupled receptors.

It is important to note that while differential desensitization of pre- and postsynaptic $G\alpha_{i/o}$-coupled GPCRs is common, it is not ubiquitous. In particular, resistance or susceptibility to desensitization by $GABA_BRs$ varies by brain region. Postsynaptic $GABA_BRs$, like presynaptic $GABA_BRs$, resist desensitization in both the VTA (Cruz et al., 2004) and the PAG (Liu et al., 2013). MORs in these same brain regions exhibit differential desensitization of pre- and postsynaptic receptors (Fyfe et al., 2010; Lowe and Bailey, 2015). Additionally, differential desensitization of pre- and postsynaptic $GABA_BRs$ exists in the hippocampus of neonatal rats, but with presynaptic receptors susceptible to desensitization and postsynaptic receptors resistant to desensitization (Tosetti et al., 2004). In GABAergic terminals presynaptic to POMC neurons there are also desensitization-resistant and –susceptible $GABA_BRs$ (Fig. 2.7).

The mechanism underlying the $GABA_BR$'s unusual patterns of desensitization is unclear. The $GABA_BR$ is a heterodimer, and one subunit of the receptor ($GABA_{BR1}$) has two known splice variants (Kaupmann et al., 1997; Kaupmann et al., 1998). Depending on the splice variant expressed, the receptor is preferentially targeted to axon terminals or the somato-dendritic region of neurons (Vigot et al., 2006). Knockout of $GABA_{BR1a}$ dramatically reduces, but does not eliminate, the ability of the receptor to inhibit glutamate release in in CA3 to CA1 synapses in the mouse hippocampus, and knockout of $GABA_{BR1b}$ reduced, but did not eliminate, the ability of the receptor to activate GIRKs on CA1 pyramidal cells. Additionally, knockout of either variant did not affect the maximal inhibition of GABA release by the $GABA_BR$ (Vigot et al., 2006). While it seems that R1a and R1b variant of the receptor exhibit preferential localization, they can both be expressed on axon terminals or on the somato-dendritic region of neurons. Differential desensitization of the $GABA_BR$ variants may explain unusual patterns of desensitization the $GABA_BR$ exhibits in some brain regions.

### 5.4 Specific Receptor-Effector Coupling Does Not Underlie Resistance or Susceptibility to Desensitization

While the mechanism underlying differential desensitization of presynaptic $GABA_BRs$ is unknown, the presence of these receptors on GABAergic terminals presynaptic to POMC neurons is useful experimentally. It has been hypothesized that resistance or susceptibility to desensitization by MOR-mediated responses is dependent on the effectors the receptor is coupled to in that system (Blanchet and Luscher, 2002). GABAergic terminals presynaptic to POMC neurons express desensitization-resistant MORs and $GABA_BRs$, as well as desensitization-susceptible $GABA_BRs$ (Fig. 2.1, 2.2, 2.7). This allowed for comparative studies between the receptor-effector coupling of desensitization-resistant and –susceptible populations of receptors.

Multiple pharmacological manipulations were used to determine what mechanisms underlie MOR- and $GABA_BR$-mediated inhibition of GABA release onto POMC neurons (Chapter 3). Presynaptic MORs commonly inhibit release from terminals via the activation voltage-dependent $K^+$ channels (VDKCs) via the phospholipase $A_2$ ($PLA_2$) pathway (Vaughan et al., 1997; Zhu and Pan, 2005). Both MORs and $GABA_BRs$ can also inhibit release via the inhibition of voltage-dependent $Ca^{2+}$ channels (VDCCs) (Hori et al., 1992) and through actions directly at the vesicular release machinery (Capogna et al., 1993; Rekling, 1993; Lupica, 1995; Capogna et al., 1996). If desensitization of presynaptic receptors is somehow dependent on the downstream effectors recruited by a given receptor, then it seemed likely that desensitization-resistant MORs and $GABA_BRs$ would couple to different effectors than desensitization-susceptible $GABA_BRs$.

Neither MORs nor $GABA_BRs$ located on GABAergic terminals presynaptic to POMC neurons were found to couple to VDKCs or GIRKs, as blockers of those channels had no effect on the ability of either receptor to inhibit release (Fig. 3.1). This result, although negative, is interesting due to the fact that desensitization-resistant MORs in the PAG (Fyfe et al., 2010) and

VTA (Lowe and Bailey, 2015) are known to couple to VDKCs (Vaughan et al., 1997; Bergevin et al., 2002). This provides evidence that coupling to this particular pathway is not necessary for MORs to resist desensitization.

MORs and $GABA_BRs$ presynaptic to POMC neurons are both able to inhibit release under conditions that prevented $Ca^{2+}$ influx or resulted in unregulated $Ca^{2+}$ influx, both of which preclude $Ca^{2+}$-dependent inhibition of neurotransmitter release (Fig. 3.2, 3.5). This suggests a $Ca^{2+}$-independent mechanism of inhibition, likely one occurring directly at the release machinery. Additionally, desensitization of $GABA_BRs$, but never MORs, was still observed when experiments were performed under these recording conditions. However, the same manipulations that were used to isolate potential $Ca^{2+}$-independent mechanisms of inhibition also prevents synchronous, $Ca^{2+}$-dependent release of neurotransmitter. Thus, these experiments only allowed us to draw conclusions about the inhibition of $Ca^{2+}$-independent spontaneous release.

Unless receptors are being studied in a system that allows direct patching of presynaptic terminals (Matthews, 1999; Borst and Soria van Hoeve, 2012) or $Ca^{2+}$-imaging of axon terminal fields (Wachowiak et al., 2005; Hamid et al., 2014), it can be difficult to study the mechanisms underlying the inhibition of $Ca^{2+}$-dependent release. This is because inhibition of this type of release often occurs via the inhibition of VDCCs near the site of release, and the same manipulations that inhibit VDCC activity (e.g. removing external $Ca^{2+}$, unregulated $Ca^{2+}$ influx, blocking VDCCs pharmacologically) also result in the inhibition of neurotransmitter release. This presents a problem when inhibition of neurotransmitter release is being used to measure receptor activity, as is the case with $G\alpha_{i/o}$-coupled GPCRs located on axon terminals. This is not an issue if terminals can be accessed directly with a micropipette, and thus $Ca^{2+}$ currents can be measured directly, or if inhibition of $Ca^{2+}$ influx is measured using $Ca^{2+}$ imaging techniques. However, the anatomy of the arcuate nucleus does not allow for either of these methods. The

axon terminals are too small for direct electrophysiological measurements, and afferents forming synapses with POMC neurons do not terminate in a field conducive to $Ca^{2+}$ imaging.

A recent study has presented a method that can be used to determine if inhibition of $Ca^{2+}$-dependent, synchronous release is occurring via inhibition of VDCCs or occurring directly at the release machinery (Hamid et al., 2014). The Gβγ G-protein subunit inhibits the vesicular release machinery by competitively inhibiting interactions between $Ca^{2+}$ bound synaptotagmin and SNAP-25 (Yoon et al., 2007; Wells et al., 2012). When $Ca^{2+}$ is replaced by $Sr^{2+}$, which can also move through VDCCs with a high conductance (Xu-Friedman and Regehr, 1999; Babai et al., 2014), the Gβγ subunit no longer competes with synaptotagmin due to conformational differences between $Ca^{2+}$ and $Sr^{2+}$ bound synaptotagmin (Hamid et al., 2014). Thus if inhibition of release by a GPCR is occurring via inhibition of VDCCs, then replacing $Ca^{2+}$ with $Sr^{2+}$ in the external recording solution will not affect inhibition of evoked release. However, if inhibition of release is occurring directly at the release machinery then the ability of that GPCR to inhibit release will be occluded due to the influx of $Sr^{2+}$ after each stimulus (Hamid et al., 2014).

Both MOR- and $GABA_BR$-mediated inhibition of GABA release onto POMC neurons was maintained when release was evoked with $Sr^{2+}$. This indicates that MOR- and $GABA_BR$-mediated inhibition of $Ca^{2+}$-dependent, evoked GABA release occurs through a $Ca^{2+}$-dependent mechanism. Additionally, desensitization of $GABA_BR$-mediated, but never MOR-mediated, inhibition of release was present in experiments that used $Sr^{2+}$ instead of $Ca^{2+}$ to evoke GABA release, demonstrating that differential desensitization between MORs and $GABA_BRs$ is present when observing $Ca^{2+}$-dependent or $Ca^{2+}$-independent inhibition of release.

Although the finding that the differential desensitization of MORs and $GABA_BRs$ was maintained in the presence of $Sr^{2+}$, there was still the possibility that the inhibition of $Ca^{2+}$ influx by desensitization-resistant and –susceptible receptors was occurring through different mechanisms. The rapid $Ca^{2+}$ influx needed to induce action potential evoked neurotransmitter release often occurs through either N-type or P/Q-type VDCCs (Meir et al., 1999). We found

that N- and P/Q-type VDCCs contribute equally to, and almost completely account for, evoked GABA release onto POMC neurons (Fig. 3.7). Therefore, the possibility that desensitization of $GABA_BR$-mediated inhibition of evoked release only occurs when the receptor is coupled to a particular type of VDCC still existed. However, both MOR- and $GABA_BR$-mediated inhibition of release were maintained under conditions that isolated N-type or P/Q-type VDCC-mediated release (Fig. 3.8), indicating that neither receptor demonstrates preferential coupling to one type of VDCC.

These findings show that MOR- and $GABA_BR$-mediated inhibition of GABA release can occur through both $Ca^{2+}$-dependent and $Ca^{2+}$-independent mechanisms, and that resistance or susceptibility to desensitization is not determined by a receptor's coupling. In addition to demonstrating that desensitization-resistant and –susceptible receptors couple to the same effectors when located in the same population of axon terminals, comparing our findings to those of others also demonstrates differential coupling of desensitization-resistant MORs exists between presynaptic MORs in the arcuate nucleus and those in the PAG (Vaughan et al., 1997) or VTA (Bergevin et al., 2002). Together, the common receptor-effector coupling of desensitization-resistant and –susceptible MORs and $GABA_BRs$, as well the differential coupling of desensitization-resistant MORs between brain regions, indicates that receptor coupling does not determine whether a receptor desensitizes. The lack of effector-delimited desensitization provides additional evidence that resistance or susceptibility to desensitization by presynaptic GPCRs is a receptor-delimited property that may be explained by some physical characteristic or presynaptic GPCRs that is absent from their postsynaptic counterparts. However, the nature of these differences, whether they be differential phosphorylation, covalent modification, or protein-protein interactions, remains to be determined by future studies.

**5.5 Mobility as a Measure of MOR Activation**

Electrophysiological and pharmacological manipulations of presynaptic MORs were sufficient to determine that they are uniquely regulated relative to postsynaptic receptors, and

eliminated several possibilities for mechanisms underlying resistance to desensitization. However, elucidating the actual mechanism behind resistance to desensitization by presynaptic MORs, and desensitization of MORs in general, will require techniques outside of brain slice electrophysiology and pharmacology. A limitation of whole-cell electrophysiological experiments is that the output the experimenter measures represents a sum of the activation or inhibition of a given effector by the entire population of MORs in that cell (or set of axon terminals when measuring activation of presynaptic receptors). If subpopulations of receptors with differential signaling exist (e.g. desensitized/non-desensitized, G-protein-mediated or β-arrestin-mediated signaling) they would not be detected using electrophysiology.

Electrophysiological measures also have to use the output provided by the activation or inhibition of a given effector as a proxy for receptor activity. The activation of the effector can be multiple steps removed from activation of the receptor. For example, measuring the activation of presynaptic receptors generally relies on measuring the reduction or increase in postsynaptic currents through ligand-gated ion channels. In the case of MORs presynaptic to POMC, an agonist binds the receptor, which results in the activation of G-protein, the active G-protein then inhibits $Ca^{2+}$ influx via inhibition of VDCCs, reduced $Ca^{2+}$ influx reduces exocytosis and GABA release, and finally the reduced GABA release results less open $GABA_A$ receptors and a reduction in the amplitude of the measured IPSC. Because there are multiple steps between activation of the receptor and current passing through the postsynaptic $GABA_A$ receptor regulation of any one of these steps can alter the output of an experiment, and such potential regulation must be taken into account when making conclusions about activity of a presynaptic receptor.

Single particle tracking (SPT) of MORs can provide a useful complement to these techniques as it can provide a real-time measure of agonist induced changes in receptor mobility, and possesses single molecule resolution. SPT can also reflect agonist-induced changes of the receptor directly, eliminating the need to rely on proteins sometimes several

steps removed from receptor activation for a functional output. There is evidence that exposure of MORs to an agonist results in robust changes in receptor mobility, and that subpopulations of MORs based on mobility are likely to exist (Sauliere et al., 2006; Sauliere-Nzeh et al., 2010). However, these experiments were performed using a technique that lacks single molecule resolution, and were performed below physiological temperature (20℃). We wanted to determine whether agonist induced changes in mobility were detectable at the level of individual receptors, and when performing experiments at 37℃.

Single particle tracking was performed in AtT20 cells stably expressing MORs modified with a FLAG epitope on their N-termini (FLAG-MORs). The AtT20 cell line is a commonly used heterologous expression system in the field of opioid pharmacology, and thus the actions of the MOR have been well characterized in AtT20 cells. MORs expressed in AtT20 cells are known to couple to native GIRKs (Celver et al., 2004; Yousuf et al., 2015), adenylyl cyclase (Thompson et al., 2016), P/Q-type VDCCs (Borgland et al., 2003), G-protein coupled receptor kinases (Dang and Christie, 2012; Thompson et al., 2016) and other non-G-protein-mediated pathways (Thompson et al., 2016). The coupling of the receptors in this system is similar, albeit not identical, to the coupling of MORs in the somato-dendritic region of neurons. Therefore, we think that AtT20 cells are likely the best choice of heterologous expression system to establish SPT of MORs before attempting experiments in neurons.

Under basal conditions FLAG-MORs exist in two distinct subpopulations based on their mobility. One population was characterized by its relatively high mobility, while the other was highly immobile and confined. Ten minutes of exposure to the MOR agonist DAMGO resulted in a shift in the size of these two populations, with a higher fraction of receptors now found in the immobile state (Fig. 4.3). Cells were treated with DAMGO for ten minutes before imaging because this is sufficient time for desensitization of the receptor to reach completion, and for most desensitized receptors to become internalized (Borgland et al., 2003). Therefore, the receptors remaining on the membrane are likely the receptors responsible for maintaining

steady-state signaling by the receptor after acute desensitization has reached completion (Fig. 1.2).

An early hypothesis regarding the effect of activation on the mobility of the MOR was that activating the receptor would drastically decrease mobility due interactions with downstream effectors (e.g. GIRKs, adenylyl cyclase, VDCCs). It was therefore expected that ten minutes of DAMGO exposure would leave a homogenous population of receptors on the plasma membrane, and these remaining receptors would represent actively signaling receptors. While DAMGO did result in a larger fraction of immobile receptors, approximately half of the receptors on the plasma membrane were still found in the mobile population. The physical basis of the mobile and immobile population of FLAG-MORs is currently unclear. It may be that both subpopulations are maintaining steady-state signaling of the receptor, but that each subpopulation is acting through different downstream effectors.

Biochemical studies of the MOR provide evidence that receptors responsible for G-protein-dependent signaling are located in Triton X-100 insoluble fractions of plasma membrane, while receptors located outside of these fractions are responsible for β-arrestin-dependent signaling (Zheng et al., 2008). A recent study examining the redistribution of FLAG-MORs after activation of the receptor with morphine or DAMGO shows that each agonist causes differential redistribution of the receptor with the plasma membrane, and that this redistribution is related to the signaling bias of each agonist (Halls et al., 2016). Although limited in number, these studies provide evidence that localization of the MOR within the plasma membrane is likely to play a role in the particular signaling cascades it recruits. Determining whether recruitment of different signaling cascades, for example G-protein- or β-arrestin-dependent signaling, corresponds to a particular mobility state of the MOR will advance SPT as true functional measure of MOR activity.

GPCRs can exist in preformed complexes with G-proteins (Tian et al., 1994; Gales et al., 2005; Nobles et al., 2005), as well as in a complex between the receptor, G-protein, and an ion

channel (Laviv et al., 2011). GPCRs also exhibit increased interactions with G-proteins after activating the receptor with an agonist (Gales et al., 2005). It may be that these interactions are facilitated by confinement of the receptor within microdomains on the plasma membrane (Baker et al., 2007), and thus MORs interacting with G-protein-dependent signaling cascades may represent the immobile population observed in our experiments. If this is the case, then the mobile population might be responsible for signaling through G-protein-independent mechanisms.

It has also been hypothesized that immobile MORs observed after extended exposure (≥10 minutes) to DAMGO represent receptors trapped within clathrin-coated pits (Sauliere-Nzeh et al., 2010). However, more recent data would suggest that interactions between the MOR and clathrin are not increased over a ten minute DAMGO treatment (Halls et al., 2016), which is identical to the treatment used in our study. Interactions between FLAG-MORs and clathrin-coated pits could be quantified, as indicated by a recent study performed with single-molecule resolution using the VDKC $K_V2.1$ (Weigel et al., 2013), and such a study would determine how much of the immobile population of FLAG-MORs can be accounted for by immobilization inside of clathrin-coated pits. Colocalization the MOR and markers of putative signaling microdomains have also been reported (Zhao et al., 2006). If the trapping of MORs in clathrin-coated pits does not account for the immobile population of MORs, measuring interactions between the MOR and such markers would be an interesting next step. Measurements of interactions between the MOR and known downstream effectors such as GIRKS, VDCCs, and β-arrestin might also be possible.

The use of FLAG-MORs expressed in AtT20 cells has allowed us to demonstrate the viability of using SPT to study agonist-dependent changes in MOR mobility. This paves the way for similar studies in neurons where the mobility of differentially desensitizing MORs located in different neuronal compartments can be compared to one another. This technique will be immediately useful as a complement to more common electrophysiological and biochemical

techniques, and will provide a useful experimental paradigm for determining how MOR mobility relates to the recruitment of particular signaling cascades.

### 5.6 Closing Remarks

Altogether, these studies provide clear evidence that regulation of desensitization-resistant MORs located on axon terminals occurs in a manner that diverges significantly from their postsynaptic counterparts. Resistance to desensitization occurs independent of receptor-effector coupling, and cannot be explained by a high receptor density on presynaptic terminals. It is unclear what physical differences between pre- and postsynaptic MORs confer resistance to desensitization, but we hypothesize that these differences may result in differential mobility between these two populations of MORs. Single-particle tracking of MORs proved to be a robust measure of basal MOR mobility, as well as of agonist-induced changes in MOR mobility, and can now be used to determine if MOR mobility varies between neuronal compartments. In addition to gaining a greater understanding of the function of presynaptic MORs in physiological systems, understanding the mechanisms underlying resistance to desensitization by presynaptic MORs could provide important insights for future drug and therapeutic design.

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**Appendix I**

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## Appendix II: Direct inhibition of hypothalamic proopiomelanocortin neurons by dynorphin A is mediated by the μ-opioid receptor [4]

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### Summary

It has recently been shown that dynorphin A (Dyn A), an endogenous agonist of the kappa opioid receptor (KOR), directly inhibits proopiomelanocortin (POMC) neurons in the hypothalamus through activation of G-protein coupled inwardly rectifying $K^+$ channels (GIRKs). This effect has been proposed to be mediated by the putative kappa-2 opioid receptor (KOR-2), and has been suggested as a possible mechanism for the orexigenic actions of KOR agonists. Using whole-cell voltage clamp recordings in brain slice preparations, the present study demonstrates that Dyn A (1 or 5 μM) induces an outward current in POMC neurons that is reversed by the highly selective mu opioid receptor (MOR) antagonist CTAP and absent in mice lacking MORs. Additionally, the KOR-2 selective agonist GR89696 binds MORs on POMC neurons but fails to induce an outward current. Similar to Dyn A, the KOR selective antagonist nor-BNI lacked specificity when used at sufficiently high concentrations. Maximal concentrations of the MOR-selective agonist DAMGO induced outward currents in POMC neurons that were completely reversed by a relatively high concentration of nor-BNI. Experiments using a half-maximal concentration of DAMGO demonstrate that nor-BNI must be used at concentrations <100 nM to avoid non-specific actions of the antagonist at MORs located on POMC neurons. These data suggest that direct inhibition of POMC neurons by Dyn A is mediated through the

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MOR, not the KOR-2, which is consistent with previous studies demonstrating that Dyn A can act at the mu opioid receptor (MOR) when present in high concentrations.

**Introduction**

Kappa opioid receptor (KOR) agonists, including the endogenous peptide dynorphin A (Dyn A), potently stimulate food intake (Morley and Levine, 1983). It was recently demonstrated that Dyn A is capable of direct inhibition of anorexigenic proopiomelanocortin (POMC) neurons of the arcuate nucleus of the hypothalamus via activation of G-protein coupled inwardly rectifying $K^+$ channels (GIRKs; (Zhang and van den Pol, 2013). This led to the hypothesis that Dyn A-induced inhibition of POMC neurons may be an important component in the stimulation of food intake by KOR agonists (Zhang and van den Pol, 2013). It was also proposed that Dyn A-induced inhibition of POMC neurons was mediated by the putative kappa-2 subtype of the kappa opioid receptor (KOR-2), the presence of which had been overlooked in previous studies characterizing opioid regulation of POMC neurons (Pennock and Hentges, 2011) due to the unique pharmacological properties of the KOR-2.

Pharmacological and receptor binding studies led to the hypothesis that two KOR subtypes are expressed in brain tissue (reviewed in (Wollemann et al., 1993). Electrophysiological studies have also observed pharmacological differences between pre- and postsynaptic KORs; the synthetic KOR-selective agonist U69593 acts as a full agonist at presynaptic KORs but as a partial agonist at postsynaptic KORs in the VTA (Ford et al., 2007). However, the gene that codes for the KOR-2 has yet to be identified, and there is evidence that putative KOR-2 binding that occurs in the brain is actually non-selective binding to other opioid receptors (Simonin et al., 2001).

If two pharmacologically distinct KOR subtypes exist on the somato-dendritic region of POMC neurons and POMC terminals, the postsynaptic population of receptors may be selectively targeted. Varying distribution of KOR subtypes could also allow for differential regulation of pre- and postsynaptic receptors, particularly as Dyn A concentrations change in

response to physiological conditions. This would make the KOR-2 an intriguing target for those wishing to develop therapies that increase food intake through the inhibition of POMC neurons, as other $G\alpha_{i/o}$-coupled receptors that directly inhibit POMC neurons through GIRK activation also disinhibit POMC neurons through the inhibition of GABA release.

Dyn A is a selective agonist of the KOR, activating the receptor at nanomolar concentrations (Goldstein and James, 1984; Zhang et al., 1998). However, Dyn A has also been shown to bind and act as an agonist at the mu opioid receptor (MOR), albeit with an $EC_{50}$ value 50-100 times greater than that observed at the KOR (Goldstein and James, 1984; James and Goldstein, 1984; Chavkin et al., 1985; Goldstein and Naidu, 1989; Mulder et al., 1989; Emmerson et al., 1994; Raynor et al., 1994). Dyn A-induced inhibition of POMC neurons occurs with an $EC_{50}$ in the micromolar range (Zhang and van den Pol, 2013), well above the concentration range that is selective for the KOR (Mulder et al., 1989; Grudt and Williams, 1993). Thus, it is possible that Dyn A-induced inhibition of POMC neurons is not mediated through either KOR subtype, but is instead due to activation of the MOR.

The present studies use pharmacological and receptor knockout approaches to show that Dyn A-induced inhibition of POMC neurons occurs at high concentrations as a consequence of MOR activation. Thus, endogenous KOR ligands most likely exert their effects via KORs located on presynaptic inputs to POMC neurons and POMC neuron terminals.

**Materials and Methods**

*Ethical approval*

All animal use procedures were approved by the Colorado State University Institutional Animal Care and Use Committee and met United States Public Health guidelines and the policies and regulations for animal experimentation described by *The Journal of Physiology*.

*Animals*

Mice expressing discosoma red (dsRed) or enhanced green fluorescent protein (eGFP) driven by the POMC promoter (Cowley et al., 1999; Hentges et al., 2009) were backcrossed onto a

C57BL/6 background for >12 generations. Mice expressing a mutant mu opioid receptor allele ($MOR^-$; (Matthes et al., 1996) were obtained from The Jackson Laboratory (B6.129S2-$Oprm1^{tm1Kff}$/J) and were backcrossed onto the C57BL/6 background for >12 generations prior to purchase. $MOR^-$ expressing mice were crossed with POMC-eGFP animals to produce mu opioid receptor knockout mice ($MOR^{-/-}$) expressing eGFP in POMC neurons (POMC-eGFP/$MOR^{-/-}$). All mice received tap water and standard rodent chow ad libitum. Animals were housed at a controlled temperature (22-24ºC) with a 12hr light/dark cycle. Transgenic mice were identified using standard PCR genotyping.

*Brain slice preparation*

58 mice were used over the course of this study. Brain slices were prepared from 6-12-week-old male and female POMC-dsRed, POMC-eGFP and POMC-eGFP/$MOR^{-/-}$ mice. Before being sacrificed, mice were deeply anesthetized using isoflurane. Mice were quickly decapitated and brains were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 $NaH_2PO_4$, 21 $NaHCO_3$ and 11 glucose. ACSF solutions were adjusted to pH 7.5 and saturated with 95% $O_2$/5% $CO_2$ mixture. Sagittal brain slices were prepared at a thickness of 240 μM using a VT 1200S vibratome (Leica). Slices containing the arcuate nucleus of the hypothalamus were transferred into warm ACSF (37ºC) containing 15 μM MK-801 immediately after collection. At least 45 min passed between collection and transfer of slices to the recording chamber.

*Electrophysiological recording*

After being transferred to the recording chamber, slices were continuously perfused (~2 mL/min) with warm (37ºC) ACSF saturated with 95% $O_2$/5% $CO_2$. Recordings were made using pipettes with a resistance of 1.5-2.5 mΩ after being filled with an internal solution containing (in mM): 57.5 K-methyl sulfate, 57.5 KCl, 20 NaCl, 1.5 $MgCl_2$, 5 HEPES ($K^+$ salt), 0.1 EGTA, 2 Mg-ATP, 0.5 Na-GTP, 10 phosphocreatine, pH 7.3. mIPSCs were recorded with an internal solution

that replaced K-methyl sulfate and KCl with Cs-methane sulfonate and CsCl, respectively. POMC neurons were identified by the presence of dsRed or eGFP fluorescence. After obtaining a seal with >1 GΩ resistance, negative pressure was used to rupture the cellular membrane and enter whole-cell mode. Recordings were made at a holding potential of -60 mV with no series resistance compensation. Postsynaptic currents were obtained in 1s sweeps taken every 11s using Axograph X software. Each sweep contained a 50 ms -10 mV voltage step to monitor access and input resistance over the course of the recording. IV curves were constructed using nine -10 mV voltage steps starting at a holding potential of -50 mV. Each step was 100 ms in length with 200 ms between steps. This protocol was repeated 200 ms after the end of the first set of voltage steps. The 2 data sweeps for each holding potential were then averaged. The currents induced by the voltage step protocol were not removed from any current traces (Fig. 1A,C,E,F; Fig. 2A-F; Fig. 3A-C). DAMGO, Dynorphin A- and baclofen-induced postsynaptic currents were recorded in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM), bicuculline methiodide (BMI; 10 μM) and tetrodotoxin (TTX; 300 nM). Miniature IPSCs were collected at 10 kHz and digitally filtered at 1 kHz. Events were collected during 15 s sweeps that were repeated every 15 s and detected using Axograph X software based on rise time kinetics. Events with a rise time <100 μs were rejected. mIPSCs were recorded in the presence of DNQX (10 uM) and TTX (300 nM).

*Drugs*

[D-Ala$^2$,N-Me-Phe$^4$,Gly$^5$-ol]-Enkephalin acetate (DAMGO; Sigma), Dynorphin A (1-13) (Dyn A; Phoenix Pharmaceuticals), R(+)-Baclofen hydrochloride (Sigma), (-)-Bicuculline methiodide (BMI; Tocris), GR 89696 fumurate (Tocris), and Tetrodotoxin citrate (TTX; Tocris) were prepared in distilled water (at least 1000:1 of the final concentration). 6,7-dinitroquinoxaline-2,3-dione (DNQX; Sigma) and (+)-MK-801 (Sigma) were prepared in DMSO

(at least 10,000:1 of the final concentration). Drugs were diluted in ACSF to achieve final concentrations.

*Statistics*

Data were analyzed using a one-sample or paired Student's t-test. Data sets containing multiple repeated measures were analyzed using a repeated-measures ANOVA and Tukey's multiple comparison post hoc test. Induced outward currents <5 pA, were not included in analyses as clear reversal was difficult to assess. Using this criteria, the number of cells excluded for each drug and concentration used was as follows: 10 μM DAMGO (2 of 17 cells), 500 nM DAMGO (4 of 11 cells), 5 μM Dyn A (2 of 21 cells), 1 μM Dyn A (10 of 18), 30 μM baclofen (1 of 14 cells). All data points are represented as the mean ± SEM. Differences were considered significant if $p<0.05$.

**Results**

*Dynorphin A (Dyn A)-induced outward currents are reversed by CTAP*

As reported previously (Zhang and van den Pol, 2013), Dyn A (1 μM or 5 μM) was found to induce a clear outward current in POMC neurons (11.4 ± 1.5 pA and 26.2 ± 3.8 pA, respectively; 8 out of 18 cells responded to 1 μM, 19 out of 21 cells responded to 5 μM, only responding cells are included in statistics; $p<0.001$, one sample t-test; Figure 1A,B,E). To determine if this outward current is mediated by activation of the putative KOR-2 (Zhang and van den Pol, 2013), or rather by actions at the MOR (Chavkin et al., 1985), the MOR-selective antagonist CTAP was applied immediately after a peak Dyn A current was observed. CTAP (1 μM) caused in a rapid reversal of the outward current induced by Dyn A (5 μM) in all cells examined (DynA=17.9 ± 3.1 pA, DynA/CTAP= -0.42 ± 1.4 pA; n=8 cells from 4 mice; $p<0.001$, paired t-test; Fig 1C,D). To further examine the possibility that the outward current induced by Dyn A was due exclusively to activation of the MOR, the experiment was repeated with a lower concentration of both Dyn A (1 μM) and CTAP (100 nM). The outward current induced by Dyn A

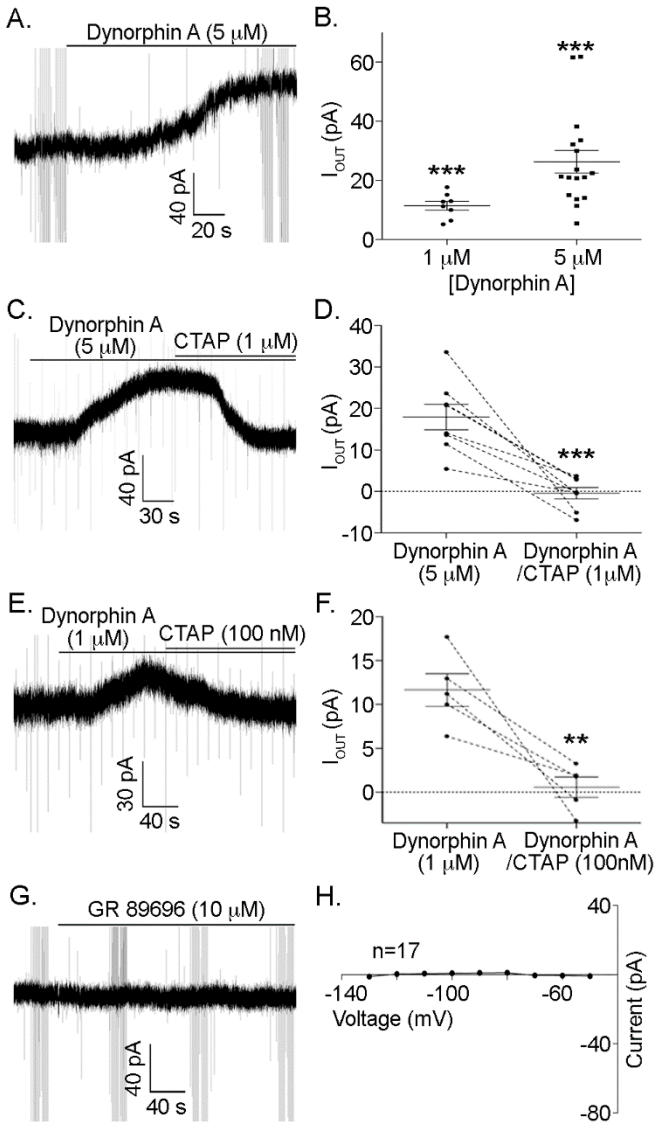


**Figure 1. Dynorphin A-induced outward currents measured from POMC neurons are reversed by the mu opioid receptor selective antagonist CTAP.** A) A sample trace of an outward current induced by Dyn A (5 μM). B) Compiled data for the magnitude of the outward current induced by Dyn A (1 or 5 μM). C) A sample trace demonstrating the reversal of an outward current induced by Dyn A (5 μM) by the MOR-selective antagonist CTAP (1 μM). D) Compiled data showing the amplitude and the Dyn A-induced current before and after addition of CTAP (1 μM). E) A sample trace demonstrating the reversal of an outward current induced by Dyn A (1 μM) by CTAP (100 nM). F) Compiled data showing the amplitude of the outward current induced by Dyn A before and after the addition of CTAP (1 μM). G) A sample trace from a recording showing that the putative KOR-2 agonist GR 89696 induces no outward current in POMC neurons. H) A subtracted IV plot showing no difference between the IV relationship of POMC neurons before and after GR 89696 application. P-values are represented by stars; ** = p<0.01, *** = p<0.001.

was reversed by CTAP in all recordings (Dyn A=11.7 ± 1.9 pA, DynA/CTAP= 0.6 ± 1.2 pA; n=5 cells from 4 mice; p=0.007, paired t-test; Fig 1E,F). Finally, the putative KOR-2 agonist GR

89696 (10 μM) was applied to determine whether any KOR-2-mediated outward currents could be detected in POMC neurons. No outward current was detected at a holding potential of -60 mV (-0.7 ± 1.1 pA; n=10 cells from 5 mice; p=0.53, one sample t-test; Fig 1G,H), and subtracted IV relationship showed no detectable currents at any of the holding potentials examined (-130 to -50 mV; n=10 cells from 5 mice; p=0.18, repeated measures ANOVA; Fig 1H). Together, these data suggest that Dyn A-induced outward currents on POMC neurons result from activation of the MOR, not the KOR-2.

*The putative KOR-2 selective agonist GR89696 binds the mu opioid receptor*

It has been previously reported that putative KOR-2 binding in the brain can be accounted for by non-selective binding of KOR-2 agonists to other opioid receptors (Simonin et al., 2001). Although GR89696 did not induce changes in the electrical properties of POMC neurons in the present study (Fig 1G,H), GR89696 does appear to bind to MORs on POMC neurons. Outward currents induced by DAMGO (10 μM) were reversed by GR89696 (10 μM) in all cells examined (DAMGO=24.2 ± 4.6 pA, DAMGO/GR89696=-1.2 ± 0.5 pA; n=5 cells from 3 mice; p=0.003, paired t-test; Fig 2A,D). Similarly, pre-application of 10 μM GR89696 occluded DAMGO-induced outward currents in POMC neurons (-0.06 ± 0.9 pA at -60 mV, n=9 cells from 5 mice; p=0.94, one sample t-test; Fig 2B,E), but not outward currents induced by the $GABA_B$ receptor agonist baclofen (32.8 ± 12.2 pA at -60 mV, n=4 cells from 2 mice; p=0.07, one sample t-test; Fig 2C,E). These data are consistent with previously reported findings (Simonin et al., 2001) that putative KOR-2 ligands can bind non-selectively to other opioid receptors.

*Dynorphin A-induced outward currents are absent in mu opioid receptor knockout mice*

To verify that Dyn A-induced outward currents are mediated by the MOR, current/voltage relationships for both the MOR-selective agonist DAMGO and Dyn A were constructed in POMC-eGFP mice and POMC-eGFP mice that were crossed with mice containing a mutant μ-opioid receptor allele that results in a loss of MOR function (POMC-eGFP/$MOR^{-/-}$). If Dyn A-

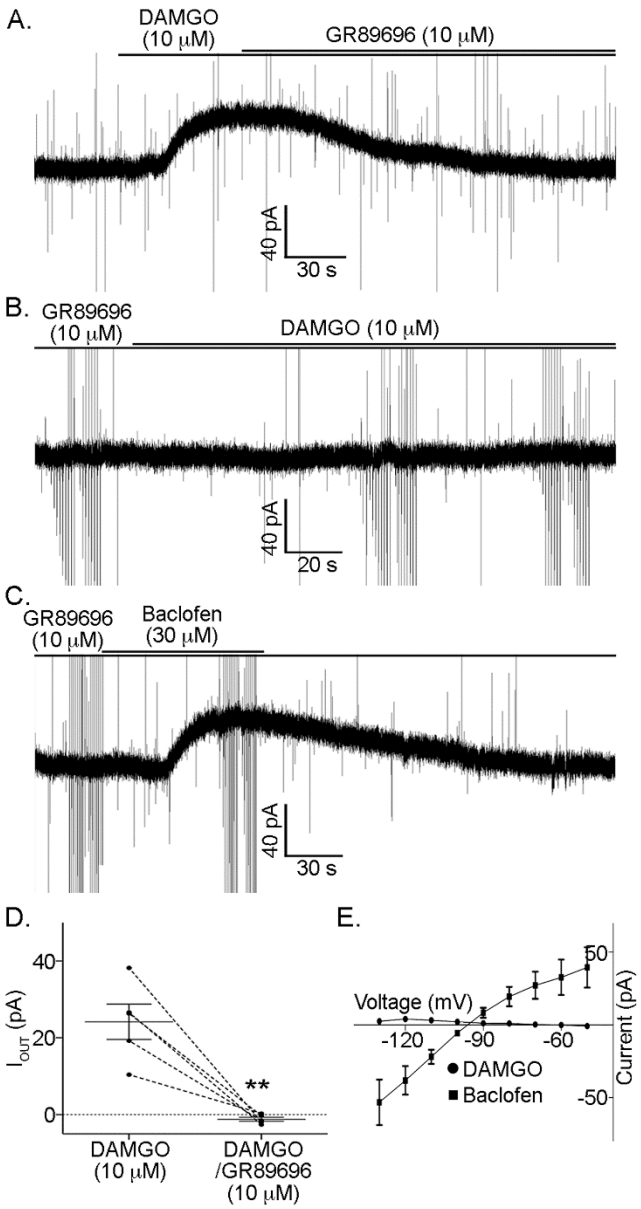


**Figure 2. GR89696 binds to mu opioid receptor.** A) A sample trace demonstrating that the outward current induced by DAMGO (10 μM) is reversed by GR89696 (10 μM). B) A sample trace demonstrating the occlusion of a DAMGO-induced outward current by pre-application of GR89696. C) A sample trace demonstrating that GR89696 pre-application does not occlude the outward current induced by baclofen (30 μM). D) Compiled data showing the amplitude of the outward current induced by DAMGO (10 μM) before and after application of GR89696 (10 μM). E) Subtracted IV plots showing the currents induced by both DAMGO and baclofen in the presence of GR89696 at voltages ranging from -50 to -130 mV in -10 mV steps. P-values are represented by stars; **= $p<0.01$.

induced inhibition of POMC neurons can be mediated by something other than the MOR, the absence of functional MORs should have no consequence on Dyn A actions. Consistent with the above studies, both DAMGO (10 μM) and Dyn A (5 μM) induced inwardly-rectifying currents

with reversal potentials near the $K^+$ equilibrium potential in POMC neurons expressing the MOR (-98.4 ± 1.2 mV for DAMGO, n=5 cells from 4 mice; -96.5 ± 4.2 mV for Dyn A, n=9 cells from 5 mice; p<0.0001, repeated measures ANOVA; Fig 3A,C,G). As expected, DAMGO did not induce a current in POMC neurons lacking MORs at when held at -60 mV (-1.4 ± 1.2 pA; p=0.29, one sample t-test) or at any other holding potential tested (-50 to -130 mV, p=0.74, repeated measures ANOVA, n=4 cells from 2 mice; Fig. 3D,H). Similarly, Dyn A did not produce a detectable current at any of the holding potentials examined (0.5 ± 0.7 pA at -60 mV, p=0.48 by one sample t-test; p=0.99 using repeated measures ANOVA on data from -50 to -130 mV holding potentials; n=6 cells from 3 mice; Fig 3B,H). To ensure that $G\alpha_{i/o}$-coupled GPCRs were still generally functional in POMC-eGFP/$MOR^{-/-}$ animals, the effect of the $GABA_B$ receptor agonist baclofen on whole cell currents were recorded in POMC neurons with and without MORs. In both populations, baclofen induced an inwardly-rectifying current with a reversal potential near the equilibrium potential of $K^+$ ($MOR^{+/+}$=-92 ± 7.4 mV, n=3 cells from 2 mice; $MOR^{-/-}$=-91.6 ± 1.6 mV, 5 cells from 4 mice; Fig 3E,F,G,H).

To ensure that the κ-opioid system was unaffected by knockout of the MOR, the inhibition of presynaptic GABA release by Dyn A was examined in $MOR^{-/-}$ and $MOR^{+/+}$ mice. In $MOR^{-/-}$ mice, Dyn A (100 nM) induced robust inhibition of mIPSC frequency measured from POMC neurons (baseline=7.5 ± 1.9 Hz, Dyn A=3.4 ± 0.7 Hz) and this was reversed by nor-BNI (50 nM, to 8.8 ± 1.7; p=0.003; repeated measures ANOVA; n=5 cells from 4 mice; Fig 4A,B). While DAMGO (10 μM) strongly inhibits mIPSC frequency in $MOR^{+/+}$ mice (~80% reduction;(Pennock and Hentges, 2011), DAMGO-induced inhibition of mIPSC frequency was essentially absent in $MOR^{-/-}$ mice (~3.8% reduction as opposed to the ~80% reduction observed in wild type mice, 7.9 ± 1.6 Hz baseline vs. 7.6 ± 1.5 Hz in 10 μM DAMGO; n=8 cells from 4 mice; p=0.02, paired t-test; Fig 4C,D). The lack of Dyn A-induced postsynaptic currents in the

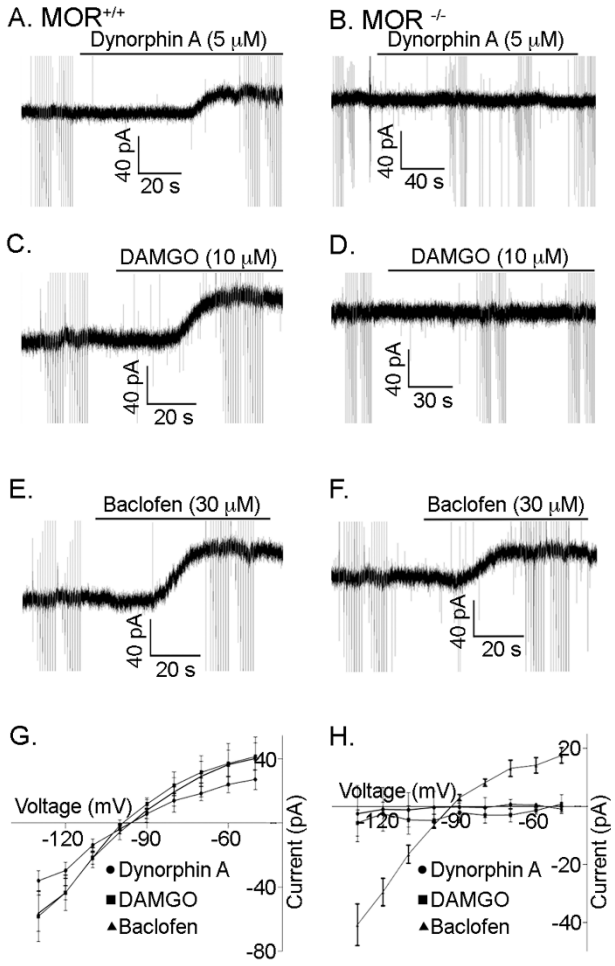


**Figure 3. Dynorphin A-induced outward currents are absent in POMC neurons lacking the mu opioid receptor.** A,B) Sample traces demonstrating the effects of Dyn A in wild type and MOR knockout mice. Dyn A induces an outward current in $MOR^{+/+}$ but not $MOR^{-/-}$ mice. Similar traces are shown for DAMGO (C,D). Sample traces demonstrating a baclofen induced outward current in both $MOR^{+/+}$ and $MOR^{-/-}$ mice. G,H) Subtracted IV relationships demonstrating the currents induced by Dyn A, DAMGO and baclofen at holding potentials ranging from -50 to -130 mV in -10 mV steps in $MOR^{+/+}$ and $MOR^{-/-}$ mice.

MOR knockout provides strong evidence that Dyn A inhibits POMC neurons via actions at the MOR.

*Nor-BNI is non-selective for the KOR at sufficiently high concentrations*

Dyn A-induced currents measured from POMC neurons have been attributed to actions at the KOR-2 in part because the current is blocked by the KOR-selective antagonist nor-BNI (Zhang and van den Pol, 2013). However, nor-BNI can also act as an antagonist of the MOR when used at sufficiently high concentrations (Emmerson et al., 1994; Raynor et al., 1994). To

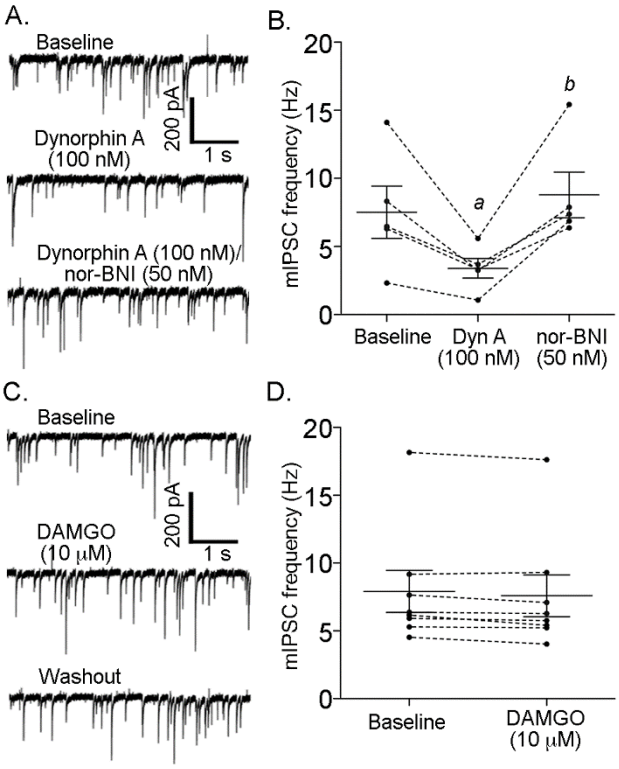


**Figure 4. Presynaptic kappa opioid receptors are still present and functional in the mu opioid receptor knockout mouse.** A) Sample traces demonstrating the inhibition of mIPSCs measured from POMC neurons by Dyn A (100 nM), as well as the reversal of Dyn A-induced inhibition of mIPSCs by the KOR selective antagonist nor-BNI (50 nM), in MOR knockout mice. B) Compiled data for the inhibition of mIPSCs by Dyn A and the reversal of Dyn A-induced inhibition by nor-BNI. Using Tukey's *post hoc* test, mIPSC frequency was found to be significantly different than the baseline mIPSC frequency (*a*) and the mIPSC frequency in the presence of nor-BNI (50 nM, *b*). The baseline mIPSC frequency and mIPSC frequency in the presence of nor-BNI (50 nM) were not significantly different. C) Sample traces demonstrating a lack of DAMGO-induced inhibition of mIPSCs measured from POMC neurons in MOR knockout mice. D) Compiled data for the effects of DAMGO on mIPSC frequency in MOR knockout mice.

determine if the concentration of nor-BNI used in previous experiments was sufficient to act on the MOR, nor-BNI (1 μM) was used to reverse outward currents induced by a maximal concentration of DAMGO (10 μM). Similar to previous studies, nor-BNI (1 μM) was sufficient to reverse the outward current induced by Dyn A (5 μM Dyn A=36.4 ± 10.4 pA, with nor-BNI=1.9 ± 1.7 pA; p=0.01, paired t-test; n=5 cells from 3 mice; Fig 5A,D). However, nor-BNI (1 μM) was also sufficient to reverse the outward current induced by a relatively high concentration of DAMGO (10 μM) in all cells examined (DAMGO=31.7 ± 9.0 pA, nor-BNI=-0.7 ± 1.3 pA; p=0.009, paired t-test; n=5 cells from 3 mice; Fig 5B,E). To determine the concentration of nor-BNI

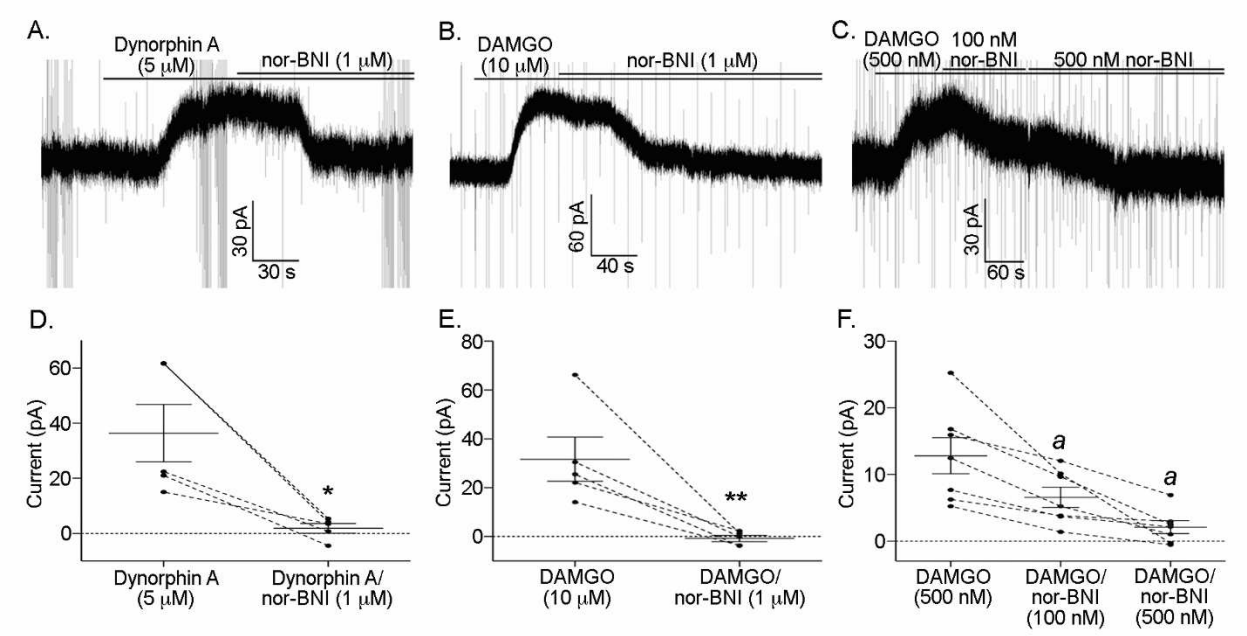


**Figure 5. MORs on POMC neurons are antagonized by nor-BNI at sufficiently high concentrations.** A) A sample trace demonstrating the reversal of an outward current induced by Dyn A (5 μM) by nor-BNI (1 μM). B) The same concentration of nor-BNI (1 μM) is also sufficient to reverse the outward current induced by higher concentration of DAMGO (10 μM). D,E) Compiled data showing the reversal of outward currents induced by Dyn A and DAMGO by nor-BNI (1 μM). C) Dose-dependent inhibition of an outward current induced by a near $EC_{50}$ concentration of DAMGO (500 nM) by 100 nM and 500 nM nor-BNI. F) Compiled data demonstrating the reversal of the outward current induced by DAMGO (500 nM) by 100 nM and 500 nM nor-BNI. The amplitude of the outward current induced by DAMGO (500 nM) in the presence of nor-BNI (500 nM) is significantly different than that of DAMGO (500 nM) alone using Tukey's *post hoc* test (*a*). P-values are represented by stars; * = $p<0.05$, ** = $p<0.01$.

needed to avoid antagonism of the MOR, outward currents were induced with an ~$EC_{50}$ concentration of DAMGO (500 nM; (Pennock and Hentges, 2011), then cells were exposed to increasing concentrations of nor-BNI. DAMGO (500 nM) induced an outward current that was partially reversed by a low concentration of nor-BNI (DAMGO=12.8 ± 2.7 pA, 100 nM nor-BNI =6.5 ± 1.5 pA; p=0.003 DAMGO vs. baseline, one sample t-test; n=7 cells from 5 mice; Fig 5C,F) and almost completely reversed by a higher concentration of nor-BNI (500 nM nor-BNI=2.1 ± 0.9 pA; p<0.001, repeated measures ANOVA; n=7 cells from 5 mice; Fig 5C,F). Thus, it appears that nor-BNI must be used at concentrations <100 nM to avoid actions at MORs on POMC neurons.

## Discussion

The present data demonstrate that Dyn A can activate an inwardly-rectifying $K^+$ current on the somato-dendritic region of POMC neurons and that this is mediated by the MOR, and not the putative KOR-2 receptor. Dyn A must be present in micromolar concentrations to induce currents in POMC neurons, which is sufficient to activate the MOR. Further, the presence of functional MORs is required for Dyn A to induce currents in POMC neurons. These results are consistent with previous studies demonstrating a lack of KOR expression on the somato-dendritic region of POMC neurons (Pennock and Hentges, 2011; Dicken et al., 2012).

*Selectivity of Dynorphin A and nor-BNI for KORs on POMC neurons*

As shown in a previous study (Zhang and van den Pol, 2013), Dyn A induced a robust outward current in POMC neurons when applied at micromolar concentrations that was reversed with nor-BNI. However, the present study demonstrates that the concentrations of Dyn A needed to induce outward currents in POMC neurons are sufficient to produce non-specific effects at the MOR. This was demonstrated by the complete reversal of Dyn A-induced outward currents by the MOR-selective antagonist CTAP, and by the complete reversal of DAMGO-induced currents by nor-BNI at the concentration (1 μM) used in the previous study to reverse the putative kappa-2 effect (Zhang and van den Pol, 2013). The mismatched binding to receptors by classical MOR or KOR ligands is consistent with previous studies demonstrating that the selectivity of Dyn A and nor-BNI diminishes when used at relatively high concentrations (Goldstein and James, 1984; James and Goldstein, 1984; Chavkin et al., 1985; Goldstein and Naidu, 1989; Mulder et al., 1989; Emmerson et al., 1994; Raynor et al., 1994). In contrast to the high concentrations of Dyn A needed to cause a postsynaptic outward current in POMC neurons, Dyn A caused robust inhibition of GABA release onto POMC neurons when present in a much lower concentration (100 nM) and this effect was reversed by a concentration of nor-BNI (50 nM) that is selective for the KOR. These concentrations are consistent with previous studies

using Dyn A and nor-BNI to selectively target KORs (Grudt and Williams, 1993; Simmons and Chavkin, 1996; Margolis et al., 2003; Ford et al., 2007).

*Dyn A-induced outward currents are absent in MOR$^{-/-}$ mice*

If Dyn A-induced currents were mediated by the KOR-2, the current should still be present in mice lacking the MOR. Although presynaptic KORs were still functional in MOR$^{-/-}$ animals, no Dyn A-induced postsynaptic currents were detected in POMC neurons lacking the MOR. There was also no outward current detected using the putative KOR-2 agonist GR89696 in wild type mice. However, it was determined that GR89696 binds, but does not activate, MORs on POMC neurons. These findings agree with previous work demonstrating that all putative KOR-2 binding can be accounted for by non-selective binding at other opioid receptors (Simonin et al., 2001).

Whereas the present work did not find a GR89696-mediated current in POMC neurons, a previous study found this putative KOR-2 agonist to hyperpolarize POMC neurons (Zhang & van den Pol, 2013). The reasons for this difference between the two studies are not completely clear. One possibility is that GR89696 acts on upstream cells to alter the activity of POMC neurons, although Zhang and van den Pol did not find GR89696 to change spontaneous IPSCs or EPSCs in POMC neurons. We cannot rule out a potential age-dependence to the GR89696 effect. The mean age of mice used in the present study was likely older than that in the previous study since the range used here was 6-12-weeks compared to 2-7.3-weeks in Zhang and van den Pol. An additional difference between the current and previous study is the use of voltage-clamp versus current-clamp. However, since the GR89696-induced hyperpolarization of POMC neurons observed by Zhang & van den Pol was reported to occur through activation of a GIRK conductance, it is unlikely that a corresponding outward current would not be detectable in experiments performed in voltage-clamp. Additional assays to detect the presence and function of KOR-2 on POMC neurons and further studies with GR89696 may help fully discern the reasons for the apparent discrepancy between these two studies.

*Possible roles for endogenous Dyn A in the regulation of POMC neurons*

Although the present study suggests that no form of KOR is present on the somato-dendritic region of POMC neurons, endogenous Dyn A may still play a role in the regulation of POMC neurons in vivo through presynaptic actions. KORs are present on the presynaptic terminals of POMC neurons and potently inhibit neurotransmitter release (Dicken et al., 2012). Inhibition of transmitter release directly at the level of POMC terminals would be expected to increase food intake since POMC-neuron-derived transmitters are largely anorexigenic (reviewed in (Cone, 2005; Mercer et al., 2013). It is also possible that Dyn A directly hyperpolarizes the somato-dendritic region of POMC neurons, albeit through the MOR. As shown in the present study, Dyn A activates somato-dendritic MORs on POMC neurons when used at a sufficient concentration. However, it is unknown whether Dyn A concentrations in vivo reach concentrations sufficient to induce activation of the MOR. The selectivity profile of Dyn A may also change depending on how the peptide is processed. For example, Dyn A (1-8) shows higher selectivity for the MOR than KOR (Goldstein and Naidu, 1989). If Dyn A- expressing neurons forming synapses with POMC neurons release a shorter fragment of Dyn A such as Dyn A (1-8) this may produce activation of the MOR.

*Conclusions*

Although Dynorphin A is capable of inducing direct inhibition of the somato-dendritic region of POMC neurons, the present study found such inhibition is mediated by activation of the MOR. There is significant evidence that the KOR system is an important endogenous regulator of energy balance, and KOR agonists have known orexigenic actions. The present results suggest that if KOR agonists act at POMC neurons increase food intake and body weight, they do so either via inhibition of presynaptic release or by activating MORs to reduce the activity of POMC neurons.

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## List of Abbreviations

| | |
|---|---|
| 4-AP | 4-aminopyridine |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| 5-HTR | Serotonin receptor |
| $A_1R$ | Adenosone $A_1$ receptor |
| aCSF | Artificial cerebrospinal fluid |
| ACTH | Adrenocorticotrophic hormone |
| ATP | Adenosine triphosphate |
| βarr | Beta-arrestin |
| β-CNA | Beta-chlornaltrexamine |
| cAMP | Cyclic adenosine monophosphate |
| CeA | Central nucleus of the amygdala |
| CGP 55845 | (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid |
| ChR2 | Channelrhodopsin-2 |
| CMT | Chronic morphine treatment |
| CTAP | D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ |
| DAMGO | [d-Ala$^2$, N-Me-Phe$^4$, Gly$^5$-ol]-enkephalin |
| DIC | Differential interference contrast |
| DMSO | Dimethyl sulfoxide |
| DNQX | 6,7-dinitroquinoxaline-2,3-dione |
| DOR | Delta opioid receptor |
| DsRed | Discosoma red |
| $EC_{50}$ | Half-maximal effective concentration |
| eGFP | Enhanced green fluorescent protein |

| | |
|---|---|
| eIPSC | Evoked inhibitory postsynaptic currents |
| ERK | Extracellular signal-regulated kinases |
| FRAP | Fluorescence recovery after photbleaching |
| FRET | Fluorescence resonance energy transfer |
| GABA | Gamma-aminobutyric acid |
| $GABA_BR$ | $GABA_B$ receptor |
| GDP | Guanosine diphosphate |
| GIRK | G-protein inwardly rectifying $K^+$ channels |
| GPCR | G-protein coupled receptor |
| GRK | G-protein-coupled receptor kinase |
| GTP | Guanosine triphosphate |
| HEK | Human embryonic kidney |
| IPSC | Inhibitory postsynaptic currents |
| KOR | Kappa opioid receptor |
| LC | Locus coeruleus |
| LTP | Long term potentiation |
| ME | [$Met^5$]-Enkephalin |
| MEF | Mouse embryonic fibroblast |
| mIPSC | Miniature inhibitory postsynaptic currents |
| MOR | Mu opioid receptor |
| MSD | Mean square displacement |
| NLX | naloxone |
| NOP | Nociceptin receptor |
| nor-BNI | Nor-binaltorphimine |

| | |
|---|---|
| PAG | Periaqueductal grey |
| PCR | Polymerase chain reaction |
| PDBu | Phorbol 12,13-dibutyrate |
| PKC | Protein kinase C |
| $PLA_2$ | phospholipase-$A_2$ |
| PMA | Phorbol 12-myristate 13-acetate |
| POMC | Proopiomelanocortin |
| Qdot | quantum dot |
| RGS | Regulators of G-protein signaling |
| SEM | Standard error of the mean |
| SNAP-25 | Synaptosome associated protein 25 |
| SNARE | Soluble NSF attachment protein receptor |
| SPT | Single-particle tracking |
| TEA | Tetraethylammonium |
| TTX | Tetrodotoxin |
| U69593 | (+)-(5α,7α,8β)-*N*-methyl-*N*[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide |
| VDCC | Voltage-dependent $Ca^{2+}$ channels |
| VDKC | Voltage-dependent $K^+$ channels |
| VTA | Ventral tegmental area |