Constitutively Active μ-Opioid Receptors

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Abstract

The μ-opioid receptor is the G protein coupled receptor (GPCR) responsible for the analgesic, rewarding and unwanted effects of morphine and similar drugs. Constitutive activity of GPCRs is a phenomenon that likely reflects receptors spontaneous adopting conformations that can activate G proteins, and is likely to be common to most if not all GPCRs. Basal constitutive activity has been observed in some systems with μ-opioid receptors, and constitutive activity is expressed by mutant μ-opioid receptors with amino acid substitutions in regions known to be important for signalling. However, μ-opioid receptors are unique in that a putative constitutively active state of the receptor, the μ*-state, has been suggested to be induced by prolonged agonist treatment. The μ*-state is thought to contribute to processes underlying adaptation to and withdrawal from opioid treatment, and may have a ligand sensitivity distinct from basal constitutive activity of the μ-opioid receptor or that exhibited by μ-opioid receptor mutants. In this Chapter we outline methods for measuring constitutively active μ-opioid receptors, including some that take advantage of the fairly direct coupling of the receptor to ion channels. We also briefly summarize the pharmacology of the different constitutively active μ-opioid receptor states, and highlight the areas where we need to know more. We hope that a better understanding of constitutive activity at the μ-opioid receptor may provide information useful in developing ligands that access subsets of receptor conformations, offering the potential to fine tune opioid pharmacotherapy.

Introduction

The constitutive activity of G protein coupled receptors (GPCR) has been recognized for some time, and is not a surprising phenomenon given the stochastic principles that underlie signalling in biology. Antagonist-sensitive, agonist-independent GPCR activity was noted from the earliest studies of purified β-adrenergic receptors (Cerione *et al.,* 1984), however, it was a comprehensive study of native δ-opioid receptors that showed G-protein activity that could be stimulated by agonists and inhibited by "inverse agonists", with both effects being blocked by neutral antagonists (Costa and Herz, 1989). The constitutive activity of GPCR is an important idea in drug action and some diseases result from mutations in GPCR that enhance basal receptor activity (Smit *et al.,* 2007). While there are varying degrees of constitutive activity measurable at different GPCR, the presumption that it arises as an inevitable consequence of continual movement of receptors through a series of different conformational states means that constitutive activity *per se* will be common to all receptors.

The μ-opioid receptor is the GPCR responsible for the analgesic and rewarding properties of morphine and similar drugs, as well as for their unwanted effects. Constitutive activity has been convincingly demonstrated for the μ-opioid receptor (e.g. Burford *et al.,* 2000; Liu and Prather, 2001; Mahmoud *et al.,* 2010), but a great deal of additional experimental interest has been generated by the proposition that chronic treatment with μ-opioid agonists induces a constitutively active form of the receptor known as the μ*-state (Wang *et al.,* 1994; Sadee *et al.,* 2005). This privileged state of the μ-opioid receptor is reported to be induced by agonist treatment *in vivo* or *in vitro* and is thought to reflect persistent phosphorylation of the receptor. The consequences of prolonged use of morphine for pain relief or other purposes are a major medical and societal challenge, and a significant research effort continues to be devoted to understanding the mechanisms underlying tolerance to the analgesic affects of

morphine and the development of dependence on opioids. The suggestion that persistent constitutive activity of the μ-receptor is responsible for some or all of the adaptations that lead to tolerance to and dependence on morphine means that it is critically important to be able to measure μ-opioid receptor activity as directly as possible, and in as wide a variety of native tissues as possible. In this chapter we will describe biochemical and electrophysiological techniques for detecting and quantifying the constitutive activity of μ-opioid receptors *in vitro*, and endeavour to outline the strengths and potential shortcomings of each assay.

Experimental Questions

The hypothesis that the constitutively active μ*-state of the μ-opioid receptor plays a pivotal role in driving cellular adaptation to prolonged opioid agonist treatment raises a number of important experimental questions specific to the μ-opioid receptor. These include whether the basal constitutive activity of μ-opioid receptors is operationally equivalent to that of the μ*-state and what functional consequences constitutive μ-opioid receptor activity has for neuronal excitability and synaptic transmission. It remains unresolved if constitutively active μ-receptors and μ*-state receptors activate the same suite of G-proteins, whether their basal activity is sensitive to the same inverse agonists or even whether the constitutive activity observed with agonist naïve μ-opioid receptors simply reflects a low level of the phosphorylation event suggested to produce the μ*-state. It has also been extremely difficult to demonstrate μ-opioid receptor constitutive activity or any antagonist effects consistent with the reversal of constitutive μ-opioid receptor activity in isolated neurons or neurons in brain slices. It remains an open question whether techniques currently used in experiments on relatively intact neurons lack sufficient sensitivity to detect constitutively active μ-opioid receptors (μ* or otherwise) or whether there is simply nothing to see.

Methods for Measuring Constitutive Activity

General Considerations

The activity of GPCR can be quantified quite directly by measuring agonist-stimulated GTPase activity or the binding of stable GTP analogs to G proteins. One of the earliest events after receptor activation is the exchange of GDP on the Gα subunit for GTP (Figure 1). Consequently, the [^{35}S]GTPγS assay, which measures binding of the radiolabelled, slowly hydrolysable, GTP analog [^{35}S]GTPγS to activated Gα proteins has been widely employed. The subsequent hydrolysis of GTP bound to the activated Gα proteins can also be measured, as can modulation of adenylyl cyclase (AC) downstream of GTP-bound Gα protein or ion channels downstream of released Gβγ subunits. It is, of course, important to define the receptor that is responsible for the activity, where possible to distinguish constitutively active receptors from constitutively active G proteins, and to ensure that the measured signal does not come from the activity of endogenous agonists remaining in the preparation. This latter consideration is less problematic for μ-opioid receptors, as the endogenous peptide agonists are likely to be scarce in the reduced preparations used for these studies, although lipophilic synthetic agonists used for pretreatment may be harder to remove.

Insert Figure 1 about here

The difficulty in measuring constitutive activity is of course that by its nature it is unstimulated. While it is possible to carefully measure basal GTPase activity while changing levels of receptor or G protein (Cerione *et al.,* 1984), this approach is not practical for addressing most experimental questions. The voltage-dependent inhibition of calcium channels (I_{Ca}) by G protein βγ subunits provides an instantaneous and repeatable measurement of constitutive activity in single cells simply by varying membrane voltage (see below), but it

is only good for isolated neurons. In general, constitutive activity is defined by the effects of ligands that stabilize poorly coupled state(s) of the μ-receptor. These compounds are called "inverse agonists" or described as ligands with "negative intrinsic efficacy" (Kenakin 2004). A list of readily available μ-opioid receptor ligands with reported negative intrinsic activity is found in Table 1. The degree to which these ligands inhibit the basal level of the output being measured is considered to reflect the amount of constitutive activity present in the system, although it is difficult to prove that any ligand completely inhibits constitutive activity in the absence of a non-pharmacological method of defining this activity. Evidence that ligands with negative intrinsic activity are acting to change signalling via their cognate receptor is provided by reversal of the inhibition of signalling by a neutral antagonist (Costa and Herz, 1989). Putative neutral opioid antagonists are also listed in Table 1. An advantage of using ligands to define constitutive activity is that the receptor specificity of the drugs is usually well defined. However, different ligands have been reported to have different amounts of negative efficacy, which presumably reflects differences in their propensity to stabilize the G-protein uncoupled states of the μ-receptor. Some laboratories report virtually all antagonists of μ-opioid receptor function have negative intrinsic activity (Sally *et al.*, 2010), with essentially no compounds being completely devoid of positive or negative efficacy. Although somewhat at odds with the observed lack of effect of μ-opioid receptor antagonists in most assays, the notion that binding to a receptor stabilizes a subset of conformations suggests that it would be difficult for a high affinity ligand not to affect the distribution of receptor-coupled conformations one way or the other (Kenakin and Onaran, 2002).

G protein measures: the [^{35}S]GTPγS/Eu-GTP assays

Two methods are employed for measuring agonist activity at the level of the G protein. They respectively take advantage of the binding of GTP to the Gα subunit following agonist-

induced dissociation of GDP and the subsequent hydrolysis of GTP bound to the Gα subunit to terminate the signal (Figure 1). Both responses show an increase following agonist occupation of a GPCR and therefore should show a response to a receptor that constitutively activates Gα in the absence of agonist. In practice, the measure of GTP binding to the Gα subunit using labelled GTP analogues is the easiest and has been much more frequently employed in the study of constitutive activity.

The most common way to measure constitutive activity of μ-opioid receptors is to assess the non-agonist-stimulated binding of labelled GTP analogues to membranes from cells or tissues expressing the receptor. The analogues used are either ^{35}S labelled GTP ([^{35}S]GTPγS) or europium labelled GTP (Eu-GTP), although the commercial availability of the latter ligand is now uncertain. Assays for the two ligands are very similar except that the amount of [^{35}S]GTPγS bound is determined by scintillation counting and the binding of Eu-GTP by time-resolved fluorescence. In assays of ligand-stimulated receptor activity μ-opioid agonists increase the binding of the GTP analogs to appropriate Gα proteins. However, in the absence of agonist and even in the presence of a high concentration of unlabelled GTPγS to define non-specific binding, there is some degree of "basal" binding of the labelled nucleotide. This basal binding of comprises binding of [^{35}S]GTPγS (or Eu-GTP) to unoccupied or constitutively active heterotrimeric G proteins, binding to small G proteins and binding caused by receptors activating Gα proteins in the absence of agonist, i.e. binding reflecting constitutive activity.

To measure labelled GTP binding a low concentration of the labelled nucleotide (usually 0.1 nM for [^{35}S]GTPγS or 1 nM for Eu-GTP, which has lower affinity for Gα subunits) is incubated in membranes from cells or tissues expressing μ-opioid receptors for sufficient time

to reach steady state (usually 60-120 mins). Specific binding of the labelled nucleotide is defined in the presence of a saturating concentration of unlabelled GTPγS. GTP-conjugate labelled Gα subunits remain bound to the cell membranes and the assay is terminated by filtration or immunoprecipitation, with signal from the retained proteins measured by traditional scintillation counting (Traynor and Nahorski, 1995) or Scintillation Proximity Assay (DeLapp, 2004) for [^{35}S]GTPγS or time-resolved fluorescence for Eu-GTP (Labrecque *et al.*, 2009)

To observe receptor driven increases in binding it is necessary to include GDP, Na and Mg in the incubation medium. GDP fills empty guanine nucleotide binding sites on the Gα protein which reduces the basal level of GTP conjugate binding and supplies GDP-bound Gα substrate for agonist-driven activation. The concentration of GDP needed to give an optimal signal-to-noise ratio must be determined experimentally for each system; it is usually between 3 and 30 μM for cells expressing cloned receptors, but may be higher for brain tissue. However, increasing the concentration of GDP increases the efficacy requirements of the system, conversely lowering GDP will raise basal binding and has been used to identify inverse agonists in other systems (Roberts and Strange 2005). Mg is necessary to promote agonist-stimulated binding of [^{35}S]GTPγS, and the concentration can be critical for the identification of inverse agonists (Wang *et al.* 2001). Higher concentrations of Mg can inhibit binding, so again, the concentration should be optimised for each system. Assays are usually performed in the presence of 100 mM Na because Na selectively promotes agonist-stimulated binding over basal binding, improving the agonist signal. However, such a high concentration of Na tends to uncouple the receptor from G proteins and so reduces spontaneous coupling. Thus, the chances of observing constitutive activity can be improved by replacement of Na with K (Szekeres and Traynor, 1997; Liu *et al.* 2001) or with N-methyl-

D-glucamine (Lin *et al.* 2006) to increase receptor-effector coupling and promote spontaneous activation of [^{35}S]GTPγS binding. This increased constitutive signal obviously allows for the easier determination of inverse agonist efficacy. Finally, endogenous adenosine can mask the signal arising from other GPCR, particularly in brain tissue, so membranes should be incubated with adenosine deaminase prior to experiments (Breivogel *et al.* 2004) or an adenosine receptor antagonist can be included in the assay (Horswill *et al.* 2007). A detailed review of the assay protocol can be found in (Harrison and Traynor, 2003), and specific experimental details in (Kara and Strange, 2010).

Several considerations need to be born in mind when using GTP analog binding assay to identify constitutive activity at μ-opioid receptors. Firstly, the assay measures an event very close to the receptor itself and therefore the response is not amplified as seen with downstream assays. Consequently it can be difficult to observe a small constitutively active signal unless receptors are over expressed or conditions such as Na concentration are altered. Further, because of the impermeable nature of the guanine nucleotides the assay is performed in isolated membranes, and this raises the possibility of disrupting the receptor-G protein coupling or scaffolding mechanisms potentially necessary to maintain constitutive activity. Finally, it is necessary to show that the constitutive effects derive from the receptor taking up active conformations and not some other process, such as changes in the spontaneous activity of the G-proteins themselves. For Gαi/o coupled receptors such as the μ-opioid receptor this can be achieved with pertussis toxin (PTX) pretreatment (usually 100 ng ml^{-1} overnight). PTX ADP ribosylates a Cys in the C-terminus of the Gα protein and prevents its coupling to receptor, thus inhibiting both agonist and constitutive activation of [^{35}S]GTPγS binding. Importantly, PTX does not alter other properties of the G protein, such as ability to bind guanine nucleotides. However, use of PTX does not identify which receptor in a system in

responsible for the observed constitutive activity. For example, PTX reduces basal [^{35}S]GTPγS binding in SH-SY5Y cells (Traynor and Nahorski, 1995), although there is no specific evidence for μ-opioid constitutive activity in these cells. The observed effect could be due spontaneous activity of one of any number of Gαi/o-coupled GPCRs expressed in these cells, including 5HT1A, ORL1 and CB1 receptors. Nonetheless, PTX is an important tool to confirm the role of receptor G protein coupling, especially in heterologous systems over-expressing one receptor.

Native μ-opioid receptors

Unlike many other GPCRs that couple to Gαi/o proteins such as the δ-opioid receptor (Costa and Herz, 1989) and the CB1 receptor (Howlett, 1994), it has proved difficult to show basal, agonist-independent, activation of [^{35}S]GTPγS binding G protein by μ-opioid receptors in naïve native tissues (Wang *et al.*, 2004; Raehal *et al.*, 2005) or often even in heterologous expression systems (Neilan *et al.*, 1999). This suggests that under normal physiological conditions there is insufficient receptor in active conformations to give a stimulation of [^{35}S]GTPγS binding that can be inhibited by ligands with negative efficacy. However, it has been demonstrated that the non-selective opioid antagonists 7-benzylidenenaltrexone (BNTX) and β-chlornaltrexamine (β-CNA) cause a small reduction in basal [^{35}S]GTPγS binding in homogenates of whole brain from wild-type mice but not their μ-opioid receptor knockout counterparts, suggesting constitutive activity of the native receptor Figure 2A (Wang *et al.*, 2004). Moreover, changes in the levels of basal, non-agonist stimulated [^{35}S]GTPγS binding have been reported in several cell lines in which the μ-opioid receptor has been over expressed. For example, an approximately 25 % increase in the basal [^{35}S]GTPγS signal is seen in HEK cells expressing a μ–opioid receptor (HEKμ) (Burford *et al.*, 2000) and a 67 % increase was reported in μ-opioid receptor expressing GH_3 cells (GH_3μ, (Liu *et al.*, 2001). By

contrast, no change was reported in μ-receptor expressing C6 glioma cells (C6μ; (Neilan *et al.,* 1999; Divin *et al.,* 2009). In general, the higher the μ-opioid receptor expression the higher the level of constitutive coupling to Gα proteins that should be observed due to increased chances of fruitful collisions occurring. However, GH_3μ cells show higher than basal [^{35}S]GTPγS binding at receptor expression levels similar to those found endogenously (0.4pmol/mg protein) and agonist-independent increases in [^{35}S]GTPγS binding have been reported in HEKμ cells expressing 1 pmol mg^{-1} receptor (Wang *et al.,* 2001). As mentioned above, the constitutive increase in basal [^{35}S]GTPγS binding is increased by removal of Na ions (Liu *et al.,* 2001) although in C6μ cells this still did not generate any constitutive activity that could be attributed to the μ-opioid receptor. The alkylating agent β-CNA inhibited the constitutive activation of [^{35}S]GTPγS binding in HEKμ cells, possibly by locking the receptor in an inactive conformation (Burford *et al.,* 2000), although this was not seen at the μ-opioid receptor in naïve GH_3 cells (Liu and Prather, 2001).

Insert Figure 2 about here

GTPγS binding after chronic agonist treatment

Chronic opioid agonist treatment produces the putative constitutively activated μ*-state of the μ-opioid receptor both in heterologous systems and following *in vivo* administration of opioid ligands (Connor, 2009). Studies in SH-SY5Y cells suggest this process involves receptor phosphorylation (Wang *et al.,* 1994). Thus, chronic treatment with agonists provides a strategy to increase the level of μ-opioid receptor constitutive activity from the rather low levels seen in naïve systems, or even to induce activity in silent systems. This makes it easier to identify compounds with inverse agonist activity. Many of these experiments have been

performed by assaying the activity of adenylyl cyclase (see below), but there are several examples that have used the [^{35}S]GTPγS binding assay.

Using brain membranes from mice, several studies found that 3 day treatment with morphine converts naltrexone and naloxone from ligands with a small positive efficacy to ones with modest negative efficacy, and this occurred in a region specific manner (Wang *et al.,* 2004; Raehal *et al.* 2005). The results suggest a induction of μ*-state receptors, however the measured responses were small, with a less than 10 % decrease in basal GTPγS binding produced by the inverse agonists. It should be noted that other studies have failed to see any increases in basal GTPγS activity after chronic morphine treatment of animals (e.g. Selley *et al.*, 1997). By contrast, basal binding of [^{35}S]GTPγS in GH_3μ cells, CHO and HEK 293 cells expressing μ-opioid receptors is increased considerably following chronic opioid treatment (Liu *et al.*, 2001; Wang *et al.,* 2007; Xu *et al.,* 2007) and there is a concomitant increase in the apparent negative efficacy of most tested antagonists (Figure 2B). Interestingly, chronic treatment with the peptide agonist [D-Ala^2,*N*-MePhe^4,Gly-ol]-enkephalin (DAMGO) did not produce a constitutively active μ-opioid receptor in C6 glioma cells as defined by increases in [^{35}S]GTPγS binding (Divin *et al.,* 2009), again highlighting the system dependence of μ opioid receptor constitutive activity. The induction of constitutive activity is ligand dependent in some studies but not others, for example Liu and Prather (2001) found that both DAMGO and morphine treatment induced constitutive activity in GH_3μ cells (although only DAMGO was reported to change basal [^{35}S]GTPγS binding), while Xu *et al*., (2007) found that herkinorin but not DAMGO pretreatment changed basal [^{35}S]GTPγS binding in CHO cells.

Of course, prolonged treatment with opioids can cause a number of changes in the cell including changes in receptor, Gα protein and accessory protein expression, and these should be born in mind when interpreting results (e.g. Liu and Prather, 2001; Xu *et al.,* 2007). Also, it is vital to demonstrate that the opioid used for chronic treatment is washed out of the preparation since residual drug will stimulate [^{35}S]GTPγS, thus giving a false impression that constitutive activity is present.

Constitutively active mutants

Experimental mutagenesis of the μ-opioid receptor has helped define regions that are important for stabilization of inactive receptor conformations as well as those that contribute to the active conformations responsible for coupling to G proteins. Some mutations appear to produce constitutively active receptors and as consequence it has been possible to use such mutations in the search for ligands that stabilize inactive conformations.

The DRY (Asp-Lys-Tyr) motif: This amino-acid sequence at the interface of TM3 and the second intracellular loop is highly conserved across GPCRs and is thought to be important for stabilization of inactive states of the receptor as well as to be playing a role in receptor activation (Rosenbaum *et al.,* 2009). Mutation of amino acids in this region leads to constitutive activity in many GPCRs. In the μ-opioid receptor the DRY motif is amino acids 164-166. Replacement of Asp^{164} with His produces a μ-opioid receptor that readily promotes a high degree of [^{35}S]GTPγS binding in the absence of agonist in both transiently transfected HEK293 cells and stably transfected CHO cells (Li *et al.,* 2001b). Indeed, addition of the efficacious agonist DAMGO to the mutant receptor produces little further increase in [^{35}S]GTPγS binding. Qualitatively similar activity is obtained with when Asp^{164} is replaced by Tyr, Glu, or Met (Li *et al.,* 2001b). The constitutive activity of these mutants is PTX-

sensitive and is seen at receptor levels close to physiological expression levels. Similar to the results in some studies of prolonged agonist treatment of wild-type μ-opioid receptors, naltrexone and naloxone acted as inverse agonists at the constitutively active mutants, but CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) and diprenorphine were neutral (Li *et al.*, 2001b). It is very important to note that expression of the constitutively active μ-opioid receptors was only detectable following prolonged incubation with high levels of naloxone (20 μM for 4 days). Naloxone stabilized the μ-opioid receptor protein and at the same time prevented constitutive internalization of the mutant receptor (Li *et al.*, 2001a).

Junction of intracellular loop 3 and transmembrane domain 6: Another site implicated in the stabilization of inactive conformations of GPCRs is a conserved XBBXXB sequence (where B is a basic amino-acid and X is non-basic) at the junction of intracellular loop 3 and transmembrane domain 6. In the μ-opioid receptor this sequence is Leu^{275}-Arg-Arg-Iso-Thr-Arg^{280}. The exchange of Thr^{279} for Lys in this sequence produces a μ-opioid receptor that shows increase basal activation of [^{35}S]GTPγS binding when stably expressed in CHO cells, although in this case there is also an additional DAMGO-induced increase (Huang *et al.*, 2001). This constitutively active μ-opioid receptor mutant behaves very similarly to the Asp^{164} mutants in that prolonged incubation with naloxone is needed for detectable protein expression and the constitutive activation of G protein is PTX sensitive.

C-terminal tail mutations: Replacement of both Cys^{348} and Cys^{353} in the C-terminal tail of the μ-opioid receptor also produces a receptor that shows increased basal activation of [^{35}S]GTPγS binding, 1.5-fold higher than wild-type receptors (Brillet *et al.*, 2003). DAMGO further increased [^{35}S]GTPγS binding, in a PTX sensitive manner. However, unlike the mutations at the junctions between transmembrane domains and intracellular loops,

spontaneous activity of the C-terminal mutant was not completely reversed by PTX, suggesting coupling to non-Gαi/o receptors or other means of promoting [^{35}S]GTPγS binding. The C-terminal mutant receptor is stable and expressed at high levels even without prolonged naloxone treatment, presumably because the C-terminus is not critical for maintaining a stability of the transmembrane domain helices. In the $Cys^{348}Cys^{353}$ double mutant naltrexone and naloxone were neutral agonists, but cyprodime and the CTAP analogue CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) showed inverse agonist activity (Brillet *et al.*, 2003).

The advantage of working with chronically μ-opioid treated systems or constitutively active mutant receptors is that they can express a high degree of constitutive activity with a large signal-to-noise ratio. On the other hand, the natural constitutively activated state(s) of the wild-type receptor may not be accurately or fully represented in these systems and so they may not identify compounds that might be inverse agonists at wild-type receptors. This problem is highlighted by the different inverse agonists identified using the different systems (Table 1). Although the molecular basis for these differences is unknown, a likely explanation for the distinct ligand efficacy profiles is that different receptor conformations are responsible for constitutive activity in the different systems, for example the constitutively active mutants do not mimic the chronic morphine-dependent μ*-state, and the DAMGO-treated system may be different from the herkinorin-treated system. Moreover, photoaffinity labelling studies have suggested that there may be differences in the profile of agonist-stimulated versus constitutively-stimulated Gα subunits (Liu *et al.*, 2001), implying that the agonist stimulated and constitutively active states may not be equivalent. This idea is reinforced by the mutant μ-opioid receptors, where constitutive activity is obtained with very different mutations - some that involve amino acids that play a role in movement of the transmembrane helices and others that causes changes in the C terminal tail downstream of helix movement.

Adenylyl cyclase assays

Adenylyl cyclase enzymes are a family of nine members (Sadana and Dessaur, 2009) that convert ATP to the second messenger cAMP. AC activity is stimulated by GTP-bound Gαs and inhibited by GTP-bound Gαi/o proteins. The AC assay is useful for studying constitutive activity and inverse agonism acutely, but since alterations are seen in the activity of the enzyme following chronic μ-opioid exposure changes in AC have been widely used in the characterization of the μ*-opioid receptor state. Resting levels of AC in a cell are generally low so to show effects of μ-opioids the direct AC activator forskolin is often included at a concentration between 10 μM and 30 μM (an approximate EC_{50} concentration) to stimulate AC activity. Alternatively, native Gαs-coupled receptors (e.g. PGE_2 or β-adrenergic receptors) can be activated by a suitable agonist to increase cAMP levels. Assays are run on adherent cells in culture medium (in the absence of serum) for between 5 and 15 mins in the presence of a phosphodiesterase inhibitor such as 3-isobutyl-1-methylxanthine (IBMX, 1 mM). Accumulation of cAMP is measured using a variety of methods including ligand binding, ELIZA, or time-resolved fluorescence resonance energy transfer. An example of a detailed assay can be found in (Clark and Traynor, 2004). Additionally, there are newer methods of analysis of cAMP, such as cAMP-mediated activation of cyclic nucleotide gated ion channels (Reinscheid *et al.*, 2003) and monitoring of cAMP signaling in neurons using genetically-encoded FRET probes that allow for real-time monitoring (Vincent *et al.*, 2008). An important advantage of cAMP measurements is that unlike G protein assays they can be made in intact cells, thereby avoiding alterations in membrane architecture and protein-protein interactions that might compromise constitutive activity. Moreover, activity at AC is downstream of G protein activation and thus benefits from amplification of the response.

In a system that expresses constitutively active Gαi/o-coupled receptors AC activity should be reduced. Although measurements of cAMP levels would appear to be an ideal assay for the of study constitutive activity it has seldom been employed to study basal constitutive activity or the constitutive activity of μ-opioid receptor mutants. In cells where the [^{35}S]GTPγS assay showed constitutive μ-opioid receptor signaling the basal (unstimulated by forskolin) level of cAMP was about half that seen with wild-type cells (Liu and Prather, 2001). The reduction in cAMP was inhibited by alkylation of the receptor with β-flunaltrexamine, this also reduced constitutive stimulation of [^{35}S]GTPγS binding (Liu and Prather, 2001). In striatal homogenates of the mouse basal cAMP levels were increased by β-CNA and BNTX (Wang *et al.* 2004). Similarly, in HEKμ cells forskolin-stimulated cAMP accumulation was increased by the irreversible antagonist β-CNA, the non-equilibrium antagonist clocinnamox, as well as BNTX, ligands that all showed negative efficacy in the [^{35}S]GTPγS assay in the same cells (Wang *et al.*, 2001).

More use has been made of assays for AC activity to define changes occurring as a result of chronic opioid exposure. Prolonged agonist treatment of μ-opioid receptor expressing cells produces cellular homeostatic changes that result in a sensitization of AC. Following challenge with an antagonist there is a rebound increase in cAMP levels (cAMP overshoot) that is absent in naïve cells (Watts and Neve 2005). This cAMP overshoot response is also seen in brain tissue from animals following chronic opioid treatment (Williams *et al.*, 2001) and has been linked to specific effectors mediating the opioid withdrawal syndrome (Bagley *et al.*, 2005b). Measures of the cAMP overshoot response in chronic morphine-dependent systems have been used to describe the μ*-state and to differentiate neutral antagonists from inverse agonists. Indeed, the first description of the μ*-state was provided by Wang and colleagues (Wang *et al.*, 1994) using this methodology. To obtain a cAMP overshoot

response cells are treated chronically with μ-opioid agonist (usually 10 μM for 24-48h), then the agonist is removed and/or the antagonist added together with an agent to stimulate AC activity (either a Gαs receptor coupled agonist or forskolin). In SH-SY5Y cells washout of chronic morphine resulted in an increase in cAMP levels compared to naive cells, but addition of naloxone resulted in a greater overshoot than simply washing off the morphine. The difference was attributed to the ability of naloxone to reverse the μ*-state form of the receptor back to the basal μ-state (Wang *et al.*, 1994). A similar degree of overshoot was seen with naltrexone and diprenorphine. In contrast the peptidic antagonists CTAP and CTOP did not increase overshoot to any greater degree than simply removing the morphine, and they blocked the effect of naloxone. Thus, as defined by this experimental protocol, naloxone, naltrexone and diprenorphine are inverse agonists that drive the μ*-state back to the resting receptor state whereas CTAP and CTOP are neutral antagonists. Similar findings have been shown in GH_3μ cells (Liu and Prather, 2001), and in HEKμ cells (Wang *et al.*, 2001). However, this effect of chronic agonists to produce a μ*-state of the receptor that is differentially sensitive to antagonists does not appear to be universal. In C6μ cells chronically exposed to morphine, all antagonists tested produced a degree of cAMP overshoot corresponding to that expected due to displacement of agonist from the receptor (Divin *et al.*, 2009) while in herkinorin or DAMGO-treated CHOμ cells CTAP and naloxone produced an equivalent degree of cAMP overshoot (Xu *et al.*, 2007).

While these experimental protocols are straightforward and appear in certain systems to provide a constitutive form of the μ-receptor, care has to be taken with the interpretation of results. In particular, complete washout of agonist has to be confirmed to ensure that findings are not the result of antagonism of residual agonist. In addition, if neutral antagonists and inverse agonists are identified then they should compete. Further, cAMP overshoot is seen

with prolonged activation of any Gαi/o coupled receptor (Watts and Neve, 2005), and can be induced after only minutes of treatment (Levitt *et al.,* 2009) so there is no guarantee that a rebound increase in cAMP levels actually represents a specific change in opioid receptor activity-dissociating these changes from those reflecting changes in the enzyme itself or other modulators of GPCR signaling is a challenge. Finally, as with the [^{35}S]GTPγS studies, whilst these experiments can inform about changes induced by chronic treatment of cells/tissues expressing μ-opioid receptors, they may not provide information on constitutively active receptors formed under other conditions.

Measuring constitutive activity using ion channels

The actions of Gα or Gβγ subunits on ion channels provide another readily measurable cellular effect that is one step removed from μ-opioid receptor activation of G-protein heterotrimers. For Gi/Go-coupled receptors, including μ-opioid receptors, the most easily measured of these interactions are the inhibition of voltage dependent calcium channels (I_{Ca}) and activation of G protein gated inwardly rectifying potassium channels (GIRK). Both processes involve the direct binding of Gi/Go-derived βγ subunits to the channel proteins, and both processes have been used as sensitive probes for changes in μ-opioid receptor activity. The excess of Gi/Go-proteins over μ-opioid receptors (Sternweis and Robishaw, 1984; Selley *et al.,* 1998) and the relatively low abundance of ion channels in neurons means that the amplification of receptor coupling to I_{Ca} and GIRK is significant, as demonstrated by the large receptor reserve for these processes (Christie *et al.,* 1987; Connor *et al.,* 1999).

Calcium channels

Although there are several pathways by which Gi/Go coupled receptors can inhibit I_{Ca}, most attention has focussed on the rapid, reversible and voltage dependent inhibition of N-type

($Ca_V2.2$) and P/Q-type ($Ca_V2.1$) I_{Ca} by G protein βγ subunits (Ikeda, 1996; Herlitze *et al.*, 1996). This pathway is defined by a characteristic voltage-dependence of the interaction between the Gβγ subunits and the channels-when the cell membrane is strongly depolarised, the Gβγ inhibition of I_{Ca} is transiently removed. This means that an index of channel inhibition can be obtained by comparing current amplitude before and after a strong depolarisation (see Figure 3). The ratio (S2:S1) of the current amplitude evoked by the control step (S1) and the step after the conditioning depolarization (S2) is often used a measure of the amount of voltage-dependent inhibition, when inhibition is present the I_{Ca} amplitude after the depolarisation is larger, and the ratio correspondingly greater. Importantly, the double pulse protocol (Figure 3) can detect constitutive activation of the voltage-dependent inhibitory pathway, in this case the basal ratio is greater than 1. Another feature of Gβγ inhibition of N- and P/Q-type I_{Ca} is an apparent slowing of channel activation when βγ subunits are bound (Ikeda, 1996; Herlitze *et al.*, 1996). Although the mechanisms underlying this slowing of whole cell P/Q- and N-type channels are distinct (Colecraft *et al.*, 2001), strong depolarisation reverses the slowing, providing another measure of relief from Gβγ inhibition. The facilitation of N-type currents by a depolarising conditioning step is usually more profound than that of P/Q-type channels, reflecting the larger inhibition of these channels by the Gβγ pathway.

Insert Figure 3 about here

The constitutive activity of several receptors has been studied using facilitation of I_{Ca} as the key assay (Pan *et al.*, 1998; Beedle *et al.*, 2004; Guo and Ikeda, 2004; Mahmoud *et al.*, 2010). In these experiments, which have largely been performed in sympathetic neurons, microinjection of appropriate receptor mRNA increases the basal facilitation ratio and this

increase is reversed by superfusion of appropriate antagonists, which also increase the absolute amplitude of the I_{Ca}. The constitutive activity of the opioid-related ORL1 receptor (NOP) has been explored in some detail in both tsA-201 HEK-293 cells and sympathetic neurons (Beedle *et al.,* 2004, Mahmoud *et al.,* 2010), with the latter study also including a few experiments on neurons injected with μ-opioid receptor mRNA (Mahmoud *et al.,* 2010; see Figure 8). In these experiments, the μ-opioid receptor ligand CTAP increased the amplitude of the N-type I_{Ca} and reduced the facilitation ratio, while DAMGO produced a further inhibition of the I_{Ca}. This shows that high levels of receptor expression can induce constitutive activity detectable at a single cell level, without use of ligands.

The detection of the constitutive activity of native receptors using I_{Ca} assays is much more unusual (Pan *et al.,* 1998). In general, little or no facilitation of baseline I_{Ca} by a positive conditioning step is observed in native cells (e.g. Connor *et al.,* 1999; Borgland *et al,* 2001; Bagley *et al.,* 2005a), although a careful study reported modest facilitation in a subpopulation of small dorsal root ganglion neurons (Beedle *et al.,* 2004). In a small population of rat pelvic ganglion neurons expressing native cannabinoid CB1 receptors, the CB1 ligand SR 141716A was shown to inhibit tonic activation of the Gβγ pathway, probably by inhibiting constitutively active CB1 receptors (Pan *et al,* 1998). The only example of constitutive inhibition of I_{Ca} by native μ-opioid receptors was demonstrated in cultured dorsal root ganglion neurons from arrestin3 knockout mice (Walwyn *et al.,* 2007). In these neurons the basal facilitation ratio of I_{Ca} was around 1, but this ratio was reduced to about 0.8 by naltrexone. The effects of naltrexone were blocked by CTAP, indicating that they were mediated via the μ receptor. In neurons from wild type mice, naltrexone had no effect on basal I_{Ca}. This study demonstrates the utility of the electrophysiological approach, even in cells with native levels of receptor expression.

The putative constitutively active μ*-state of the μ-opioid receptor should also produce a tonic inhibition of I_{Ca} in appropriate neurons, assuming that it activates a similar subset of G proteins as agonist-activated receptors. However, no differences in facilitation ratio were found between locus coeruleus neurons from chronically morphine treated (CMT) and vehicle rats (Connor *et al.,* 1999), or in periaqueductal gray neurons from CMT and vehicle mice (Bagley *et al.,* 2005a). Both cell types exhibit an increased facilitation ratio and I_{Ca} activation when opioid agonists are superfused, indicating that the molecular machinery appropriate for expression of constitutive activity is present. Similarly, superfusion of naloxone and simply washing off morphine produced an equivalent increase in I_{Ca} in chronically morphine treated SH-SY5Y cells, indicating that μ*-state receptors could not be detected using electrophysiology in this preparation (Kennedy and Henderson, 1992).

The Gβγ subunit modulation of I_{Ca} is ubiquitous and easy to measure using standard whole cell patch clamp techniques. There are, however, a few possible confounds that need to be kept in mind. Firstly, many studies have shown that depolarising steps can facilitate I_{Ca} independently of the Gβγ pathway, although the I_{Ca} affected is usually L- ($Ca_V1.x$) or P/Q-type rather than N-type (Dolphin 1996). The facilitation can be associated with channel phosphorylation (Dolphin 1996) or be mediated by a direct effect of Ca (Chaudhuri *et al.,* 2007). Ca-dependent facilitation can be minimized without interfering with Gβγ-mediated channel inhibition by using Ba as the charge carrying cation (Chaudhuri *et al.,* 2007). Conversely, it can be difficult to obtain a basal facilitation ratio of around 1, owing to the propensity of I_{Ca} to desensitise. Ca-dependent desensitization can again be reduced by using Ba as a charge carrier while voltage-dependent processes can be attenuated by limiting the duration and amplitude of the test steps and the conditioning depolarisation, and by allowing

sufficient time between pulse sets to minimize the accumulation of channel desensitization. We and others have found that a conditioning depolarisation to +80 mV for 50-80 ms is sufficient to produce a robust facilitation of μ-receptor inhibited I_{Ca} in a variety of central and peripheral neurons, without any significant channel desensitization (Connor *et al.,* 1999; Bagley *et al.,* 2005a; Walwyn *et al.,* 2007; Mahmoud *et al.,* 2010). Of course, it is also possible that a Gβγ-mediated inhibition of I_{Ca} results from constitutive activity of G-proteins in a cell, quite independent of any altered G-protein/receptor coupling. Given that Go-type G proteins are much more abundant than either μ-opioid receptors or I_{Ca}, small changes in basal G-protein activity could produce significant effects on I_{Ca}, although in this situation the facilitation would not be sensitive to receptor antagonists.

Another possible pitfall is a change in the mix of I_{Ca} produced by a treatment. For example, in trigeminal ganglion neurons from morphine-treated mice there is a significant reduction in the amount of P/Q-type I_{Ca} (Johnson *et al.,* 2006). If there were constitutively active receptors present in these cells, the basal facilitation ratio may actually increase, because the contribution of N-type I_{Ca} to the total current is greater, and N-type I_{Ca} are inhibited more effectively by the Gβγ pathway (Bourinet *et al.,* 1996; Connor and Christie, 1998).

Potassium channels

GIRK (Kir 3.x) channels are directly gated by Gβγ subunits, and they also provide an easily measurable output very closely tied to G protein activation. Unlike measuring I_{Ca}, which is episodic and rarely sustainable for more than about 30 minutes, continuous recordings of current or membrane potential can be used to monitor GIRK activity for periods of hours. GIRK currents are also most readily measured in brain slices, preparation of which generally requires less tissue handling or exposure to unphysiological solutions than dissociation (and

culturing) of neurons for recording I_{Ca}. There are a few reports of constitutively active GIRK in mammalian cells (Chen and Johnston, 2005; Dobrev *et al.*, 2005), but we are not aware that constitutive activity associated with a specific GPCR has been detected in a mammalian cell using measurements of GIRK function. Several studies have examined coupling of GPCR to GIRK in sympathetic neurons and HEK 293 cells transfected with both receptors and channels, and constitutive activation of heterologously expressed GIRK was small and infrequently observed (Ruiz-Velasco and Ikeda, 1998; Guo and Ikeda, 2004; Johnson *et al.*, 2006).

There are several reasons why constitutively active GIRK may be rarely reported, and activation of GIRK mediated by constitutively active receptors not at all. Firstly, maximal Gβγ activation of the GIRK channels requires binding of multiple Gβγ subunits (Sadja *et al.*, 2002), which is in contrast to the single βγ subunit required for inhibition of I_{Ca} (Dascal, 2001). Thus, more constitutively active receptors may be required to produce a detectable Gβγ signal at GIRK than at I_{Ca}. Secondly, there is no obvious ligand-free strategy for detecting basally active GIRK currents; there are no specific GIRK channel blockers and Gβγ modulation of the currents is not intrinsically voltage-dependent (Doupnik *et al.*, 1995).

Despite the necessity of using ligands to detect constitutive receptor activation of GIRK, μ*-state stimulation of GIRK should be apparent when opioids which inhibit μ*-state signalling are superfused onto neurons from chronically morphine treated animals. Reversal of μ*-state activity would be seen as ligand-precipitated closing of a K conductance or a membrane depolarization. This has not been observed in studies where naloxone has been washed onto brain slices from morphine treated animals (Christie *et al.*, 1987), and studies that have reported opioid antagonist-mediated depolarization in PAG have attributed this to non-GIRK

conductances (Chieng *et al.,* 1996; Bagley *et al.,* 2005b). Opioid receptor modulation of cAMP-regulated conductances or neurotransmitter release onto neurons in brain slices are a potential source of significant confounds in studies of constitutive activity in these preparations.

Conclusions

Constitutive activity reflects fundamental properties of GPCR. μ-opioid ligands are extremely important therapeutic drugs, with significant unwanted effects and a better understanding of constitutive activity at the μ-opioid receptor may provide information useful in developing ligands that access subsets of receptor conformations, offering the potential to fine tune opioid pharmacotherapy. The unique role suggested for the putative μ*-state of the receptor also provides an intriguing possibility for therapeutic exploitation. While readily amenable to standard methods of measuring constitutive activity, μ-opioid receptors also couple to G proteins that modulate ion channels, providing the opportunity for studying receptor states in single neurons in real time. Together with emerging techniques for relatively direct studies of conformational states within GPCR (Yao *et al.* 2009), and access to purified μ-opioid receptors (Kuszak *et al.* 2009), the methods outlined in this Chapter should provide the tools for many fruitful investigations of this intriguing property of the μ-opioid receptor.

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References

Bagley, E. E., Chieng, B. C. H., Christie M. J., and Connor, M. (2005a). Opioid tolerance in periaqueductal gray neurons isolated from mice chronically treated with morphine. *Brit. J. Pharmacol.* **146**, 68-76.

Bagley, E. E., Gerke, M. B., Vaughan, C. W., Hack, S. P., and Christie, M. J. (2005). GABA transporter currents activated by protein kinase A excite midbrain neurons during opioid withdrawal. *Neuron* **45**, 433-445.

Beedle, A. M., McRory, J. E., Poirot, O., Doering, C. J., Altier, C., Barrere, C., Hamid, J., Nargeot, J., Bourinet, E., and Zamponi, G. W. (2004). Agonist-independent modulation of N-type calcium channels by ORL1 receptors. *Nat. Neurosci.* **7**, 118-125.

Borgland, S. L., Connor, M., and Christie, M. J. (2001). Nociceptin inhibits calcium channel currents in a subpopulation of small nociceptive trigeminal ganglion neurons in mouse. *J. Physiol.* **536**, 35-47.

Brillet, K., Kieffer, B. L., and Massotte, D. (2003). Enhanced spontaneous activity of the mu opioid receptor by cysteine mutations: characterization of a tool for inverse agonist screening. *BMC Pharmacol.* **3**, 14.

Breivogel, C. S., Walker, J.M., Huang, S.M., Roy, M.B., and Childers, S.R. (2004). Cannabinoid signaling in rat cerebellar granule cells: G-protein activation, inhibition of glutamate release and endogenous cannabinoids. *Neuropharmacology* **47**, 81-91.

Burford, N.T., Wang, D., and Sadee, W. (2000). G-protein coupling of μ-opioid receptors (OP_3): elevated basal signalling activity. *Biochem. J.* **348**, 531-537.

Cerione, R. A., Codina, J., Benovic, J. L., Lefkowitz, R. J., Birnbaumer, L., and Caron, M. G. (1984). The mammalian β2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry* **23**, 4519-4525.

Chaudhuri, D., Issa, J. B. and Yue, D. T. (2007). Elementary mechanisms producing facilitation of $Ca_V2.1$ (P/Q-type) channels. *J. Gen. Physiol.* **129**, 385-401.

Chen X., and Johnston, D. (2005). Constitutively active G-protein-gated inwardly rectifying K^+ channels in dendrites of hippocampal CA1 pyramidal neurons. *J. Neurosci.* **25**, 3787-3792.

Chieng, B., and Christie, M. J. (1996). Local opioid withdrawal in rat single periaqueductal gray neurons in vitro. *J. Neurosci.* **16**, 7128-36.

Christie, M. J., Williams, J. T. and North, R. A. (1987). Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Mol. Pharmacol.* **32**, 633-638.

Clark, M. J. and Traynor, J. R. (2004). Assays for G-protein-coupled receptor signaling using RGS-insensitive Galpha subunits. *Methods Enzymol.* **389**, 155-169.

Colecraft, H. M., Brody, D. L., and Yue D. T. (2001). G-protein inhibition of N- and P/Q-type calcium channels: distinctive elementary mechanisms and their functional impact. *J. Neurosci.* **21**, 1137-1147.

Connor, M. (2009). Shadows across μ-star? Constitutively active μ-opioid receptors revisited. *Br. J. Pharmacol.* **156**, 1041-1043.

Connor, M., Borgland, S. L., and Christie, M. J. (1999). Continued morphine modulation of calcium channel currents in acutely isolated locus coeruleus neurons from morphine-dependent rats. *Brit. J. Pharmacol.* **128**, 1561-1569.

Costa T., and Herz A. (1989). Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* **86**, 7321-7325.

Dascal, N. (2001). Ion-channel regulation by G proteins. Trends. Endocrin. Metab. 12, 391-398.

DeLapp, N. W. (2004). The antibody-capture [^{35}S]GTPγS scintillation proximity assay: a powerful emerging technique for analysis of GPCR pharmacology. *Trends Pharmacol. Sci.* **25**, 400-401.

Divin, M. F., Bradbury, F. A., Carroll F. I., and Traynor, J. R. (2009). Neutral antagonist activity of naltrexone and 6β-naltrexol in naive and opioid-dependent C6 cells expressing a μ-opioid receptor. *Br. J. Pharmacol.* **156**, 1044-1053.

Dobrev, D., Friedrich, A., Voight, N., Jost, N., Wettwer, E., Christ, T., Knaut, M., and Ravens, U. (2005). The G protein-gated potassium current IK,ACH is constitutively active in patients with chronic atrial fibrillation. *Circulation* **112**, 3697-3706.

Dolphin, A. C. (1996). Facilitation of Ca^{2+} current in excitable cells. *Trends Neurosci.* **19**, 35-43.

Doupnik, C. A., Lim, N. F., Kofuji, P., Davidson, N., and Lester, H. A. (1995). Intrinsic gating properties of a cloned G protein-activated inward rectifier K^+ channel. *J. Gen. Physiol.* **106**, 1-23.

Guo, J., and Ikeda, S. R. (2004). Endocannabinoids modulate N-type calcium channels and G-protein-coupled inwardly rectifying potassium channels via CB1 cannabinoid receptors heterologously expressed in mammalian neurons. *Mol. Pharmacol.* **65**, 665-674.

Harrison, C. and Traynor, J. R. (2003). The [^{35}S]GTPgammaS binding assay: approaches and applications in pharmacology. *Life Sci.* **74**, 489-508.

Herlitze, S., Garcia, D. E., Mackie, K., Hille, B., Scheuer, T., and Catterall, W. A. (1996). Modulation of Ca^{2+} channels by G-protein βγ subunits. *Nature* **380**, 258-262.

Horswill, J. G., Bali, U., Shaaban, S., Kelly, J.F., Jeevaratnam P, Babbs, A.J., Reynet, A.J., In, P.W.K. (2007). PSNCBAM-1, a novel allosteric antagonist at cannabinoid CB1 receptors with hypophagic effects in rats. *Br. J. Pharmacol* **152**, 805-814.

Howlett, A. C. (2004). Efficacy in CB1 receptor-mediated signal transduction. *Brit. J. Pharmacol.* **142**, 1209-1218.

Huang, P., Li, J., Chen, C., Visiers, I., Weinstein, H., and Liu-Chen, L. Y. (2001). Functional role of a conserved motif in TM6 of the rat mu opioid receptor: constitutively active and inactive receptors result from substitutions of Thr6.34(279) with Lys and Asp. *Biochemistry* **40**, 13501-13509.

Ikeda, S. R. (1996). Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. *Nature* **380**, 255-258.

Johnson, E. A., Oldfield, S., Brakstor, E., Gonzalez-Cuello, A., Couch, D., Hall, K. J., Mundell, S. J., Bailey, C. P., Kelly, E., and Henderson, G. (2006). Agonist-selective mechanisms of μ-opioid receptor desensitization in human embryonic kidney 293 cells. *Mol. Pharmacol.* **70**, 676-685.

Kara, E., and Strange. P. (2010). Use of the [35S]GTPγS binding assay to determine ligand efficacy at G-protein coupled receptors. In: Poyner, D., Wheatley, M. G protein-coupled receptors: essential methods.Wiley-VCH.

Kenakin T. (2004). Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol. Pharmacol.* **65**, 2-11.

Kenakin T., and Onaran O. (2002). The ligand paradox between affinity and efficacy: can you be there and not make a difference ? *Trends Pharmacol. Sci.* **23**, 275-280.

Kennedy, C., and Henderson, G. (1992). Chronic exposure to morphine does not induce dependence at the level of the calcium current in human SH-SY5Y cells. *Neuroscience* **49**, 937-944.

Kuszak, A.J., Pitchiaya, S., Anand, J.P., Mosberg, H.I., Walter, N.G., and Sunahara, R.K. (2009). Purification and functional reconstitution of monomeric mu-opioid receptors: allosteric modulation of agonist binding by Gi2. *J. Biol. Chem.* **284**, 26732-26741.

Labrecque, J., Wong, R.S., and Fricker, S.P. (2009). A time-resolved fluorescent lanthanide (Eu)-GTP binding assay for chemokine receptors as targets in drug discovery. *Methods Mol. Biol.* **552**, 153-169.

Levitt, E. S., Clark, M.J., Jenkins, P.M., Martens, J.R., and Traynor, J.R. (2009). Differential effect of membrane cholesterol removal on mu- and delta-opioid receptors: a parallel comparison of acute and chronic signaling to adenylyl cyclase. *J. Biol. Chem.* **284**, 22108-22122.

Li, J., Chen, C., Huang, P., and Liu-Chen, L-Y. (2001a). Inverse agonist up-regulates the constitutively active D3.49(164)Q mutant of the rat μ-opioid receptor by stabilizing the structure and blocking constitutive internalization and down-regulation. *Mol. Pharmacol.* **60**, 1064-1075.

Li, J., Huang, P., Chen, C., de Reil, J. K., Weinstein, H., and Liu-Chen, L-Y. (2001b). Constitutive activation of the mu opioid receptor by mutation of D3.49(164), but not

D3.32(147): D3.49(164) is critical for stabilization of the inactive form of the receptor and for its expression. *Biochemistry* **40**, 12039-12050.

Lin, H., Saisch, S.G., Strange, P.G. (2006). Assays for enhanced activity of low efficacy partial agonists at the D(2) dopamine receptor. *Br J Pharmacol* **149**, 291-299.

Liu, J-G., and Prather, P. L. (2001). Chronic exposure to μ-opioid agonists produces constitutive activation of the μ-opioid receptors in direct proportion to the efficacy of the agonist used for pretreatment. *Mol. Pharmacol.* **60**, 53-62.

Liu, J. G., Ruckle, M. B., and Prather, P. L. (2001). Constitutively active μ-opioid receptors inhibit adenylyl cyclase activity in intact cells and activate G-proteins differently than the agonist [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin. *J. Biol. Chem.* **276**, 37779-37786.

Mahmoud, S., Margas, W., Trapella, C., Calo, G., and Ruiz-Velasco, V. (2010). Modulation of silent and constitutively active nociceptin/orphanin FQ receptors by potent receptor antagonists and Na^+ ions in rat sympathetic neurons. *Mol. Pharmacol*. **77**, 804-817.

Neilan, C. L., Akil, H., Woods, J. H,. and Traynor, J. R. (1999). Constitutive activity of the δ-opioid receptor expressed in C6 glioma cells: identification of non-peptide δ-inverse agonists. *Br. J. Pharmacol.* **128**, 556-562.

Pan, X., Ikeda, S.R., and Lewis , D. L. (1998). SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca^{2+} currents by reversal of tonic CB1 cannabinoid receptor activity. *Mol. Pharmacol.* **54**, 1064-1072.

Raehal, K. M., Lowery, J. J., Bhamidipati, C. M., Paolino, R. M., Blair, J. R., Wang, D., Sadee, W., and Bilsky, E. J. (2005). In vivo characterization of 6beta-naltrexol, an opioid ligand with less inverse agonist activity compared with naltrexone and naloxone in opioid-dependent mice. *J. Pharmacol. Exp. Ther.* **313**, 1150-1162.

Reinsheid, R. K., Kim, J., Zeng, J., and Civelli, O. (2003). High-throughput real-time monitoring of Gs-coupled receptor activation in intact cells using cyclic-nucleotide gated channels. *Eur. J. Pharmacol.* **478**, 27-34.

Roberts, D. J., and Strange, P. G. (2005). Mechanisms of inverse agonist action at D2 dopamine receptors. *Br. J. Pharmacol.* **145**, 34-42.

Rosenbaum, D. M., Rasmussen, S. G., and Kobilka B. K. (2009). The structure and function of G-protein-coupled receptors. *Nature* **459**, 356-363.

Ruiz-Velasco, V., and Ikeda, S.R. (1998). Heterologous expression and coupling of G protein-gated inwardly rectifying K^+ channels in adult rat sympathetic neurons. *J Physiol.* **513**, 761-773.

Sadana, R., and Dessauer, C. W. (2009). Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. *Neurosignals* **17**, 5-22.

Sadee, W., Wang, D., and Bilsky, E. J. (2005). Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. *Life Sci.* **76**, 1427-1437.

Sadja, R., Alagem, N., and Reuveny, E. (2002). Graded contribution of the Gβγ binding domains to GIRK channel function. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 10783-10788.

Sally, E. J., Xu, H., Dersch, C. M., Hsin, L.-W., Chang, L.-T., Prisinzano, T. E., Simpson, D. S., Giuvelis, D., Rice, K. C., Jacobson, A. E., Cheng, K., Bilsky, E. J., and Rothman, R. B. (2010). Identification of a novel "almost neutral" μ-opioid receptor antagonist in CHO cells expressing the cloned μ-opioid receptor. *Synapse* **64**, 280-288.

Selley, D. E., Liu, Q., and Childers, S. R. (1998). Signal transduction correlates of *mu*-opioid agonist intrinsic efficacy: receptor-stimulated [^{35}S]-GTPγS binding in mMOR-CHO cells and rat thalamus. *J. Pharmacol. Exp. Ther.* **285**, 496-505.

Selley, D. E., Nestler, E. J., Brievogel, C. S., and Childers, S. R. (1997). Opioid receptor-coupled G proteins in rat locus coeruleus membranes: decrease in activity after chronic morphine treatment. *Brain Res.* **746**, 10–18.

Smit, M. J., Vischer, H. F., Bakker, R. A., Jongejan, A., Timmerman, H., Pardo, L., and Leurs, R. (2007). Pharmacogenomic and structural analysis of constitutive G protein-coupled receptor activity. *Annu. Rev. Pharmacol. Toxicol.* **47**, 53-87.

Szekeres, P. G. and Traynor, J.R. (1997). Delta opioid modulation of the binding of guanosine-5'-O-(3-[35S]thio)triphosphate to NG108-15 cell membranes: characterization of agonist and inverse agonist effects. *J. Pharmacol. Exp. Ther.* **283**, 1276-1284.

Traynor, J. R., and Nahorski, S. R. (1995). Modulation by μ-opioid agonists of guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol*. **47**, 848-854.

Vincent, P., Gervasi, N., and Zhang, J. (2008). Real-time monitoring of cyclic nucleotide signalling in neurons using genetically encoded FRET probes. *Brain Cell Biol*. **36**, 3-17.

Walwyn, W., Evans, C. J., and Hales, T. G. (2007). β-Arrestin and c-Src regulate the constitutive activity and recycling of μ-opioid receptors in dorsal root ganglion neurons. *J. Neurosci.* **27**, 5092-5104.

Wang, D., Raehal, K. M., Bilsky, E. J., and Sadee, W. (2001). Inverse agonists and neutral antagonists at μ opioid receptor (MOR): possible role of basal receptor signaling in narcotic tolerance. *J. Neurochem.* **77**, 1590-1600.

Wang, D., Raehal, K. M., Lin, E. T., Lowery, J. J., Keiffer, B. L., Bilsky, E. J., and Sadee, W. (2004). Basal signalling activity of μ opioid receptor in mouse brain: role in narcotic dependence. *J. Pharmacol. Exp. Ther.* **308**, 512-520.

Wang, D., Sun, X., and Sadee, W. (2007). Different effects of opioid antagonists on μ-, δ-, and κ-opioid receptors with and without agonist pretreatment. *J. Pharmacol. Exp. Ther*. **321,** 544-552.

Wang, Z., Bilsky, E.J., Porreca, F., and Sadee, W. (1994). Constitutive μ opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci.* **54**, PL339-350.

Watts, V. J. and Neve, K. A. (2005). Sensitization of adenylate cyclase by Gαi/o-coupled receptors. *Pharmacol. Ther.* **106**, 405-421.

Williams, J. T., Christie, M. J., and Manzoni, O. (2001). Cellular and synaptic adaptations mediating opioid withdrawal. *Physiol. Rev.* **81**, 299-343.

Xu, H., Partilla, J. S., Wang, X., Rutherford, J. M., Tidgewell, K., Prisinzano, T. E., Bohn, L. M., and Rothman, R. B. (2007). A comparison of noninternalizing (herkinorin) and internalizing (DAMGO) μ-opioid agonists on cellular markers related to opioid tolerance and dependence. *Synapse* **61**, 166-175.

Yao, X. J., Velez Ruiz, G., Whorton, M.R., Rasmussen, S.G.F., DeVree, B.T., Deupi, X., Sunahara, R.K., and Kobilka, B. (2009). The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. *Proc. Natl. Acad. Sci. U S A* **106**, 9501-9506.

Figure Legends

Figure 1

Common (and potential) assay points for constitutively active μ-opioid receptors. A diagram of where μ-opioid receptor activity can be assayed relatively straightforwardly, with respect to the G protein cycle. Brief comments about the nature of each assay are included.

Figure 2

Detection of constitutively active μ-opioid receptors using the [^{35}S]GTPγS assay.

A) Basal [^{35}S]GTPγS binding in homogenates (10μg) of whole brains from wild-type (MOR +/+) or μ-opioid receptor knockout (MOR-/-) mice in the absence or presence of the opioid antagonists β-CNA (1 μM) or BNTX (10μM). β-CNA and BNTX reduced basal binding only in brain homogenates from the wild-type animals (**$p < 0.01$). The assays were performed using 0.1nM [^{35}S]GTPγS in the presence of 10μM GDP, 100mM NaCl and 4mM $MgCl_2$ for 30 mins at 30^{o}C as described in (Wang *et al.* 2004). B) In homogenates of untreated HEKμ cells (5μg), BNTX (1μM) acts as an inverse agonist to reduce basal [^{35}S]GTPγS binding; naloxone (10μM) and 6β-naltrexol (10μM) reverse the effect of BNTX. In DAMGO-treated HEKμ cells (5μg) (1μM, 24h) naloxone is converted to an inverse agonist, but 6β-naltrexol is still a neutral antagonist (**$p < 0.01$; *$p < 0.0$). Assays were performed as above but in the presence of 150mM KCL and 1 mM $MgCl_2$ (Wang *et al.* 2007). It should be noted from a practical standpoint that the differences observed are very small and the assay components critical, emphasizing the low level of basal μ-opioid receptor constitutive activity and the difficulty of studying this phenomenon. The figure was redrawn from data in Wang *et al*., (*J. Pharmacol. Exp. Ther*. 308, 512-520, 2004) 2004 (A) and Wang *et al*., (*J. Pharmacol. Exp. Ther*. 321, 544-552, 2007), http://www.jpet.aspetjournals.org, with permission.

Figure 3

Detection of constitutively active μ-opioid receptors using voltage-gated calcium channels as a reporter. These patch clamp recordings were made from cultured sympathetic neurons injected with μ-opioid receptor mRNA. I_{Ca} were elicited using a double pulse protocol, neurons were stepped from -80mV to +10 mV to elicit control currents (prepulse) and then βγ subunit-mediated inhibition of the channels was transiently relieved by a long step to +80 mV, the current amplitude was retested by another step to +10 mV following a brief rest at -80 mV (postpulse). **A)** A representative time course of a typical experiment showing plotting pre- and postpulse amplitudes. **B)** Representative current traces illustrating the effects of superfusion of the μ-opioid antagonist CTAP and the agonist DAMGO. Note that even before drug application the amplitude of the postpulse is greater than that of the prepulse, indicating constitutive inhibition of I_{Ca}, which is reversed by the highly selective μ-opioid ligand CTAP and mimicked by a subsequent application of the agonist DAMGO. The Figure is based on Figure 8 in Mahmoud *et al.*, (*Mol. Pharmacol.* **77**, 840-817, 2010, used with permission), with the traces kindly provided by Dr Victor Ruiz-Velasco.

Table 1: The activity of ligands for the μ-opioid receptor as modulators of [^{35}S]-GTPγS binding in basal and agonist pretreated conditions, and to constitutively active mutants of the μ-opioid receptor. Ligand efficacy is reported as positive (stimulates binding), negative (inhibits binding) or neutral (no significant effect on binding) according to the results presented in the cited studies. Because of differing experimental conditions and methods of presenting data, we have assigned just direction to efficacy, not a strength. Not all ligands tested in these studies are reported here, full references can be found at the end of the manuscript.

Drug	Basal	Agonist pretreated ("μ*")	Constitutively active mutants	Comment
naloxone	positive [b,e,i,j] neutral [a,c,d,g] negative [h]	neutral [c] negative [e,f,h,i,j]	negative, D164Q [d] neutral C348/353A [a]	
naltrexone	positive [i] neutral [a,b,d] negative [h]	neutral [c] negative [f,h,i,j]	negative, D164Q [d] neutral C348/353A [a]	
6β-naltrexol	weak positive [i] neutral [c,h]	neutral [h] negative [f]		
CTAP	neutral [c,d,e,h]	neutral [c,e,h,j] negative [f]	neutral, D164X [d]	CTAP had negative efficacy in both conditions in Hi K buffer in [c]
CTOP	positive [a]	neutral [j]	negative, C348/353A [a]	
β-flunaltrexamine	neutral [h]	neutral [h]		alkylating agent
β-chlornaltrexamine	neutral [e] negative [b,h]	negative [e,h]		alkylating agent
BNTX	negative [h,i]	negative [f,h,i]		poorly correlated with μ-receptor affinity [h,i]
naltrindole	positive [a]	negative [f]	neutral, C348/353A [a]	
diprenorphine	neutral [d]	negative [f,j]	neutral, D164Q [d]	
cyprodime	positive [a]		negative, C348/353A [a]	

a) Brillet *et al.,* 2003
b) Burford *et al.,* 2000
c) Divin *et al.,* 2009
d) Li *et al.,* 2001a
e) Liu and Prather 2001
f) Sally *et al.,* 2010
g) Traynor and Nahorski, 1995
h) Wang *et al.,* 2001
i) Wang *et al.,* 2007
j) Wang *et al.,* 1994

Figure 1

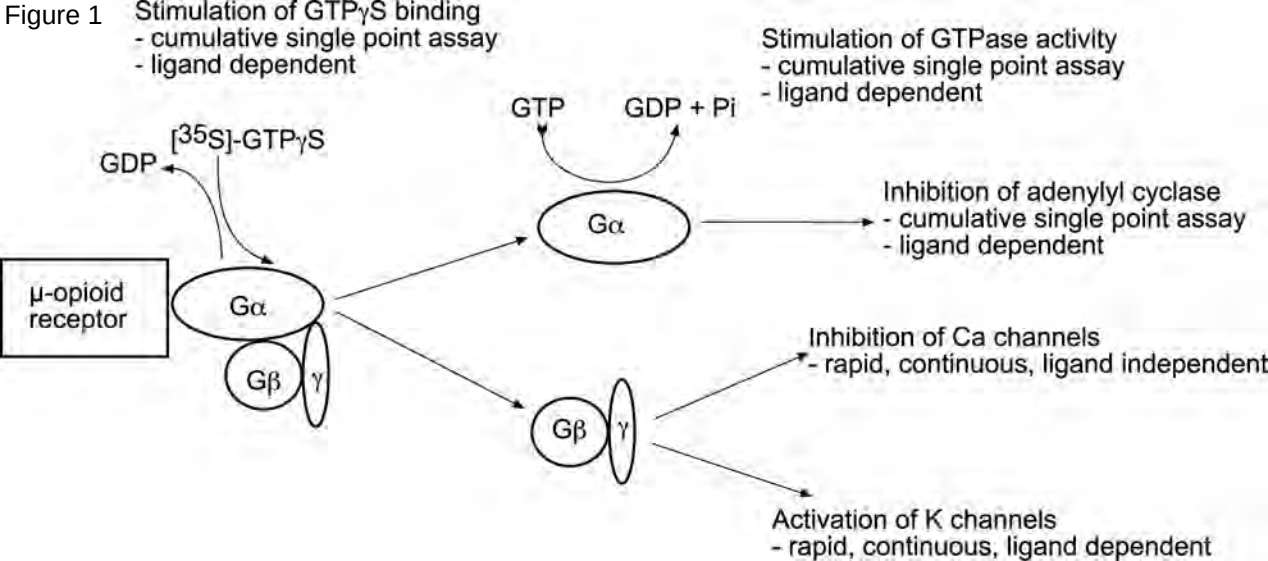

Stimulation of GTPγS binding
- cumulative single point assay
- ligand dependent
Stimulation of GTPase activity
- cumulative single point assay
- ligand dependent
GTP
GDP + Pi
[35S]-GTPγS
GDP
Gα
Inhibition of adenylyl cyclase
- cumulative single point assay
- ligand dependent
μ-opioid receptor
Gα
Gβ
γ
Inhibition of Ca channels
- rapid, continuous, ligand independent
Gβ
γ
Activation of K channels
- rapid, continuous, ligand dependent

Figure 2

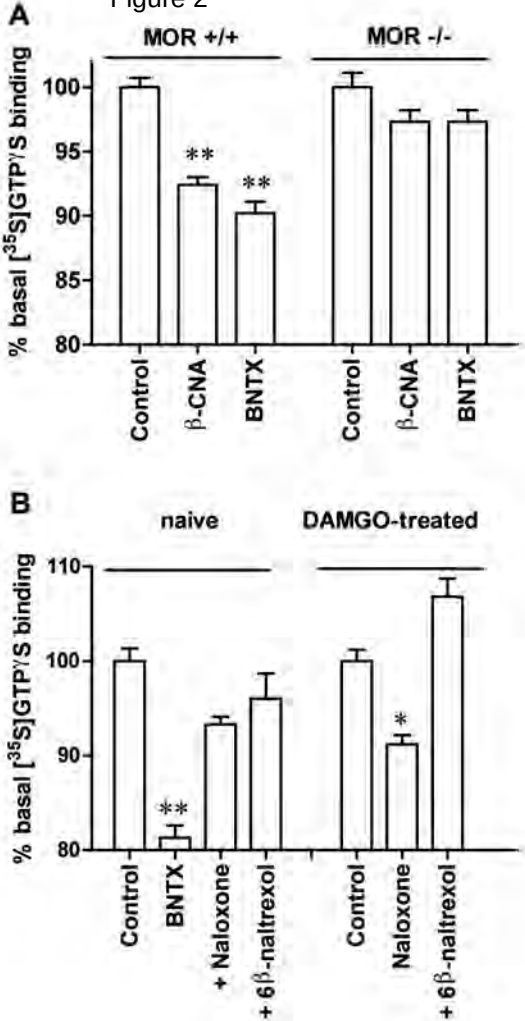
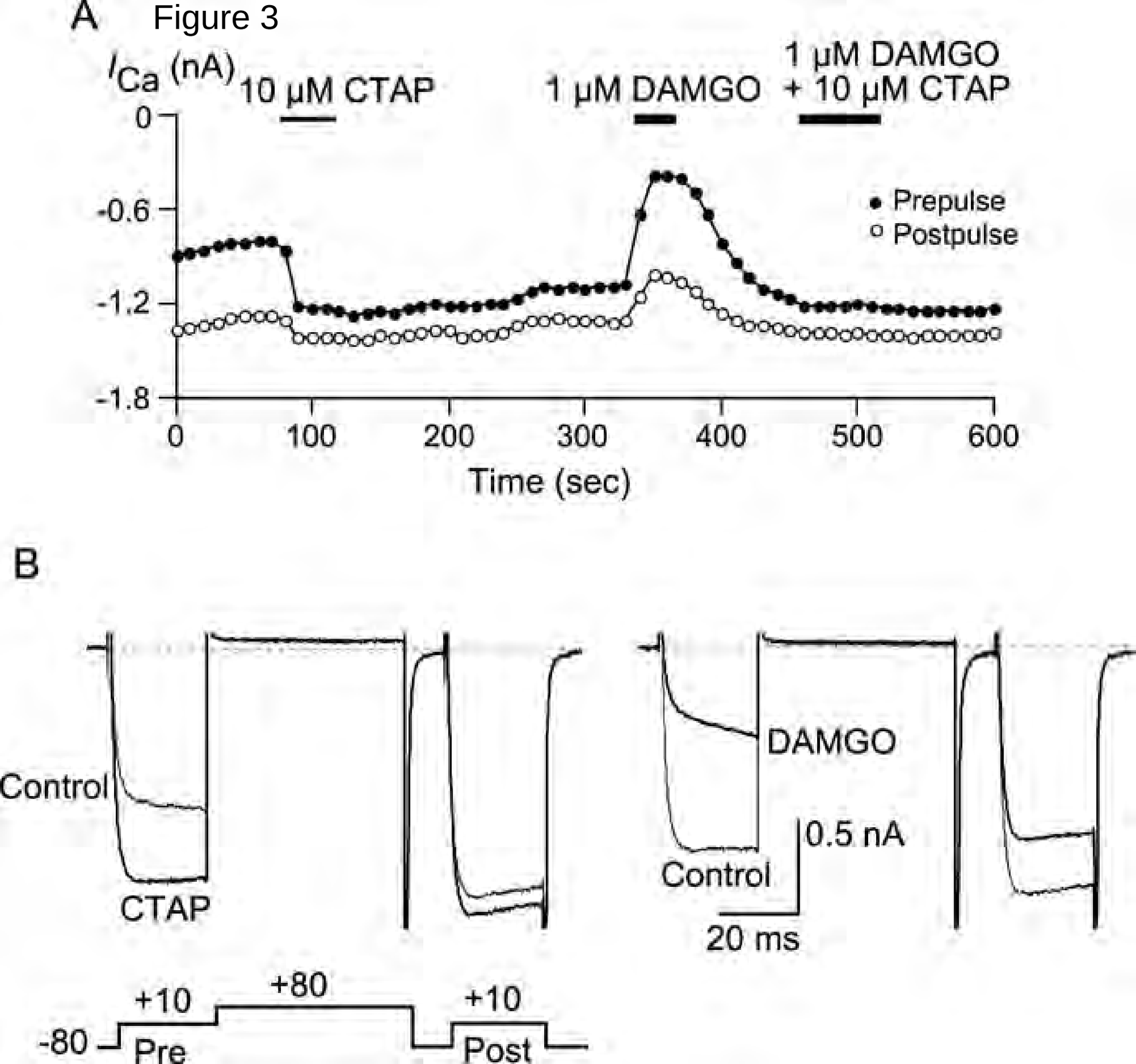

A
MOR +/+
MOR -/-
% basal [35S]GTPγS binding
100
95
90
85
80
**
**
Control
β-CNA
BNTX
Control
β-CNA
BNTX
B
naive
DAMGO-treated
% basal [35S]GTPγS binding
110
100
90
80
**
*
Control
BNTX
+ Naloxone
+ 6β-naltrexol
Control
Naloxone
+ 6β-naltrexol

Figure 3


A
I_{Ca} (nA)
10 μM CTAP
1 μM DAMGO
1 μM DAMGO + 10 μM CTAP
0
-0.6
-1.2
-1.8
Prepulse
Postpulse
0
100
200
300
400
500
600
Time (sec)
B
Control
CTAP
DAMGO
0.5 nA
Control
20 ms
-80
+10
Pre
+80
+10
Post