

**STUDIES ON THE ROLE OF G PROTEIN ALPHA SUBUNITS IN OPIOID
SIGNALING AND BEHAVIOR**

by

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A la morphine

Prends, s'il le faut, docteur, les ailes de Mercure
Pour m'apporter plus tôt ton baume précieux!
Le moment est venu de faire la piqûre
Qui, de ce lit d'enfer, m'enlève vers les cieux.

Merci, docteur, merci! Qu'importe si la cure
Maintenant se prolonge en des jours ennuyeux!
Le divin baume est là, si divin qu'Epicure
Aurait dû l'inventer pour l'usage des Dieux!

Je le sens qui circule en moi, qui me pénètre!
De l'esprit et du corps ineffable bien-être,
C'est le calme absolu dans la sérénité.

Ah! Perce-moi cent fois de ton aiguille fine
Et je te bénirai cent fois, Sainte Morphine,
Dont Esculape eût fait une Divinité.

Jules Verne
Poésies inédites, 1886

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To my parents, Dan and Cathie
For my husband Jason

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List of Abbreviations

δ	delta
κ	kappa
μ	mu
% MPE	percent maximum possible effect
[³⁵ S]GTP γ S	guanosine-5'-O-(3-[³⁵ S]thio)triphosphate
129P3	129P3/J
129S1	129S1/SvImJ
129S6	129S6/SvEvTac
AC	adenylate cyclase
ANOVA	analysis of variance
B _{max}	maximal radioligand binding
CI	confidence interval
DAMGO	[D-Ala ² ,N-Me-Phe ⁴ ,Gly ⁵ -ol]-enkephalin
DPDPE	[D-Pen ^{2,5}]-enkephalin
DRG	dorsal root ganglion
EC ₅₀	50% effective concentration (<i>in vitro</i>)
ED ₅₀	50% effective dose (<i>in vivo</i>)
ED ₈₀	80% effective dose (<i>in vivo</i>)
eIPSCs	evoked GABA-mediated inhibitory post-synaptic currents
E _{max}	maximal agonist-stimulated response
GAP	GTPase accelerating protein
GIRK	G protein-coupled inwardly-rectifying K ⁺ channel
GPCR	G protein-coupled receptor
G α_{i2} -/-	G α_{i2} homozygous null
G α_{i2} +/-	G α_{i2} heterozygous null
G α_{i3} -/-	G α_{i3} homozygous null
G α_{i3} +/-	G α_{i3} heterozygous null
G α_o -/-	G α_o homozygous null
G α_o +/-	G α_o heterozygous null
G α_o +/GS	G α_o RGSi heterozygous knock-in
G α_o GS/GS	G α_o RGSi homozygous knock-in
G α_o RGSi	RGS-insensitive G α_o mutant G α_o ^{G184S}
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
K _D	radioligand binding affinity
MAPK	mitogen-activated protein kinase
ME	[Met ⁵]-enkephalin
NOP	nociceptin receptor
ODN	oligodeoxynucleotide

PAG	periaqueductal gray
PLC	phospholipase C
PTX	pertussis toxin
RGS	regulator of G protein signaling
RGSi	RGS-insensitive
RH	RGS homology
RNAi	RNA interference
RVM	rostromedial medulla
s.c.	subcutaneous
SEM	standard error of the mean

Abstract

Opioids (e.g. morphine) are powerful analgesics that are used clinically to treat a variety of pain conditions. However, chronic use of opioids is associated with the development of adaptations such as tolerance and dependence, which limit their utility as long-term pain therapeutics. Opioids produce analgesia by activating mu (μ) opioid receptors that are located on both central and peripheral nerve terminals. The μ opioid receptor is a G protein-coupled receptor that activates inhibitory heterotrimeric G proteins composed of a $G\alpha_{i/o}$ subunit and a $\beta\gamma$ heterodimer *via* nucleotide exchange. Once activated, these G protein subunits modulate the activity of a number of downstream effectors, including adenylate cyclase and various ion channels. G protein signaling is terminated by the intrinsic GTP hydrolysis of the $G\alpha$ subunit, although this process is accelerated *in vivo* by regulator of G protein signaling (RGS) proteins.

The goal of the work described in this thesis was to evaluate the role of $G\alpha_o$ protein and its regulation by RGS proteins in opioid-mediated behaviors. $G\alpha_o$ is the most abundant $G\alpha_{i/o}$ subtype in the brain and has been shown to couple preferentially to the μ opioid receptor. In addition, individual RGS subtypes have been demonstrated to play important roles in opioid-mediated behaviors, but it is not known if endogenous RGS proteins exert their effects through direct binding to $G\alpha_{i/o}$ subunits or by some other mechanism. To study this, opioid-mediated signaling and behaviors were measured in two different transgenic mouse models, one with a one with targeted knockout of $G\alpha_o$ protein ($G\alpha_o$ knockout mice) and the other with targeted knock-in of an RGS-insensitive mutant $G\alpha_o$ protein ($G\alpha_o$ RGSi mice). Together, these studies demonstrated that μ opioid receptor coupling to $G\alpha_o$ is important for the production of opioid antinociception, and that RGS proteins regulate this response by directly interacting with $G\alpha_o$ subunits. This work also showed that $G\alpha_o$ is protective against morphine tolerance and dependence. Overall, these findings suggest that the RGS: $G\alpha_o$ interface could be a potential target for the development of improved analgesics that are devoid of unwanted adaptations.

Chapter 1

General Introduction

Opioid receptors and ligands

Opioid analgesics are the most commonly prescribed pain medications on the market (Melnikova, 2010). Although drugs within this class produce effective analgesia, they also elicit several unwanted side effects, including constipation and respiratory depression (Harris, 2008). The clinical utility of long-term opioid drug treatment is further limited by the development of adaptations such as tolerance, in which larger doses of drug are required to produce the desired effect (Buntin-Mushock et al., 2005), and dependence, which is manifested as a mild withdrawal syndrome upon drug discontinuation (Farrell, 1994). Moreover, opioids modulate brain reward systems, and therefore many opioid analgesics carry significant abuse liability (Mendelson et al., 2008; Rosenblum et al., 2008). Thus, a better understanding of the cellular mechanisms that underlie the behavioral effects of opioids is required in order to identify new targets for the development of improved analgesics with reduced side effects.

Humans have been using opioids for thousands of years in the form of opium, which is a crude preparation of the sap from the opium poppy *Papaver somniferum*. Morphine, the primary active component of opium, was originally isolated in 1804 by the pharmacist Freidrich Sertürner and was the first plant alkaloid to be successfully purified. Opium also contains other active alkaloids including codeine and thebaine that are actually the precursors for morphine biosynthesis. Identification of the chemical structures of the opium alkaloids allowed for synthesis of many related compounds, including the agonists hydrocodone, oxycodone and nalbuphine and the antagonists naloxone and naltrexone. In addition, there are a number of fully synthetic opioid agonists (e.g. fentanyl, methadone) that produce morphine-like analgesia yet differ greatly in structure from the natural alkaloids (Figure 1.1) (Trescot et al., 2008).

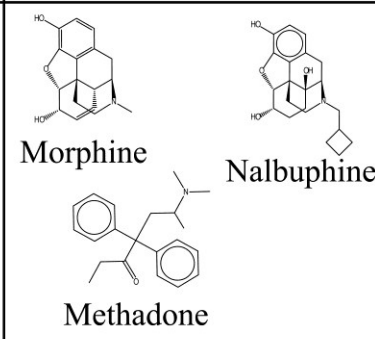
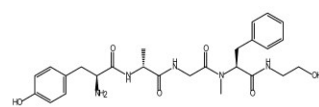
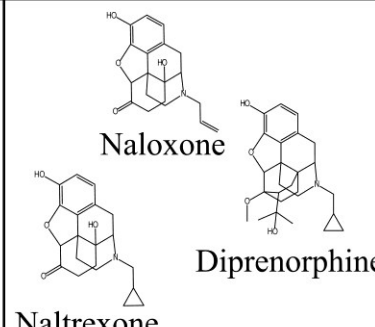
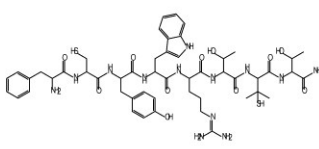
	Small Molecules	Peptides
<i>Agonists</i>	 <p>Morphine</p> <p>Nalbuphine</p> <p>Methadone</p>	 <p>[D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO)</p>
<i>Antagonists</i>	 <p>Naloxone</p> <p>Naltrexone</p> <p>Diprenorphine</p>	 <p>Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP)</p>

Figure 1.1: Structures of the main opioid ligands utilized in this thesis.

Morphine is the primary active component of opium and is structurally related to the semi-synthetic opioid agonist nalbuphine. Methadone is a fully synthetic opioid agonist whose structure is unlike other opium alkaloids. Morphine, methadone and nalbuphine exert their antinociceptive effects primarily by activating the μ opioid receptor, but morphine and nalbuphine have some affinity at other opioid receptors and methadone has activity at non-opioid sites. Morphine and methadone are generally considered full agonists while nalbuphine is usually described as a partial agonist. DAMGO is a μ opioid receptor-selective full agonist whose structure is derived from that of the endogenous opioid peptide enkephalin. Naloxone, naltrexone and diprenorphine are structurally similar to morphine yet act as antagonists of μ , δ and κ opioid receptors. CTAP is a μ opioid receptor-selective antagonist that is not structurally related to the endogenous opioid peptides.

Though the pharmacological actions of opioids have been known for a century (Watkyn-Thomas, 1912; Macht et al., 1915), opioid binding sites were not identified until the 1970s (Goldstein et al., 1971; Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Shortly thereafter, three distinct opioid receptors were described, and each receptor was named after the agonist or assay system with which it was characterized: the mu (μ) receptor for morphine, the kappa (κ) receptor for ketocyclazocine (Martin et al., 1976), and the delta (δ) receptor for the mouse vas deferens (Lord et al., 1977). Identification of the cellular targets of opioid analgesics also led to the discovery of the endogenous opioid peptide system comprised of the enkephalins (Hughes et al., 1975), the endorphins (Mains et al., 1977), and the dynorphins (Goldstein et al., 1979). The structures of the endogenous opioid peptides eventually became the basis for the development of opioid receptor-selective ligands, including the μ -selective agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) (Figure 1.1) (Handa et al., 1981) and the δ -selective agonist [D-Pen^{2,5}]-enkephalin (DPDPE) (Mosberg et al., 1983). These ligands, as well as the κ agonist bremazocine (Romer et al., 1980), were utilized in autoradiography studies in rat brain (Mansour et al., 1988) to support the existence of three distinct opioid receptor types with separate, yet overlapping, functional roles (Dhawan et al., 1996; Kieffer and Evans, 2009).

Despite the early identification of opioid receptor-selective ligands, opioid receptors were classified primarily on the basis of physiology and pharmacology until the δ receptor was cloned in 1992 (Evans et al., 1992; Kieffer et al., 1992; Kieffer, 1995). This was soon followed by the cloning and molecular characterization of μ and κ opioid receptors (Reisine and Bell, 1993; Satoh and Minami, 1995). Later, the nociceptin receptor (NOP) was classified as a fourth opioid receptor type based on close amino acid sequence homology (Bunzow et al., 1994; Mollereau et al., 1994). Molecular cloning of the opioid receptors allowed for a more detailed analysis of opioid receptor anatomy (Mansour et al., 1995), as well as the use of gene knockout techniques to more fully characterize behavioral responses to opioid agonists (Kieffer, 1999). Although all four opioid receptors modulate pain signaling (Mogil and Pasternak, 2001; Martin et al., 2003), the analgesic effects of morphine – the prototypical opioid agonist – are mediated

almost exclusively by the μ opioid receptor, as demonstrated using μ receptor knockout mice (Matthes et al., 1996).

In simple terms, opioids produce analgesia by blocking incoming pain signals. By definition, pain is an experience that includes an affective component, and therefore the measurement of pain and analgesia in humans includes both subjective and objective measures. Given that subjective measures are not possible in rodents, experimental models actually test nociception, which is the basic neuronal processing of noxious stimuli (Loeser and Treede, 2008). The measurement of analgesia in animal models is therefore referred to as antinociception. The following section will describe our current knowledge of nociceptive pathways and discuss how opioids interact with these pathways to produce antinociception.

Opioid antinociception

Ascending and descending nociceptive pathways

The concept of nociception has been around for centuries, having first been described in 1662 by René Descartes who hypothesized that behavioral responses to noxious stimuli were somehow centrally-coordinated. The notion that a neural process indeed underlies nociception was solidified by Sir Charles Scott Sherrington who in 1906 reported the existence of a primary afferent nociceptive neuron, or nociceptor (Sherrington, 1906). It is now known that nociception is mediated by specific afferent nerve fibers in the periphery whose cell bodies reside in the ganglia and project to the spinal cord (Figure 1.2). In particular, lightly myelinated A δ and unmyelinated C fibers are responsible for sensing the majority of noxious stimuli, including mechanical, thermal and chemical insults (Julius and Basbaum, 2001). Thus, like other sensory processes, nociception occurs when neurons respond to a particular stimulus, in this case tissue injury.

Primary sensory neurons that respond to noxious stimulation transmit nociceptive information along a well-defined ascending pathway (Figure 1.2). These primary nociceptors synapse in the dorsal horn of the spinal cord onto second-order neurons that then carry nociceptive information to higher brain centers including the thalamus and cortex (Heinricher and Morgan, 1999; Julius and Basbaum, 2001; Fields, 2004).

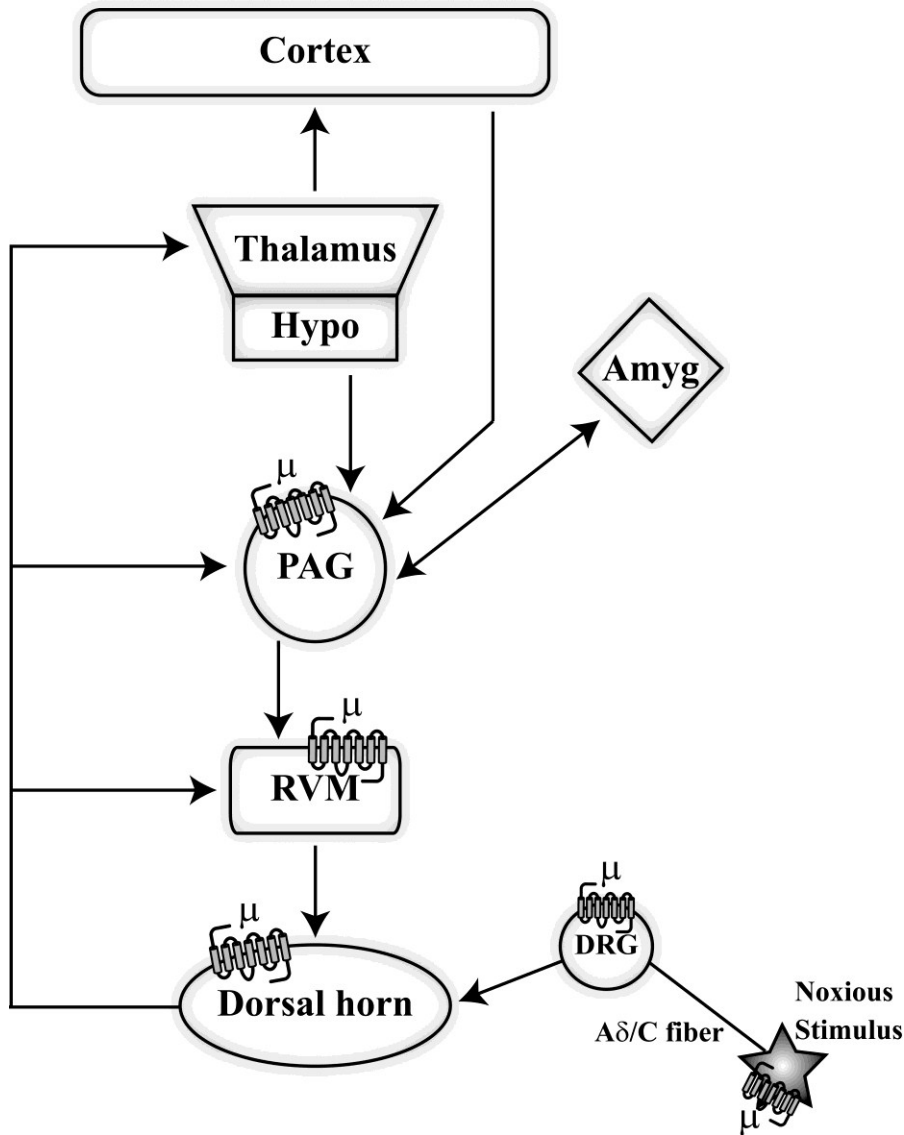


Figure 1.2: Ascending and descending nociceptive pathways and primary sites of opioid antinociception.

A peripheral noxious stimulus is sensed by primary afferent nociceptors (usually A δ or C fibers) whose cell bodies are located in the dorsal root ganglion (DRG) and project to the dorsal horn of the spinal cord. Nociceptive information is then relayed by second-order neurons to higher brain centers including the thalamus and cortex. These neurons also provide feedback at the level of the rostroventral medulla (RVM) and periaqueductal gray (PAG). Descending modulation of nociception is controlled by outputs from the cortex, hypothalamus (Hypo) and amygdala (Amyg) to the dorsal horn *via* synapses in the PAG and RVM. Primary sites of opioid action are denoted by the presence of the μ opioid receptor. Major supraspinal sites of opioid antinociception include the PAG and RVM. Opioids can also have direct effects within the dorsal horn. Finally, opioids are able to elicit antinociception by acting peripherally, either in the DRG or at the site of injury itself.

In addition to the ascending pathway, there is also a well-defined nociception modulating circuit that provides descending control of nociception (Figure 1.2). The descending pathway is controlled by several supraspinal sites (e.g. cortex, amygdala, and hypothalamus), and projections from these higher brain areas modulate the activity of primary afferent nociceptors in the dorsal horn *via* relays through the periaqueductal gray (PAG) and rostroventral medulla (RVM) (Heinricher and Morgan, 1999; Fields, 2004).

Opioid modulation of nociceptive pathways

In general, the antinociceptive effects of opioids are mediated through actions within the descending nociception modulatory pathway. Using microinjection techniques in rats, researchers have been able to identify the major brain areas that are responsible for generating opioid antinociception (Yaksh et al., 1988). In particular, the most important and well-studied supraspinal sites of opioid action are the PAG and RVM (Figure 1.2) (Heinricher and Morgan, 1999). Microinjection of the μ opioid receptor-selective agonist DAMGO, but not ligands selective for other opioid receptor types, into either the PAG (Smith et al., 1988; Fang et al., 1989) or the RVM (Fang et al., 1986; Heinricher et al., 1994) produces significant antinociception. Importantly, both the PAG and the RVM express high levels of μ opioid receptors (Mansour et al., 1988; Mansour et al., 1995). Overall, this work suggests that the μ opioid receptor is the main target of opioid analgesic drugs in these brain areas.

The primary effect of opioids acting in the PAG and RVM is to activate descending projection neurons which in turn inhibit afferent nociceptors at the level of the spinal cord (Heinricher and Morgan, 1999). This is thought to occur *via* removal of tonic GABA inhibition of descending projection neurons, a process known as GABA disinhibition (Moreau and Fields, 1986; Chieng and Christie, 1994b; Heinricher and Tortorici, 1994). However, there may also be a small contribution from the ability of opioids to directly suppress descending neurons that facilitate nociception (Heinricher et al., 1992; Chieng and Christie, 1994a).

In addition to effects on supraspinal sites, opioids can produce antinociception through direct actions at the dorsal horn (Cesselin et al., 1999), which also expresses a high density of μ opioid receptors (Figure 1.2) (Besse et al., 1990). Specifically, there are several peptides and transmitters released onto second-order projection neurons that serve

to facilitate nociceptive transmission; these include substance P, cholecystokinin, and glutamate, among others (Millan, 2002). The primary consequence of opioids acting in the spinal cord is to inhibit the release of these neurotransmitters, with the net result of decreasing ascending nociceptive transmission (Cesselin et al., 1999). Lastly, opioids can also act peripherally to elicit antinociception (Figure 1.2), especially in inflamed tissues (Stein and Lang, 2009); this mechanism will not be addressed in the present work.

Experimental models of nociception in rodents

Experimental animal models have been used for decades to address mechanisms of nociception and antinociception; such models were recently reviewed by Barrot (2012) and Le Bars et al. (2001). In general, models of mechanical and thermal nociception involve exposure to a noxious stimulus followed by the measurement of a response, such as withdrawal latency or threshold to avoidance. In contrast, behavioral scoring is usually employed for chemical or inflammatory stimulus modalities, for example in the formalin test an experimenter will count the number of paw licks, bites, or shakes following intradermal formalin injection.

The measurement of opioid antinociception has classically been accomplished using either the hot plate or the tail flick test, both of which involve the measurement of responses to an acute thermal stimulus. The tail flick, or tail withdrawal test was one of the first nociceptive tests described (D'Amour and Smith, 1941). To perform the test, the animal is first lightly restrained, and then the distal part of the tail is exposed to either a water bath or a focused infrared beam. In both cases, the response measured is the latency for the animal to flick the tail. The hot plate is another classic nociceptive test (Woolfe and MacDonald, 1944; O'Callaghan and Holtzman, 1975). In this test, the animal is allowed to move freely atop a heated plate, and the response measured is the latency to paw licking or often the latency to any first response (e.g. hind paw shaking, jumping). In both the hot plate and the tail flick test, administration of an opioid causes prolongation of the response latency, and this is defined as an antinociceptive effect.

One reason why these tests are utilized so frequently in studies of opioid antinociception is because they are highly sensitive to μ opioid agonists. This is not surprising given that μ opioid receptors are located on thermosensitive primary afferent C fibers (Scherrer et al., 2009). On the other hand, both the hot plate and the tail flick test

are faced with a few limitations. In particular, both tests are sensitive to stress and/or stress-induced analgesia, which could affect the experimental results. This is especially true for the tail flick test, in which animals are generally restrained in order to expose the tail to the desired stimulus. However, stress exposure can be reduced by prior habituation to the testing apparatus. Another drawback to these tests is that small changes in temperature can have large effects on response latency, and therefore the stimulus temperature must be accurately maintained. Nevertheless, when properly performed by a trained experimenter, both the hot plate and the tail flick test are able to provide stable measures of nociception and opioid antinociception.

There are several additional factors that can affect response latency in either the hot plate or the tail flick test; these factors are important to consider when interpreting the results of these tests. Since response latency is partially a measure of the time it takes for the skin temperature to increase above a certain threshold, anything that affects the rise in skin temperature will ultimately influence the observed response latency. The rate of rise in skin temperature can be affected by both the type of heat and the stimulus intensity. For example in the tail flick test, the rise in skin temperature is much more gradual when the tail is exposed to an infrared beam than when it is immersed in a water bath. In the hot plate test, the rise in paw temperature can differ depending on the material from which the hot plate is constructed. Furthermore, increases or decreases in the stimulus intensity, or temperature, can affect both nociceptive and antinociceptive responses in these tests.

In addition to being affected by stimulus perception, response latency is also influenced by neuronal processing of the response itself. Specifically, responses in the hot plate test are considered supraspinal because they involve the expression of complex, coordinated behaviors, such as paw licking or jumping. As such, baseline response latencies in the hot plate test generally range from 10 to 20 seconds, depending upon the hot plate temperature. In contrast, the response in the tail flick test is primarily a spinal reflex. The reflexive nature of the tail flick response is perhaps best illustrated by studies in which chronic spinal animals were evaluated in the tail flick test. Despite having a short segment of spinal cord removed, these animals retain the ability to produce a tail flick response (Irwin et al., 1951). Since spinal transection eliminates both ascending and

descending nociceptive pathways, this suggests that the minimal circuit required to elicit a tail flick response involves only primary afferent nociceptors, dorsal horn interneurons, and efferent motor neurons (Figure 1.2). Thus, baseline tail flick latencies are usually on the order of 1 to 5 seconds, depending on the stimulus intensity. Nevertheless, tail flick responses can also be modulated by descending projections from supraspinal areas (Yaksh and Rudy, 1978), and therefore the tail flick response should not be considered exclusively spinal.

Effects of chronic opioid exposure

Unfortunately, the chronic use of opioids leads to adaptations that detract from the usefulness of these drugs as analgesics. These adaptations, including tolerance and dependence, are thought to be mediated by homeostatic and/or compensatory mechanisms that serve to counteract the effects of continuous opioid receptor activation (Williams et al., 2001; Bailey and Connor, 2005; Christie, 2008). The first of these adaptations, tolerance, is defined as a loss of effectiveness of a drug during repeated exposure such that increasing doses are needed to produce the desired effect. Tolerance occurs for many of the behavioral effects of opioids, but the primary clinical concern is the development of analgesic tolerance, which in some cases necessitates dose escalation of greater than 10-fold in order to maintain analgesic efficacy (Buntin-Mushock et al., 2005). In experimental models, antinociceptive tolerance can be observed using standard nociceptive tests as a decrease in the ability of opioids to produce antinociception with repeated administration. Antinociceptive tolerance to opioids can be observed in rodents after only a single treatment, also called acute tolerance (Cochin and Kornetsky, 1964; Kornetsky and Bain, 1968). Antinociceptive tolerance continues to develop over more long-term opioid administration (Cochin and Kornetsky, 1964), and this can lead to prolonged changes within nociceptive pathways, even after drug treatment has stopped (Williams et al., 2001).

Adaptive changes within opioid-sensitive networks also underlie the development of opioid dependence (Williams et al., 2001; Bailey and Connor, 2005; Christie, 2008). Dependence is defined as an altered homeostasis in which the presence of a drug is required to prevent symptoms of withdrawal. In the case of opioid dependence in humans, common withdrawal symptoms include nausea and vomiting, sweating, yawning

and fatigue, tremors, and diarrhea (Farrell, 1994). Some of these same behaviors (e.g. tremors, diarrhea) are observed in rodent experimental models, in addition to jumping and wet dog shakes (Way and Loh, 1976; Maldonado et al., 1992). In an experimental setting, opioid physical dependence is usually quantified by scoring these and other behavioral signs after rapidly precipitating withdrawal with an opioid antagonist, such as naloxone (Way and Loh, 1976).

Though the exact mechanistic basis of opioid dependence is still unknown, studies have clearly demonstrated that the development of morphine dependence, like most other morphine-induced behaviors, is mediated by the μ opioid receptor (Maldonado et al., 1992; Matthes et al., 1996). Given that both the desired antinociceptive effects and unwanted side effects produced by opioids are mediated by a single receptor, viable strategies toward developing safer analgesics have attempted to identify differences in downstream signaling pathways.

Opioid receptor signaling

Opioid receptors belong to the family of seven-transmembrane, G protein-coupled receptors (GPCRs). GPCRs convert extracellular signals (e.g. neurotransmitters, hormones, etc.) into intracellular responses by coupling to heterotrimeric G proteins composed of a $G\alpha$ subunit bound to a $\beta\gamma$ heterodimer (Gilman, 1987; Milligan and Kostenis, 2006). In the inactive state, the $G\alpha$ subunit is bound to the guanine nucleotide GDP. Agonist activation of a GPCR leads to a conformational change within the receptor that is propagated to the G protein (Chung et al.; Westfield et al., 2011), resulting in the exchange of GTP for GDP on the $G\alpha$ subunit. This leads to the dissociation of $G\alpha$ -GTP from the $\beta\gamma$ heterodimer and subsequent modulation of downstream effector proteins (Figure 1.3).

Termination of G protein signaling is achieved *via* intrinsic hydrolysis of GTP to GDP by the GTPase domain of the $G\alpha$ subunit, which results in the reassociation of $G\alpha$ -GDP and $G\beta\gamma$. The intrinsic GTP hydrolysis rate for some $G\alpha$ subtypes is slow (~ 2 - 5 min^{-1}); however, this process is significantly accelerated *in vivo* by regulator of G protein signaling (RGS) proteins, which enhance the GTPase activity of $G\alpha$ subunits (De Vries et al., 2000; Lan et al., 2000; Ross and Wilkie, 2000; Hollinger and Hepler, 2002).

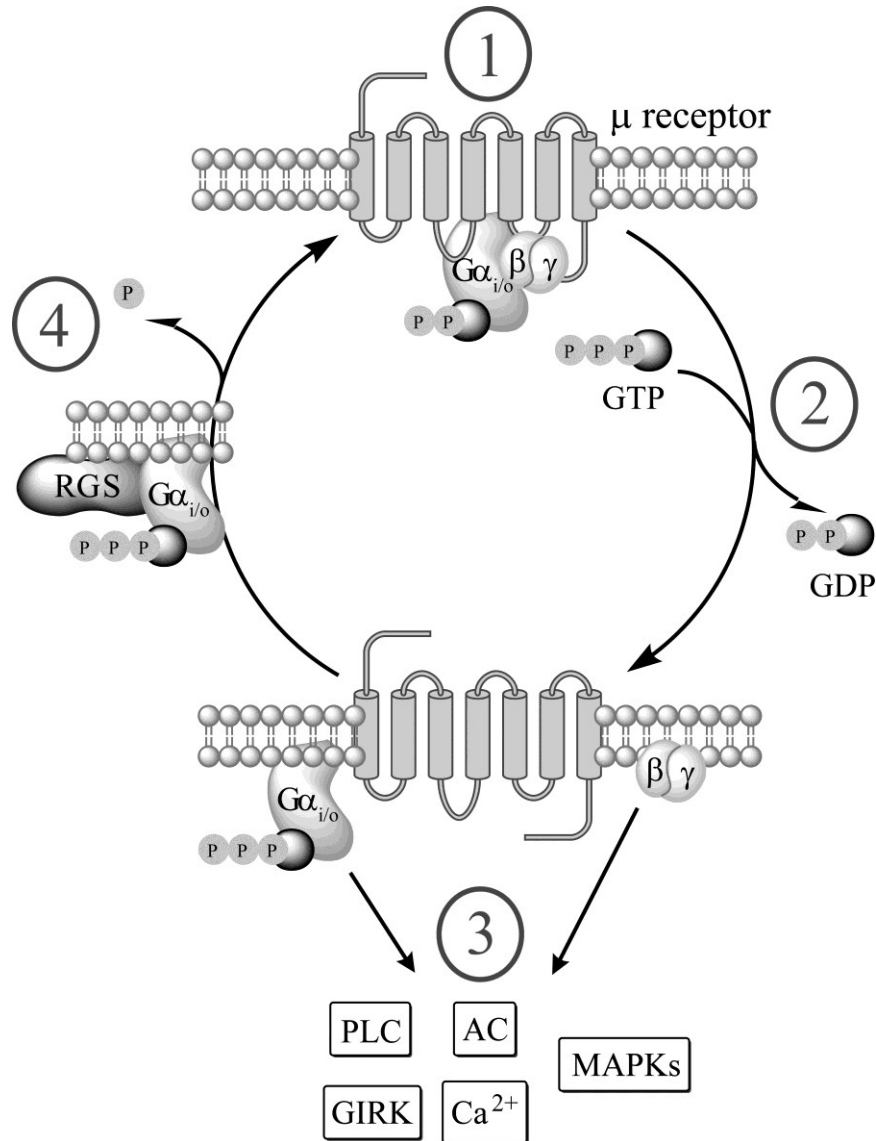


Figure 1.3: Cycle of G protein activation and inactivation at the μ opioid receptor. Agonist stimulation of the μ opioid receptor (1) results in the activation of associated heterotrimeric $G_{i/o}$ proteins *via* nucleotide exchange (2). Once activated, $G\alpha_{i/o}$ -GTP dissociates from the $\beta\gamma$ heterodimer, and both subunits signal to downstream effectors (3), including adenylate cyclase (AC), Ca^{2+} channels (Ca^{2+}), G protein-coupled inwardly-rectifying K^+ (GIRK) channels, mitogen-activated protein kinases (MAPKs), and phospholipase C (PLC). G protein signaling is terminated by GTP hydrolysis, which is enhanced by regulator of G protein signaling (RGS) proteins (4).

Thus, RGS proteins negatively regulate G protein signaling by decreasing levels of active $G\alpha$ -GTP and $\beta\gamma$ subunits (Figure 1.3).

Early stages of G protein signaling, including both the activation and inactivation of G proteins, likely play an essential role in the expression of behavioral responses to morphine. The remainder of this chapter will review what is known regarding the roles of G proteins and RGS proteins in the pharmacology of the μ opioid receptor, the primary target of the prototypical opioid agonist morphine.

G proteins and μ opioid receptor pharmacology

Heterotrimeric G proteins are generally defined by the identity of the $G\alpha$ subunit, and each $G\alpha$ protein may associate with one of 5 β and one of 12 γ subunits (Milligan and Kostenis, 2006). There exist over 15 genetically distinct $G\alpha$ subtypes that are divided into four major families based upon structural and functional homology: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Wilkie et al., 1992; Wettschureck and Offermanns, 2005). The μ opioid receptor couples to G proteins of the $G\alpha_{i/o}$ family, including both the pertussis toxin (PTX)-sensitive G proteins $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ and the PTX-insensitive G protein $G\alpha_z$ (Standifer and Pasternak, 1997; Connor and Christie, 1999).

It was hypothesized early on that receptor-activated $G\alpha_{i/o}$ proteins were responsible for the inhibition of adenylate cyclase (AC) enzymes (Gilman, 1987), and that this activity was sensitive to ADP-ribosylation by PTX (Katada et al., 1982). Using PTX, it was therefore possible to identify and characterize both $G\alpha_i$ (Bokoch et al., 1983; Codina et al., 1983) and $G\alpha_o$ (Neer et al., 1984; Sternweis and Robishaw, 1984) from partially purified tissue preparations. Not long after the identification of $G\alpha_{i1}$ and $G\alpha_o$, another AC inhibitory $G\alpha$ subtype, $G\alpha_z$, was discovered (Fong et al., 1988; Matsuoka et al., 1988; Wong et al., 1992). Whereas $G\alpha_{i1-3}$ proteins are expressed fairly ubiquitously (Milligan and Kostenis, 2006), $G\alpha_o$ and $G\alpha_z$ tend to be localized to the brain (Gierschik et al., 1986; Worley et al., 1986; Hinton et al., 1990), although $G\alpha_o$ is also enriched in the heart (Huff et al., 1985; Valenzuela et al., 1997).

Gα subtypes and μ opioid receptor signaling

It is well-documented that μ opioid receptor coupling to Gα_{i/o} proteins produces AC inhibition in addition to Ca²⁺ channel inhibition, G protein-coupled inwardly-rectifying K⁺ (GIRK) channel activation, phospholipase C (PLC) stimulation, and mitogen-activated protein kinase (MAPK) activation (Figure 1.3) (Standifer and Pasternak, 1997; Law et al., 2000). Using immunoprecipitation techniques, the μ opioid receptor has been shown to interact with multiple Gα_{i/o} subtypes (Chalecka-Franaszek et al., 2000). Indeed, μ opioid receptor signaling is generally thought to be mediated by all Gα_{i/o} subtypes indiscriminately, including Gα_z (Connor and Christie, 1999). This has been demonstrated using assays for receptor and/or G protein activity in artificially reconstituted systems (Ueda et al., 1988; Stanasila et al., 2000; Massotte et al., 2002), heterologous cell expression models (Laugwitz et al., 1993; Chakrabarti et al., 1995; Chan et al., 1995; Clark et al., 2006; Clark and Traynor, 2006), and rodent tissue (Murthy and Makhlouf, 1996; Garzon et al., 1997a). In contrast, several lines of evidence have suggested that the μ opioid receptor couples preferentially to Gα_o over other Gα_{i/o} subtypes. In particular, Gα_o was shown to efficiently mediate μ receptor inhibition of Ca²⁺ currents in both cells (Hescheler et al., 1987) and dorsal root ganglion (DRG) neurons (Moises et al., 1994). Moreover, knockdown of Gα_o, but not Gα_{i1-3}, resulted in a significant reduction in DAMGO-mediated inhibition of AC activity in both cells and rat striatum (Carter and Medzihradsky, 1993).

Coupling of the μ opioid receptor to Gα_{i/o} subunits has also been examined by *in vivo* knockdown of individual Gα_{i/o} isoforms in rodents using selective antibodies, targeted antisense oligodeoxynucleotides (ODNs) (Garzon et al., 2000), or genetic knockout techniques (Jiang et al., 2002; Wettschureck et al., 2004). In general, these studies have shown that knockdown of either Gα_{i2} or Gα_z (intracerebroventricular; i.c.v.) results in reduced [³H]DAMGO binding to μ receptors (Sanchez-Blazquez et al., 1993; Rossi et al., 1995) and decreased μ opioid-stimulated G protein activation (Garzon et al., 1994) in the PAG, as well as decreased μ opioid inhibition of AC the striatum (Shen et al., 1998). In addition, exogenous application of Gα_{i2} protein appeared to reverse the deficit in morphine-stimulated G protein activation caused by knockdown of Gα_{i2}

(Garzon et al., 1999). These data suggest that $G\alpha_{i2}$ and/or $G\alpha_z$ may be the most important $G\alpha_{i/o}$ subtypes for μ opioid receptor signaling *in vivo*. On the other hand, there are conflicting reports demonstrating that $G\alpha_o$ knockout mice exhibit decreased DAMGO-stimulated G protein activation across several brain regions (Jiang et al., 2001) and reduced DAMGO-induced Ca^{2+} current inhibition in DRG neurons (Jiang et al., 1998).

G α proteins and μ opioid receptor-mediated behaviors

The same *in vivo* knockdown and knockout techniques have also been applied to study the function of $G\alpha_{i/o}$ subunits in μ opioid-induced behaviors (Table 1.1). Evaluation of morphine antinociception in the tail flick test in either mice or rats with reduced $G\alpha_{i/o}$ protein expression has also implicated $G\alpha_{i2}$ and/or $G\alpha_z$ in the control of μ opioid-induced spinal antinociception (Sanchez-Blazquez et al., 1993; Raffa et al., 1994; Rossi et al., 1995; Sanchez-Blazquez et al., 1995; Raffa et al., 1996; Standifer et al., 1996; Shen et al., 1998; Garzon et al., 1999; Sanchez-Blazquez et al., 1999; Yoburn et al., 2003). However, there are again conflicting reports in the literature suggesting that $G\alpha_o$ is the primary $G\alpha_{i/o}$ subtype mediating morphine antinociception in the tail flick test (Standifer et al., 1996; Karim and Roerig, 2000). Moreover, when $G\alpha_z$ was completely knocked out in mice, there was either no change (Hendry et al., 2000) or a small decrease (Yang et al., 2000; Leck et al., 2004) in morphine antinociception in the hot plate test. Thus, there are clearly inconsistencies in our understanding of the contribution of individual $G\alpha_{i/o}$ isoforms to μ receptor-mediated antinociception; Chapter 2 of this thesis attempts to address these discrepancies by evaluating opioid antinociception in a single, reproducible mouse model using two noxious tests.

Though there are only a few reported studies that evaluate the role of $G\alpha_{i/o}$ proteins in the development of adaptations following chronic opioid exposure, these publications generally report consistent results. In particular, morphine antinociceptive tolerance appears to be reduced by knockdown of $G\alpha_{i2}$ (Garzon and Sanchez-Blazquez, 2001; Yoburn et al., 2003) but enhanced by knockout of $G\alpha_z$ (Hendry et al., 2000; Leck et al., 2004), suggesting that $G\alpha_{i2}$ is protective against morphine tolerance while $G\alpha_z$ is facilitative.

Table 1.1: Role of individual G α subunits in μ opioid receptor-mediated behaviors.

Behavior	Mouse model	G $\alpha_{i/o}$ subunit	Effect on behavior	References
Morphine antinociception (tail flick test)	Antisense ODN knockdown	G α_{i1}	No change	(Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Raffa et al., 1996; Standifer et al., 1996; Shen et al., 1998; Sanchez-Blazquez et al., 1999; Karim and Roerig, 2000; Yoburn et al., 2003)
		G α_{i2}	↓	
		G α_{i3}	No change	
		G α_o	↓ / No change	
		G α_z	↓ / No change	
	Gene knockout	G α_z	No change	(Hendry et al., 2000)
Morphine antinociception (hot plate test)	Gene knockout	G α_z	↓ / No change	(Hendry et al., 2000; Yang et al., 2000; Leck et al., 2004)
Morphine antinociceptive tolerance (tail flick test)	Antisense ODN knockdown	G α_{i2}	↓	(Yoburn et al., 2003)
Morphine antinociceptive tolerance (hot plate test)	Gene knockout	G α_z	↑	(Hendry et al., 2000; Leck et al., 2004)
Withdrawal from chronic morphine	Antibody knockdown	G α_{i2}	↓	(Sanchez-Blazquez and Garzon, 1994)
		G α_o	↓	
		G α_z	↓	
	Antisense ODN knockdown	G α_{i2}	No change	(Raffa et al., 1996)
		G α_o	↓	(Kest et al., 2009)
Morphine constipation	Antisense ODN knockdown	G α_{i2}	No change	(Raffa et al., 1996)

↓, decrease; ↑, increase; ODN, oligodeoxynucleotide

With regards to morphine dependence, knockdown of $G\alpha_o$, $G\alpha_z$, or $G\alpha_{i2}$ reduced the severity of opioid antagonist-precipitated withdrawal (Sanchez-Blazquez and Garzon, 1994; Garzon and Sanchez-Blazquez, 2001; Kest et al., 2009), suggesting that $G\alpha_{i/o}$ signaling does play a role in this adaptation but that more than one $G\alpha_{i/o}$ isoform may be involved. Chapter 4 of this thesis is focused on better understanding the role of the $G\alpha_{i/o}$ subtype $G\alpha_o$ in both morphine tolerance and dependence.

RGS proteins and μ opioid receptor pharmacology

Although μ opioid receptor activation of G proteins initiates signaling, the receptor and G protein are also regulated by the activity of RGS proteins. There are over 30 known proteins that contain the conserved RGS homology (RH) domain; these are divided into several families based upon the structure of the RH domain and the presence of other protein-protein binding domains (Ross and Wilkie, 2000).

“Canonical” RGS proteins of the R4, RZ, R7 and R12 families (Figure 1.4) are best-known for their ability to serve as GTPase-accelerating proteins, or GAPs, for $G\alpha$ subunits by enhancing GTP hydrolysis and reducing the lifetime of activated $G\alpha$ (Figure 1.3). Structural characterization of $G\alpha$:RGS complexes has revealed that RGS proteins enhance GTP hydrolysis by binding to active $G\alpha$ -GTP proteins and stabilizing the transition state during GTP hydrolysis (Tesmer et al., 1997). Whereas the smaller RGS proteins (R4 and RZ families) function primarily as GAPs, some of the larger RGS proteins (R7 and R12 families) have been shown to serve additional regulatory functions that are mediated through various protein-protein interacting domains. These activities include scaffolding, facilitation of G protein signaling, regulation of non-G protein signaling, and signal transduction at non-GPCRs (Hollinger and Hepler, 2002; Sethakorn et al., 2010). “Non-canonical” RGS proteins of the RA and RL families, including G protein-coupled receptor kinase 2 (GRK2), contain an RH domain yet do not possess GAP activity. However, these proteins are also able to regulate GPCR signaling by directly interacting with and/or scaffolding G proteins (Sethakorn et al., 2010).





RGS Family	Structure	Members
R4		RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16, RGS18, RGS21
RZ		RGS17 (RGSZ2) RGS19 (GAIP) RGS20 (RGSZ1)
R7		RGS6, RGS7 RGS9, RGS11
R12		RGS10, RGS12 RGS14

Figure 1.4: Basic domain structures of the “canonical” regulator of G protein signaling (RGS) proteins from the R4, RZ, R7 and R12 families.

Shown for each family is the common domain structure with the N-terminus oriented to the left, as well as a list of individual family members. The R4 and RZ families contain relatively simple RGS proteins, with short N- and C-terminal extensions. R4 family members contain the RGS homology (RH) domain, together with a short N-terminal amphipathic helix. The RZ family is comprised of proteins with an N-terminal cysteine string (Cys) preceding the conserved RH domain. The R7 and R12 families are more complex, and contain several domains for protein-protein interaction in addition to the RH domain. Members of the R7 family contain both a DEP (Disheveled, Egl-10, Pleckstrin) domain with a helical extension (DHEX) and a GGL (G protein gamma-like) domain. The R12 family contains proteins with a PDZ (PSD95, Dlg1, ZO-1) domain, a phosphotyrosine binding (PTB) domain, two Ras binding domains (RBD), and a GoLoco motif that binds $G\alpha$ -GDP subunits.

Contribution of individual RGS isoforms

Approaches utilizing knockdown, overexpression, or constitutive gene knockout of individual RGS proteins have demonstrated the importance of these regulatory proteins for μ opioid receptor signaling and behavior (Kimple et al., 2011). RGS4, a member of the R4 family of RGS proteins (Figure 1.4), functions as a negative regulator of μ opioid receptor signaling in heterologous expression systems. For example, in HEK293 cells expressing the μ opioid receptor, introduction of RGS4 has been shown to reduce μ agonist-mediated inhibition of AC and activation of MAPK (Garnier et al., 2003; Georgoussi et al., 2006; Xie et al., 2007; Leontiadis et al., 2009; Talbot et al., 2010). However, RGS4 knockout mice do not exhibit changes in morphine antinociception or antinociceptive tolerance (Grillet et al., 2005; Han et al., 2010). In contrast, knockout of RGS9-2, a member of the R7 family (Figure 1.4), resulted in increased morphine antinociception in the hot plate test (Zachariou et al., 2003b), suggesting that RGS9-2 negatively regulates μ opioid receptor-mediated antinociception. RZ and R12 family RGS proteins (Figure 1.4) have not been studied in great detail, although there are isolated reports. In particular, ODN knockdown (i.c.v.) of RZ family RGS proteins, including RGS17, 19, and 20, resulted in enhanced morphine antinociception and antinociceptive tolerance (Garzon et al., 2004; Garzon et al., 2005; Sánchez-Blázquez et al., 2005). Alternatively, knockdown of RGS12 (R12 family) was shown to increase μ opioid antinociception (Garzon et al., 2001; Garzon et al., 2003).

Role of endogenous RGS proteins

RGS proteins have been hypothesized to show varying degrees of selectivity for specific GPCRs, G proteins, and/or signaling pathways (Ross and Wilkie, 2000), yet it is often difficult to observe phenotypic effects of eliminating a single RGS protein due to the diversity of the mammalian RGS proteins family (Grillet et al., 2005). Therefore, some evidence demonstrating that RGS proteins regulate μ opioid receptor signaling comes from studies using RGS-insensitive (RGSi) mutant $G\alpha_{i/o}$ proteins (Traynor, 2011). A mutant $G\alpha$ protein resistant to RGS GAP activity was initially discovered in yeast (DiBello et al., 1998). This mutation, a Gly to Ser substitution at amino acid position 183 in $G\alpha_{i1}$ (or the analogous position in other $G\alpha_{i/o}$ subtypes), does not alter intrinsic

GTPase activity yet prevents RGS protein binding and GAP activity (Lan et al., 1998). Thus, evaluation of signaling mediated by RGSi $G\alpha_{i/o}$ proteins in cell systems (Clark and Traynor, 2004; Fu et al., 2004) or *in vivo* (Kaur et al., 2011) can illuminate the role of endogenous RGS proteins in a response or behavior of interest.

The majority of the work utilizing RGSi $G\alpha_{i/o}$ proteins in the context of μ opioid receptor signaling has evaluated cellular responses to μ agonists in heterologous expression systems. In cells expressing RGSi $G\alpha_o$ subunits, endogenous RGS proteins negatively regulate μ opioid receptor signaling to AC and MAPK (Clark et al., 2003; Clark et al., 2008). In the absence of RGS regulation of $G\alpha_o$, the μ receptor agonists morphine and DAMGO produced increased levels of cellular tolerance (Clark and Traynor, 2005) and dependence, as measured by AC supersensitization (Clark et al., 2004), indicating that RGS-mediated inactivation of $G\alpha$ subunits is protective against these adaptations. Chapter 3 of this thesis furthers these preliminary studies by evaluating antinociceptive responses to opioids in mice that express RGSi $G\alpha_o$ protein.

Overall goal and specific aims

The overall goal of the work described in this thesis is to better understand the role of the $G\alpha$ subunit $G\alpha_o$ in the behavioral effects of morphine and other opioids acting at the μ receptor. $G\alpha_o$ is the most abundant $G\alpha_{i/o}$ subtype expressed in the brain (Gierschik et al., 1986) and is therefore poised to play an important role in signaling pathways activated by neurotransmitter GPCRs, including the μ opioid receptor (Brown and Sihra, 2008; Jiang and Bajpayee, 2009). Moreover, although RGS proteins are known to play important roles in μ opioid receptor-mediated behaviors (Traynor, 2011), it is not known whether this is mediated *via* direct interaction with $G\alpha_{i/o}$ proteins or through another RGS protein function, such as scaffolding. Given that RGS proteins have been proposed as targets for the therapeutic management of nociception (Neubig and Siderovski, 2002), a better understanding of the relationship between μ opioid receptors, $G\alpha_o$ proteins and RGS proteins will aid the development of improved analgesics that are devoid of deleterious side effects. In particular, this work will further inform drug development strategies that exploit the receptor and G protein selectivity of RGS proteins

and/or the ability of RGS proteins to exert greater control over the effects of partial agonists (Clark et al., 2008).

Aim 1: To determine the role of $G\alpha_o$ in μ opioid-induced antinociception.

The first data chapter of this thesis (Chapter 2) addresses the contribution of $G\alpha_o$ subunits to the antinociceptive effects of morphine and other opioid agonists. Though $G\alpha_o$ is the most abundant $G\alpha_{i/o}$ subtype in the brain (Gierschik et al., 1986), previous studies utilizing ODN knockdown of individual $G\alpha$ isoforms (i.c.v.) have implicated $G\alpha_{i2}$ and/or $G\alpha_z$ in the control of μ opioid receptor-mediated signaling and behavior (Garzon et al., 2000). However, these studies have reported inconsistent results, which is not surprising given that the effectiveness of ODN knockdown strategies can vary across brain regions, mice and/or experiments (Standifer et al., 1996; Connor and Christie, 1999). Thus, the studies in this chapter utilize a transgenic mouse that constitutively lacks expression of $G\alpha_o$ protein as a model in which the manipulation of the $G\alpha$ isoform is consistent. These mice were evaluated for the ability of several μ opioid agonists, including morphine, methadone, and nalbuphine, to produce antinociception against noxious thermal stimuli using the hot plate test and/or the warm-water tail withdrawal test. To determine the contribution of $G\alpha_o$ to μ opioid receptor signaling *in vivo*, homogenates prepared from whole brain or spinal cord of mice lacking $G\alpha_o$ protein were evaluated for μ opioid receptor and G protein expression levels, as well as μ opioid agonist-stimulated G protein activation.

Aim 2: To evaluate how endogenous RGS regulation of $G\alpha_o$ contributes to μ opioid-induced antinociception.

Chapter 3 of this thesis evaluates the hypothesis that endogenous RGS proteins regulate μ opioid antinociception by interacting with $G\alpha_o$. The studies in this chapter utilize a novel knock-in mouse model that expresses the RGSi mutant $G\alpha_o$ protein, $G\alpha_o^{G184S}$. Given the high level of redundancy within the RGS family, knockout of individual RGS isoforms often produces qualitatively minor and/or inconsistent results (Grillet et al., 2005). In contrast, this issue of redundancy is eliminated in mice expressing RGSi $G\alpha_o$ subunits because the $G\alpha$ protein of interest is insensitive to the actions of all endogenous RGS proteins. Mice expressing RGSi $G\alpha_o$ protein were tested

for the ability of morphine or methadone to induce antinociception using the hot plate test and/or the tail withdrawal test. In addition, the ability of morphine and [Met⁵]-enkephalin (ME) to modulate GABAergic neurotransmission in the PAG was examined as a biochemical correlate of antinociception. Finally, homogenates prepared from whole brain or spinal cord of mice expressing RGSi G α_o protein were evaluated for μ opioid receptor expression, G protein expression, and μ opioid agonist-stimulated G protein activation to determine whether the RGSi mutation had any effect on μ opioid signaling *in vivo*.

Aim 3: To examine the role of G α_o in morphine tolerance and dependence.

Using the same transgenic mouse model as in Aim 1 that lacks G α_o protein expression, the final data chapter of this thesis (Chapter 4) examines the involvement of G α_o in the development of tolerance and dependence following chronic exposure to morphine. The role of individual G α subunits in the development of adaptations following chronic morphine exposure has not been extensively studied. Thus, the goal of the studies in this section was to characterize the role of one G α isoform, G α_o , in morphine tolerance and dependence in a comprehensive manner. In particular, mice with reduced G α_o expression were administered repeated injections of morphine and then evaluated for the development of morphine antinociceptive tolerance in the hot plate test and the expression of morphine dependence by measuring the severity of opioid antagonist-precipitated withdrawal. In addition, μ opioid receptor expression and μ opioid agonist-stimulated G protein activation were determined in several brain regions to evaluate if chronic morphine exposure alters μ opioid signaling pathways and whether any observed changes are dependent upon G α_o .

Chapter 2

μ Opioid Receptor Coupling to $G\alpha_o$ Plays An Important Role in Opioid Antinociception

Summary

Opioid analgesics elicit their effects *via* activation of the μ opioid receptor, a GPCR known to interact with $G\alpha_{i/o}$ -type G proteins. Work *in vitro* has suggested that the μ receptor couples preferentially to the abundant brain $G\alpha_{i/o}$ isoform, $G\alpha_o$. However, studies *in vivo* evaluating morphine-mediated antinociception have not supported these findings. The aim of the present work was to evaluate the contribution of $G\alpha_o$ to μ receptor-dependent signaling by measuring both antinociceptive and biochemical endpoints in a $G\alpha_o$ null transgenic mouse strain. Male wild type and $G\alpha_o$ heterozygous null ($G\alpha_o$ +/-) mice were tested for opioid antinociception in the hot plate test or the warm-water tail withdrawal test as measures of supraspinal or spinal antinociception, respectively. Reduction in $G\alpha_o$ levels attenuated the supraspinal antinociception produced by morphine, methadone and nalbuphine, with the magnitude of suppression dependent upon agonist efficacy. This was explained by a reduction in both high-affinity μ receptor expression and μ agonist-stimulated G protein activation in whole brain homogenates from $G\alpha_o$ +/- and $G\alpha_o$ homozygous null ($G\alpha_o$ -/-) mice, compared with wild type littermates. On the other hand, morphine spinal antinociception was not different between $G\alpha_o$ +/- and wild type mice and high-affinity μ receptor expression was unchanged in spinal cord tissue. However, the action of the partial agonist nalbuphine was compromised, showing that reduction in $G\alpha_o$ protein does decrease spinal antinociception, but suggesting a higher $G\alpha_o$ protein reserve. These results provide the

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first *in vivo* evidence that $G\alpha_o$ contributes to maximally efficient μ opioid receptor signaling and antinociception.

Introduction

Opioid analgesics are prescribed for the management of moderate to severe pain. Clinically used opioids elicit their effects by stimulation of the μ opioid receptor, a member of the GPCR superfamily that interacts with heterotrimeric G proteins ($G\alpha\beta\gamma$), which are defined in terms of the $G\alpha$ subunit. Specifically, the μ receptor couples to $G\alpha$ proteins of the PTX-sensitive $G\alpha_{i/o}$ family, comprised of $G\alpha_o$ (including splice variants $G\alpha_{o1}$ and $G\alpha_{o2}$), $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ (Laugwitz et al., 1993; Chakrabarti et al., 1995), as well as PTX-insensitive $G\alpha_z$ (Garzon et al., 1997b). In the inactive state, $G\alpha\beta\gamma$ exists in complex with the receptor. Upon agonist stimulation, GDP bound to the $G\alpha$ subunit is exchanged for GTP, resulting in dissociation of active $G\alpha$ -GTP from the $G\beta\gamma$ heterodimer (reviewed in Brown and Sihra, 2008); both $G\alpha$ -GTP and $G\beta\gamma$ modulate effectors downstream of the μ opioid receptor, including AC (Yu and Sadee, 1988) and calcium channels (Hescheler et al., 1987; Moises et al., 1994). It has been shown that specific $G\alpha_{i/o}$ subunits differentially contribute to μ receptor-dependent behavioral responses, including morphine-mediated antinociception (Raffa et al., 1994; Sanchez-Blazquez et al., 2001). However, findings are inconsistent due to the variety of methods and models utilized in previous work, such that the contribution of each $G\alpha$ subunit to these responses is controversial.

$G\alpha_o$ is highly expressed in brain (Gierschik et al., 1986). Multiple lines of evidence suggest that opioid agonists can activate μ opioid receptor-G protein complexes in a non-selective manner, especially in heterologous expression systems (Laugwitz et al., 1993; Clark et al., 2006; Clark and Traynor, 2006). On the other hand, the μ -selective agonist DAMGO was found to activate $G\alpha_o$ to a greater extent than either $G\alpha_{i2}$ or $G\alpha_{i3}$ (Clark et al., 2008). Furthermore, in cultured neurons or neuronal-like cells, the μ receptor has been shown to couple to AC (Carter and Medzihradsky, 1993) and N-type Ca^{2+} channels (Hescheler et al., 1987; Moises et al., 1994) primarily *via* activation of $G\alpha_o$ (for review, see Jiang and Bajpayee, 2009).

Despite the abundance of $G\alpha_o$ in the brain and evidence from *in vitro* studies that $G\alpha_o$ modulates signaling downstream of the μ receptor, together with a recent report that $G\alpha_o$ may be involved in opioid dependence (Kest et al., 2009), findings *in vivo* have primarily implicated $G\alpha_{i2}$ and/or $G\alpha_z$ proteins as mediators of opioid agonist antinociception (Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Standifer et al., 1996; Sanchez-Blazquez et al., 2001). These studies utilized mice administered i.c.v. antisense ODNs against a specific $G\alpha$ subunit prior to antinociceptive testing of opioid agonists (i.c.v.) in the tail flick test (reviewed in Garzon et al., 2000). However, there are a number of inherent difficulties with this technique, including proper verification of the extent of protein knockdown. For most of these studies, knockdown of $G\alpha$ protein did not exceed ~50% in peri-ventricular regions (e.g. PAG) (Sanchez-Blazquez et al., 1995), while ODNs were less effective in brain regions more distal to the site of infusion (e.g. thalamus), presumably due to poor diffusion (Sanchez-Blazquez et al., 1995; Standifer et al., 1996). However, in one study, in which greater (~60-80%) knockdown of $G\alpha$ subunits was achieved, ODNs directed against $G\alpha_o$, in addition to other $G\alpha$ isoforms, suppressed morphine antinociception (Standifer et al., 1996). Clearly, inconsistencies in the efficacy and selectivity of $G\alpha$ protein knockdown complicate the interpretation of these studies. This previous work is further limited in that only a single measure of opioid antinociception was evaluated.

The present study was designed to test the hypothesis that μ opioid receptor coupling to $G\alpha_o$ is necessary for opioid antinociception using a constitutive $G\alpha_o$ knockout mouse strain (Duan et al., 2007). To probe the role of $G\alpha_o$ in μ receptor-mediated antinociception, opioid spinal and supraspinal antinociception were evaluated in response to noxious thermal stimuli; this is the first time that mice null for $G\alpha_o$ have been evaluated for alterations in μ receptor-dependent antinociception. Furthermore, to directly relate changes in opioid antinociception to alterations in μ opioid receptor function, membrane homogenates from either whole brain or spinal cord of $G\alpha_o$ transgenic mice were evaluated for μ opioid receptor expression and μ agonist-stimulated G protein activity. These studies demonstrate that the abundant brain G protein, $G\alpha_o$, is the primary $G\alpha$ subtype responsible for μ opioid receptor-mediated signaling and antinociception.

Methods

Transgenic mice

Transgenic mice null for *Gnao1*, *Gnai2* or *Gnai3* were generated as previously described (Mortensen et al., 1992; Sowell et al., 1997; Duan et al., 2007) and were backcrossed onto the 129S6/SvEvTac (129S6) strain for four generations. Transgenic mice and wild type littermates were obtained by heterozygous breeding to control for genetic background. Adult, opioid-naïve male mice, matched for age, were utilized for all experiments. Mice were group-housed with food and water available *ad libitum*. Lights were maintained on a 12-h light/dark cycle (lights on at 7:00), and all testing was performed during the light phase. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and all experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Antinociceptive tests

The hot plate test was used to evaluate supraspinal antinociception. Mice were given two injections of saline (intraperitoneal; i.p.) to determine baseline latency, followed by three cumulative doses of agonist (i.p.) in 15 min intervals (nalbuphine) or 30 min intervals (morphine, methadone). Where four doses of drug were used, dose-effect curves were generated by pooling data from two overlapping, cumulative dose-effect measurements. Mice were placed on a 52°C or 55°C hot plate at the appropriate interval following each injection and the latency to lick forepaw(s) or jump was measured with a cutoff time of 60 s or 45 s for the 52°C or 55°C hot plate temperatures, respectively, in order to prevent tissue damage.

The warm-water tail withdrawal test was used to evaluate spinal antinociception. Mice were given a single injection of saline (i.p.) to determine baseline latency, followed by four cumulative doses of agonist (i.p.) in 15 min intervals (nalbuphine) or 30 min intervals (morphine). The distal tip of the mouse's tail was placed in a 50°C or 55°C warm-water bath at the appropriate interval following each injection and the latency to tail flick was measured with a cutoff time of 20 s or 15 s for 50°C or 55°C water, respectively, in order to prevent tissue damage.

For both antinociceptive tests, agonist-stimulated antinociception is expressed as percent maximum possible effect (% MPE), where $\% \text{ MPE} = (\text{post-drug latency} - \text{baseline latency}) \div (\text{cutoff latency} - \text{baseline latency}) \times 100$.

Membrane preparation

Mice were euthanized by cervical dislocation. Whole brain tissue, minus cerebellum, or thoracic and lumbar spinal cord was removed, immediately chilled in ice-cold 50 mM Tris base, pH 7.4, and membrane homogenates were prepared as previously described (Lester and Traynor, 2006). Final membrane pellets were resuspended in 50 mM Tris base, pH 7.4, aliquoted and stored at -80°C . Protein content was determined using the method of Bradford (Bradford, 1976).

Western blot analysis

Membranes from whole brain (20 μg protein) were mixed with sample buffer (63 mM Tris base, pH 6.8, 2% SDS, 10% glycerol, 0.008% bromophenol blue, 50 mM dithiothreitol) and separated by SDS-PAGE on 10% (for detection of $\text{G}\alpha_{\text{o}}$, $\text{G}\alpha_{\text{z}}$, $\text{G}\alpha_{\text{i1}}$, $\text{G}\alpha_{\text{i2}}$, $\text{G}\alpha_{\text{i3/1}}$ or $\text{G}\beta_{\text{1-4}}$) or 15% polyacrylamide gels (for detection of $\text{G}\gamma_{\text{2}}$). Proteins were then transferred to nitrocellulose membranes (Pierce, Rockford, IL) and probed with either rabbit polyclonal anti- $\text{G}\alpha_{\text{o}}$ (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti- $\text{G}\alpha_{\text{z}}$ (1:200; Santa Cruz), rabbit polyclonal anti- $\text{G}\alpha_{\text{i1}}$ (1:100; Santa Cruz), mouse monoclonal anti- $\text{G}\alpha_{\text{i2}}$ (1:1000; Millipore, Billerica, MA), rabbit polyclonal anti- $\text{G}\beta_{\text{1-4}}$ (1:500; Santa Cruz) or rabbit polyclonal anti- $\text{G}\gamma_{\text{2}}$ (1:200; Santa Cruz). Membranes from spinal cord (20 μg protein) were also evaluated for $\text{G}\alpha_{\text{o}}$ protein content, as above. All membranes were probed with mouse monoclonal anti- α -tubulin (1:1000; Sigma-Aldrich, St. Louis, MO) as a loading control. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:10,000; Santa Cruz). Antibody immunoreactivity was detected by enhanced chemiluminescence using an EpiChem3 Benchtop Darkroom (UVP, Upland, CA) and band densities were quantified using Image J software (<http://rsbweb.nih.gov/ij/index.html>). Specifically, after background chemiluminescence was subtracted, G protein band densities were normalized to respective α -tubulin band densities and used to calculate expression relative to wild type for each G protein.

Radioligand binding assays

For [³H]diprenorphine binding, membranes from whole brain (100 µg protein) or spinal cord (100-200 µg protein) were incubated for 60 min at 25°C with 4 nM [³H]diprenorphine in 50 mM Tris base, pH 7.4, with or without the µ-selective antagonist CTAP (300 nM) to define µ opioid receptors. For [³H]DAMGO saturation binding, membranes from whole brain (100 µg protein) were incubated for 60 min at 25°C with increasing concentrations of [³H]DAMGO (0.09-12 nM) in 50 mM Tris base, pH 7.4. Membranes from spinal cord (100-200 µg protein) were incubated for 60 min at 25°C with 12 nM [³H]DAMGO in 50 mM Tris base, pH 7.4. For all radioligand binding assays, non-specific binding was evaluated in the presence of 10 µM naloxone. Reactions were stopped by rapid filtration through a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD), and bound radioligand was collected on GF/C filtermats (Whatman, Kent, UK) and rinsed three times with ice-cold 50 mM Tris base, pH 7.4. Filters were dried, saturated with EcoLume scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity was counted using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

Agonist-stimulated [³⁵S]GTPγS binding assays

To measure binding of the non-hydrolyzable GTP analog guanosine-5'-O-(3-[³⁵S]thio)triphosphate ([³⁵S]GTPγS) to Gα proteins, membranes from whole brain (10 µg protein) or spinal cord (25-50 µg protein) were pre-incubated for 10 min at 25°C with or without various concentrations of the opioid agonists DAMGO, methadone, morphine or nalbuphine in [³⁵S]GTPγS binding buffer (50 mM Tris base, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 100 µM GDP and 0.4 U/mL adenosine deaminase). After pre-incubation, 0.1 nM [³⁵S]GTPγS was added and reactions were further incubated for 90 min at 25°C. For saturation analysis of [³⁵S]GTPγS binding, membranes from whole brain (10 µg protein) were pre-incubated for 10 min at 25°C with or without 10 µM DAMGO in [³⁵S]GTPγS binding buffer, followed by incubation for 90 min at 25°C with 0.1 nM [³⁵S]GTPγS, with or without various concentrations of unlabeled GTPγS (0.8 – 50 nM). For all [³⁵S]GTPγS binding assays, non-specific binding was evaluated in the presence of 10 µM GTPγS. Binding reactions were stopped by rapid

filtration, rinsed three times with ice-cold wash buffer (50 mM Tris base, pH 7.4, 5 mM MgCl₂, 100 mM NaCl), and bound radioactivity was measured by liquid scintillation counting, as above.

Drugs

Morphine sulfate was from RTI (Research Triangle Park, NC). Methadone and nalbuphine were obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). For behavioral experiments, all drugs were diluted in sterile water. [³H]diprenorphine, [³H]DAMGO and [³⁵S]GTPγS were purchased from PerkinElmer. Adenosine deaminase was obtained from Calbiochem (San Diego, CA). DAMGO, CTAP, GDP, GTPγS and all other chemicals were obtained from Sigma-Aldrich, unless otherwise noted.

Data analysis

All data were analyzed using GraphPad Prism software, version 5.0 (GraphPad, San Diego, CA). Differences between genotypes were evaluated using Students' *t*-tests or one-way or two-way analysis of variance (ANOVA) with Bonferroni's post-tests, where appropriate. For all statistical tests, significance was set at $p < 0.05$. *In vivo* potency (50% effective dose; ED₅₀) values were calculated by fitting the compiled data to an agonist *versus* normalized response curve (Hill slope=1), and values are expressed as the mean (95% confidence interval; CI). Where antinociception was near or below 50% MPE, ED₅₀ values were extrapolated from the fitted data. Maximal radioligand binding (B_{max}) and radioligand binding affinity (K_D) values were derived by fitting each experiment to a one-site saturation binding curve fit (Hill slope=1), while maximal [³⁵S]GTPγS stimulation (maximal agonist-stimulated response; E_{max}) and *in vitro* potency (50% effective concentration; EC₅₀) values were calculated by fitting individual experiments to an agonist *versus* response curve fit (Hill slope=1); values are expressed as the mean ± standard error of the mean (SEM).

Results

Characterization of transgenic mice lacking Gα_o protein

The full knockout, Gα_o -/- mice did not often survive until weaning (~21 days), whereas wild type and Gα_o +/- mice were obtained at frequencies predicted by Mendelian

genetics (Table 2.1) ($\chi^2=11.07$, $df=1$, $p<0.001$). Peri-natal lethality was also noted in the initial reports of two independently-generated $G\alpha_o$ null mouse strains (Valenzuela et al., 1997; Jiang et al., 1998). These previous studies also reported several neurological abnormalities in $G\alpha_o$ $-/-$ animals, including hyperactivity, tremor and turning behavior; however, no such gross behavioral abnormalities were noted for the $G\alpha_o$ $+/-$ or $G\alpha_o$ $-/-$ mice used in this study (Duan et al., 2007; unpublished observations). In adulthood (>8 weeks), body weight varied as a function of genotype (Table 2.1) ($F(2,23)=14.54$, $p<0.001$). *Post hoc* analysis revealed that those $G\alpha_o$ $-/-$ mice that did survive weighed significantly less than their wild type littermates, whereas $G\alpha_o$ $+/-$ mice did not differ from wild type controls.

Supraspinal antinociception in $G\alpha_o$ transgenic mice

To determine whether $G\alpha_o$ is involved in opioid antinociception, $G\alpha_o$ $+/-$ mice were evaluated for morphine antinociception in the hot plate test (Figure 2.1). In the 52°C hot plate test, the baseline nociceptive threshold was not significantly different between wild type (12.6 ± 0.6 s; $n=30$) and $G\alpha_o$ $+/-$ mice (12.2 ± 0.5 s; $n=32$; $t(60)=0.4885$, $p=0.627$). Morphine produced a dose-dependent increase in antinociception that was significantly reduced (~4-fold) in $G\alpha_o$ $+/-$ mice when compared with wild type controls, with ED_{50} values of 47.7 mg/kg (31.2 – 72.9) and 11.4 mg/kg (5.9 – 22.1), respectively (Figure 2.1a). Although there was no significant interaction, there were significant main effects of dose and genotype (dose: $F(2,36)=19.88$; $p<0.001$; genotype: $F(1,36)=15.76$, $p<0.001$).

Increasing the efficacy requirements of the nociceptive system might further exaggerate this observed genotype difference; thus, the hot plate temperature was raised to 55°C and $G\alpha_o$ transgenic mice were again evaluated for morphine supraspinal antinociception (Figure 2.1b). As expected, a decreased baseline nociceptive threshold was observed at the elevated hot plate temperature, and there were no significant differences between wild type (7.5 ± 0.8 s; $n=9$) and $G\alpha_o$ $+/-$ mice at baseline (6.6 ± 0.6 s; $n=10$; $t(17)=0.8940$, $p=0.384$). Morphine dose-dependently produced antinociception in both wild type and $G\alpha_o$ $+/-$ mice, but the ED_{50} was shifted ~6-fold for $G\alpha_o$ $+/-$ mice, with a value of 62.7 mg/kg (42.9 – 91.5) compared with 9.9 mg/kg (5.8 – 17.1) for wild type littermates (Figure 2.1b).

Table 2.1: Physical characteristics of wild type and $G\alpha_o$ transgenic mice.

Genotype	Body Weight (g)	Genotype Frequency at Weaning (%)	
		<i>Expected</i>	<i>Observed (n=347)</i>
Wild type	28.6 ± 1.3 (n=10)	25.0	35.7 (n=124)
$G\alpha_o$ +/-	27.6 ± 0.8 (n=12)	50.0	59.4 (n=206)
$G\alpha_o$ -/-	18.2 ± 1.4 (n=4)*	25.0	4.9 (n=17)

Asterisk indicates a statistical difference *versus* wild type by Bonferroni's post-test ($p < 0.001$).

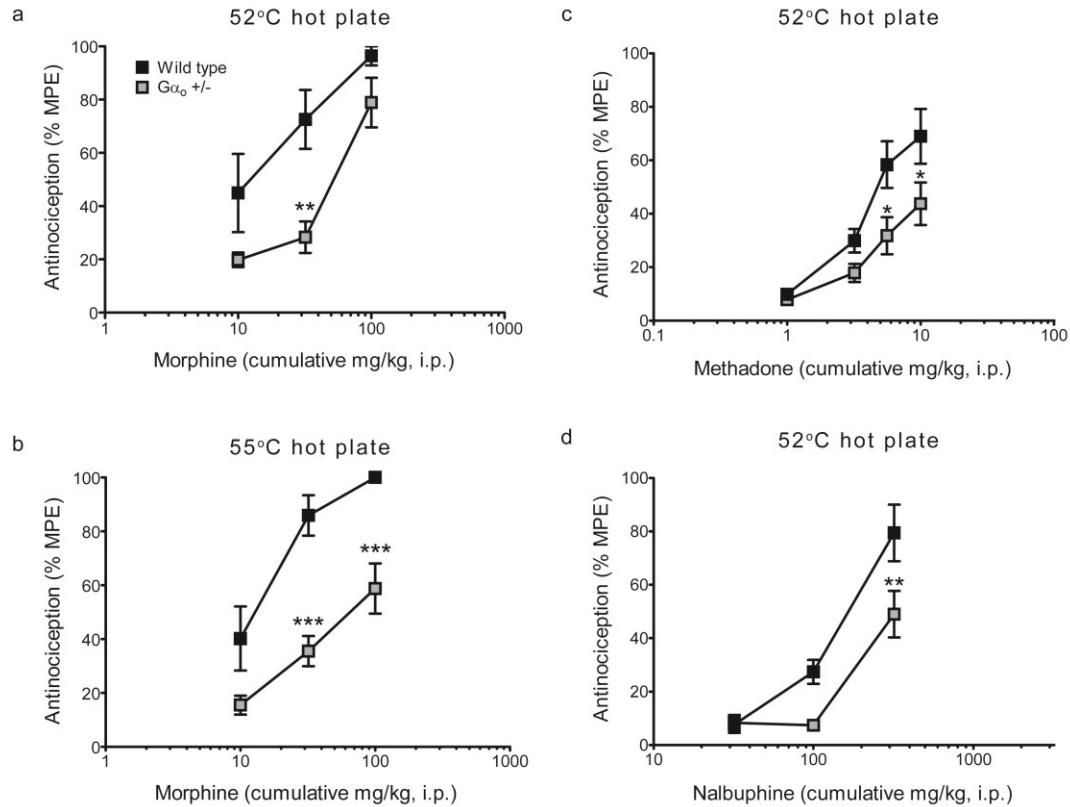


Figure 2.1: Supraspinal antinociception produced by morphine, methadone and nalbuphine in the hot plate test in $G\alpha_o$ transgenic mice.

Antinociception was measured in wild type and $G\alpha_o$ +/- mice 30 min following morphine in the (a) 52°C or (b) 55°C hot plate test, (c) 30 min following methadone in the 52°C hot plate test, and (d) 15 min following nalbuphine in the 52°C hot plate test. Data represent the mean \pm SEM for morphine at 52°C ($n=7$) and 55°C ($n=9-10$), for methadone ($n=7-15$) and for nalbuphine ($n=8-10$). Legend in panel (a) also describes panels (b) through (d). Asterisks indicate a statistical difference *versus* wild type by Bonferroni's post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

There were significant main effects of both dose ($F(2,51)=25.37$; $p<0.001$) and genotype ($F(1,51)=41.98$, $p<0.001$), although there was no significant interaction.

To determine whether $G\alpha_o$ plays a role in the antinociception produced by opioid agonists other than morphine, $G\alpha_o$ transgenic mice were evaluated for either methadone or nalbuphine antinociception in the 52°C hot plate test (Figure 2.1c,d). Like morphine, methadone produced a dose-dependent increase in antinociception (Figure 2.1c). The ED_{50} value for wild type mice was 13.0 mg/kg (10.1 – 16.8), which was ~2-fold higher than the extrapolated ED_{50} value for $G\alpha_o$ +/- mice of 5.8 mg/kg (4.4 – 7.6). There was no significant interaction; however, there were significant main effects of both dose ($F(3,82)=33.12$, $p<0.001$) and genotype ($F(1,82)=19.87$, $p<0.001$). The partial agonist nalbuphine also produced a dose-dependent stimulation of antinociception that was significantly reduced for $G\alpha_o$ +/- mice, compared with wild type littermates (Figure 2.1d), with an ED_{50} value of 170.2 mg/kg (108.3 – 267.6) for wild type mice. Extrapolation of the dose-response curve for $G\alpha_o$ +/- mice gave an ED_{50} value of 432.0 mg/kg (289.0 – 645.9), representing a ~3-fold shift. There were significant main effects of both dose ($F(2,48)=48.62$, $p<0.001$) and genotype ($F(1,48)=11.09$; $p=0.002$), as well as a significant dose \times genotype interaction ($F(2,48)=3.377$, $p=0.043$).

Spinal antinociception in $G\alpha_o$ transgenic mice

$G\alpha_o$ transgenic mice were also evaluated in the warm-water tail withdrawal test (Figure 2.2), the same antinociceptive measure that was utilized in the majority of antisense ODN studies (Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Sanchez-Blazquez et al., 2001). In the 50°C tail withdrawal test, the baseline tail flick latency was not significantly different between wild type (3.2 ± 0.4 s; $n=13$) and $G\alpha_o$ +/- mice (4.2 ± 0.6 s; $n=15$; $t(26)=1.399$, $p=0.174$). Morphine produced a dose-dependent increase in antinociception in both wild type and $G\alpha_o$ +/- mice, with ED_{50} values of 5.2 mg/kg (2.7 – 9.8) and 4.1 mg/kg (2.3 – 7.2), respectively (Figure 2.2a). There was a significant main effect of dose ($F(3,44)=14.79$, $p<0.001$), although the main effect of genotype ($F(1,44)=0.1024$, $p=0.751$) and the dose \times genotype interaction were not significant.

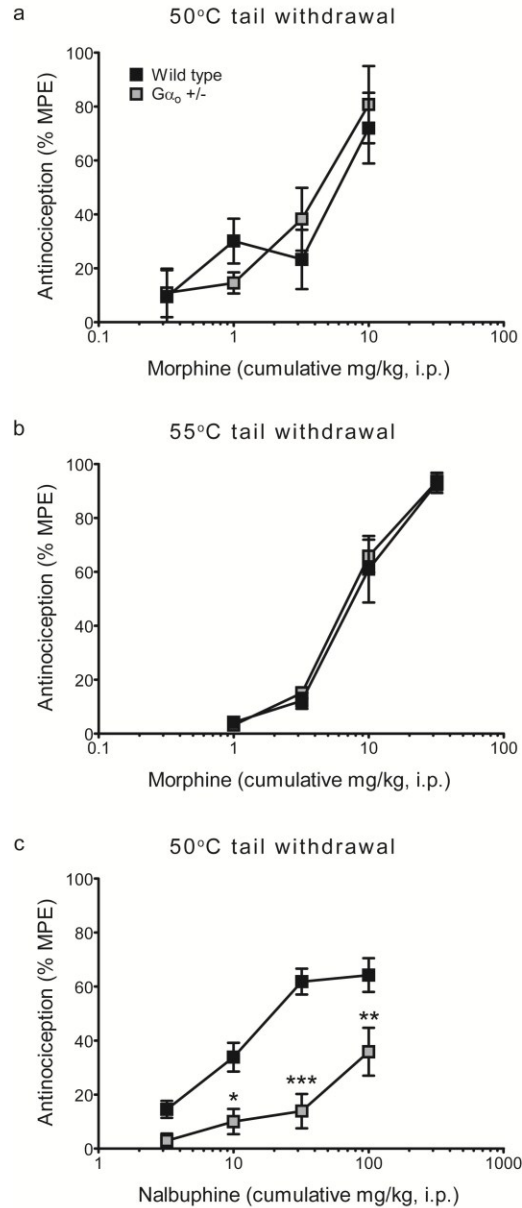


Figure 2.2: Ability of morphine and nalbuphine to induce spinal antinociception in the warm-water tail withdrawal test in $G\alpha_0$ transgenic mice.

Antinociception was measured in wild type and $G\alpha_0$ +/- mice 30 min following morphine in the (a) 50°C or (b) 55°C warm-water tail withdrawal test and (c) 15 min following nalbuphine in the 50°C warm-water tail withdrawal test. Data represent the mean \pm SEM for morphine at 50°C ($n=6-7$) and 55°C ($n=8$) and for nalbuphine ($n=7-8$). Legend for panels (b) and (c) is the same as for panel (a). Asterisks indicate a statistical difference *versus* wild type by Bonferroni's post-test (* $p<0.05$, *** $p<0.001$).

Given that morphine behaves as a full agonist in this test, which may preclude the identification of small differences between genotypes, it was hypothesized that increasing the efficacy requirement of the system by raising the water bath temperature to 55°C (Figure 2.2b) should allow for the identification of such differences. Again, as predicted, a decreased baseline nociceptive threshold was observed at the elevated water temperature, and there were also no significant differences between wild type (1.9 ± 0.1 s; $n=8$) and $G\alpha_o$ +/- mice in this test (1.8 ± 0.2 s; $n=8$; $t(14)=0.6932$, $p=0.500$). Against the 55°C stimulus, morphine produced a dose-dependent increase in antinociception that was equivalent between wild type and $G\alpha_o$ +/- mice, with ED_{50} values of 8.2 mg/kg (5.5 – 12.5) and 7.4 mg/kg (5.5 – 10.0), respectively (Figure 2.2b). There was no significant interaction or significant effect of genotype (genotype: $F(1,56)=0.2371$, $p=0.628$), but there was a significant main effect of dose (dose: $F(3,56)=124.0$, $p<0.001$).

As an alternative method of evaluating whether the efficacious antinociception produced by morphine was masking a mediatory role for $G\alpha_o$, spinal antinociception was measured in the 50°C warm-water tail withdrawal test in response to the low-efficacy agonist, nalbuphine (Figure 2.2c). Nalbuphine produced a dose-dependent stimulation of spinal antinociception that was significantly reduced (~7-fold) in $G\alpha_o$ +/- mice when compared with wild type littermates, with wild type mice exhibiting an ED_{50} value of 24.2 mg/kg (17.5 – 33.4) (Figure 2.2a). Extrapolation of the nalbuphine dose-response for $G\alpha_o$ +/- mice revealed an ED_{50} value of 176.1 mg/kg (113.3 – 273.8). There were significant main effects of dose and genotype (dose: $F(3,52)=20.35$, $p<0.001$; genotype: $F(1,52)=48.62$, $p<0.001$), as well as a significant dose \times genotype interaction ($F(3,52)=3.552$, $p=0.021$).

Antinociception in $G\alpha_{i2}$ and $G\alpha_{i3}$ transgenic mice

To confirm the importance of $G\alpha_o$ for opioid antinociception, transgenic mice lacking either $G\alpha_{i2}$ ($G\alpha_{i2}$ heterozygous null, $G\alpha_{i2}$ +/-; $G\alpha_{i2}$ homozygous null, $G\alpha_{i2}$ -/-) or $G\alpha_{i3}$ ($G\alpha_{i3}$ heterozygous null, $G\alpha_{i3}$ +/-; $G\alpha_{i3}$ homozygous null, $G\alpha_{i3}$ -/-), together with their respective wild type littermates, were evaluated in the 52°C hot plate and 50°C warm-water tail withdrawal tests (Figure 2.3).

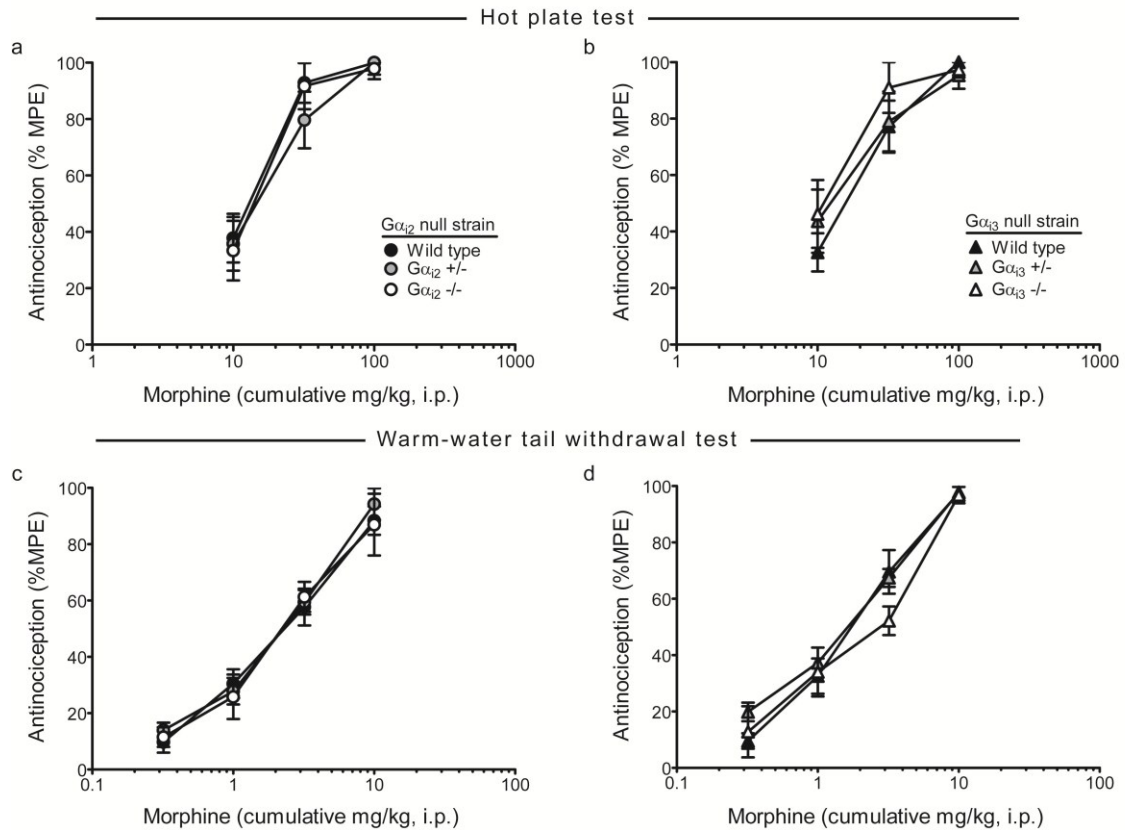


Figure 2.3: Morphine supraspinal and spinal antinociception in $G\alpha_{i2}$ and $G\alpha_{i3}$ transgenic mice.

Antinociception produced 30 min following morphine was evaluated in the 52°C hot plate test in (a) $G\alpha_{i2} +/-$ and $G\alpha_{i2} -/-$ mice and (b) $G\alpha_{i3} +/-$ and $G\alpha_{i3} -/-$ mice and in the 50°C warm-water tail withdrawal test in (c) $G\alpha_{i2} +/-$ and $G\alpha_{i2} -/-$ mice and (d) $G\alpha_{i3} +/-$ and $G\alpha_{i3} -/-$ mice, together with their respective wild type littermates. Data represent the mean \pm SEM for $G\alpha_{i2}$ mice in the hot plate ($n=8-10$) and tail withdrawal tests ($n=6-9$) and for $G\alpha_{i3}$ mice in the hot plate ($n=8-9$) and tail withdrawal tests ($n=6-9$). Legends for panels (c) and (d) are the same as for panels (a) and (b), respectively.

Both the $G\alpha_{i2}$ and the $G\alpha_{i3}$ transgenic mouse strains were generated in parallel with $G\alpha_o$ transgenic mice, and inactivation of the appropriate $G\alpha$ subunit has been previously confirmed by Western blot analysis (Sowell et al., 1997; Duan et al., 2007). In the 52°C hotplate test (Figure 2.3a,b), the baseline response latency was equivalent among all $G\alpha_{i2}$ transgenic mouse genotypes (wild type: 12.0 ± 0.7 s, $n=10$; $G\alpha_{i2} +/-$: 11.6 ± 0.7 s, $n=9$; $G\alpha_{i2} -/-$: 12.9 ± 0.7 s, $n=8$; $F(2,24)=0.8112$, $p=0.456$). Morphine produced a dose-dependent increase in antinociception that was not different between wild type, $G\alpha_{i2} +/-$ and $G\alpha_{i2} -/-$ mice, with ED_{50} values of 9.4 mg/kg (5.8 – 15.3), 12.0 mg/kg (6.9 – 20.8) and 10.6 mg/kg (6.0 – 18.8), respectively (Figure 2.3a). There was a significant main effect of dose ($F(2,72)=59.03$, $p<0.001$), but the main effect of genotype ($F(2,72)=0.3274$, $p=0.722$) and the dose \times genotype interaction were not significant. Similarly, in $G\alpha_{i3}$ transgenic mice, morphine produced a dose-dependent increase in antinociception that was equivalent between wild type, $G\alpha_{i3} +/-$ and $G\alpha -/-$ mice, with ED_{50} values of 13.2 mg/kg (8.5 – 20.4), 10.6 mg/kg (6.0 – 18.9) and 8.1 mg/kg (4.4 – 15.0), respectively (Figure 2.3b). There was a significant main effect of dose ($F(2,69)=37.38$, $p<0.001$), but not genotype ($F(2,69)=0.7686$, $p=0.468$), and no significant interaction. There were also no genotype-dependent differences observed in the baseline nociceptive threshold for these mice (wild type: 13.3 ± 1.4 s, $n=9$; $G\alpha_{i3} +/-$: 16.8 ± 1.2 s, $n=9$; $G\alpha_{i3} -/-$: 16.2 ± 1.4 s, $n=8$; $F(2,23)=2.139$, $p=0.141$).

$G\alpha_{i2}$ and $G\alpha_{i3}$ transgenic mice were also evaluated for spinal antinociception in the 50°C tail withdrawal test (Figure 2.3c,d). Baseline response latencies in this test were equivalent among all $G\alpha_{i2}$ (wild type: 5.0 ± 0.4 s, $n=9$; $G\alpha_{i2} +/-$: 4.4 ± 0.6 s, $n=8$; $G\alpha_{i2} -/-$: 3.9 ± 0.5 s, $n=6$; $F(2,20)=1.050$, $p=0.368$) and $G\alpha_{i3}$ transgenic mouse genotypes (wild type: 5.6 ± 0.4 s, $n=8$; $G\alpha_{i3} +/-$: 4.2 ± 0.5 s, $n=9$; $G\alpha_{i3} -/-$: 4.4 ± 0.8 s, $n=6$; $F(2,20)=1.990$, $p=0.163$). Morphine produced a dose-dependent increase in antinociception that was not different between wild type, $G\alpha_{i2} +/-$ and $G\alpha_{i2} -/-$ mice, with ED_{50} values of 2.2 mg/kg (1.6 – 2.9), 2.0 mg/kg (1.6 – 2.6) and 2.2 mg/kg (1.5 – 3.3), respectively (Figure 2.3c). There was no significant interaction or main effect of genotype ($F(2,80)=0.2412$, $p=0.786$), but there was a significant main effect of dose ($F(3,80)=113.5$, $p<0.001$). Similarly, in $G\alpha_{i3}$ transgenic mice, morphine produced a dose-dependent increase in antinociception that was equivalent in wild type, $G\alpha_{i3} +/-$ and $G\alpha_{i3} -/-$ mice, with ED_{50}

values of 1.6 mg/kg (1.2 – 2.2), 1.4 mg/kg (1.2 – 1.7) and 2.0 mg/kg (1.4 – 3.1), respectively (Figure 2.3d). Although there was no significant interaction or main effect of genotype ($F(2,80)=1.936, p=0.151$), there was a significant main effect of dose ($F(3,80)=171.1, p<0.001$).

G protein expression in $G\alpha_o$ transgenic mouse brain

Western blot analysis of G protein expression in whole brain membrane samples confirmed the loss of $G\alpha_o$ protein in $G\alpha_o$ $-/-$ mice (Figure 2.4a). Quantification of Western blot images for $G\alpha_o$ revealed that, in comparison with wild type controls, $G\alpha_o$ $+/-$ mice express ~60% less $G\alpha_o$ protein, which is close to the expected 50% reduction (Figure 2.4a). Across a panel of G protein subunits, including $G\alpha_{i/o}$, $G\beta$ and $G\gamma$ proteins (Figure 2.4), the expression of $G\alpha_o$ (Figure 2.4a) ($F(2,6)=527.9, p<0.001$), $G\beta_{1-4}$ (Figure 2.4f) ($F(2,6)=46.53, p<0.001$) and $G\gamma_2$ (Figure 2.4g) ($F(2,6)=18.45, p=0.003$) were significantly decreased as a function of genotype. In contrast, there were no compensatory changes noted for the expression of either $G\alpha_z$ (Figure 2.4b) ($F(2,6)=0.0548, p=0.947$), $G\alpha_{i1}$ (Figure 2.4c) ($F(2,6)=0.6938, p=0.536$) or $G\alpha_{i2}$ (Figure 2.4d) ($F(2,6)=0.0189, p=0.981$).

μ Receptor expression in $G\alpha_o$ transgenic mouse brain and spinal cord

To evaluate whether the reduction in opioid antinociception observed in $G\alpha_o$ $+/-$ mice could be explained by alterations at the receptor level, μ opioid receptor expression was measured in membranes from whole brain or from spinal cord of $G\alpha_o$ transgenic mice (Table 2.2). Binding of a maximal concentration (4 nM) of the radiolabeled opioid antagonist [3 H]diprenorphine, representing the entire pool of μ , δ and κ opioid receptors, was unaffected by genotype in either whole brain ($F(2,5)=0.3542, p=0.718$) or spinal cord ($t(4)=0.0097, p=0.993$). To measure total μ receptor expression, maximal [3 H]diprenorphine binding was displaced using the μ -selective antagonist CTAP (300 nM). Total μ receptor expression was also not different between genotypes (Table 2.2) in either whole brain ($F(2,5)=0.6832, p=0.547$) or spinal cord ($t(4)=0.7611, p=0.489$).

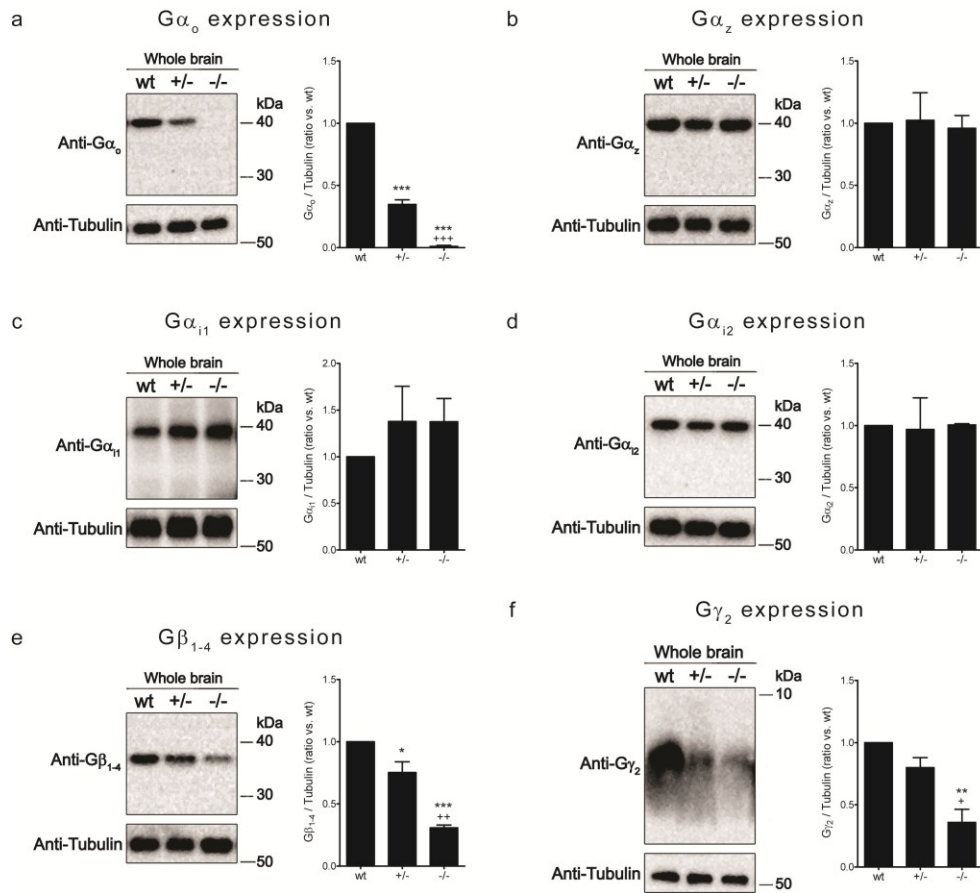


Figure 2.4: G protein expression in whole brain homogenates from $G\alpha_0$ transgenic mice.

Membranes from whole brain of wild type (wt), $G\alpha_0$ +/- (+/-) and $G\alpha_0$ -/- (-/-) mice were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed for the expression of (a) $G\alpha_0$, (b) $G\alpha_z$, (c) $G\alpha_{i1}$, (d) $G\alpha_{i2}$, (e) $G\beta_{1-4}$ or (f) $G\gamma_2$ using selective antibodies (see Materials and Methods); membranes were also probed for tubulin as a loading control. G protein expression was quantified in Image J by normalizing G protein band intensity to tubulin band intensity, and data are plotted as a ratio of wt expression. Data represent the mean \pm SEM ($n=3$). Symbols indicate a statistical difference *versus* wt (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) or +/- ($^+p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$) by Bonferroni's post-test.

Table 2.2: Properties of agonist and antagonist radioligand binding in membranes from whole brain or spinal cord of wild type and $G\alpha_o$ transgenic mice.

Tissue	Genotype	$[^3\text{H}]$ Diprenorphine Binding		$[^3\text{H}]$ DAMGO Binding	
		Total (fmol/mg protein)	μ Receptors ^a (fmol/mg protein)	B_{max} (fmol/mg protein)	K_D (nM)
Whole brain	Wild type	366 ± 24	218 ± 13 ^b	246 ± 29	2.5 ± 0.4
	$G\alpha_o$ +/-	391 ± 38	216 ± 3	181 ± 16	1.9 ± 0.5
	$G\alpha_o$ -/-	407 ± 38	233 ± 12	121 ± 17*	3.5 ± 1.3
Spinal cord	Wild type	161 ± 20	95 ± 5	84 ± 5 ^c	ND
	$G\alpha_o$ +/-	161 ± 14	101 ± 6	93 ± 6 ^c	ND

ND, not determined. ^a μ Receptor expression was evaluated as the amount of bound $[^3\text{H}]$ diprenorphine at a maximal concentration that was displaced by the μ -selective antagonist CTAP (300 nM). ^b In wild type whole brain, there is a trend for total μ receptor number to be less than high-affinity μ opioid receptor number because binding was measured indirectly (see Materials and Methods). ^c In spinal cord, B_{max} values were estimated using a single maximal concentration of $[^3\text{H}]$ DAMGO. Data represent the mean ± SEM ($n=2-3$ performed in at least duplicate). Asterisk indicates a statistical difference *versus* wild type whole brain by Bonferroni's post-test ($p<0.05$).

In whole brain, maximal binding (B_{max}) of [3 H]DAMGO (Table 2.2), which, as an agonist, recognizes only high-affinity μ opioid receptors, was significantly decreased in $G\alpha_o$ +/- and $G\alpha_o$ -/- mice when compared with wild type controls ($F(2,5)=10.44$; $p=0.016$). There was no change across genotypes in the affinity (K_D) of [3 H]DAMGO for high-affinity μ receptor sites ($F(2,5)=1.398$; $p=0.330$). In contrast, maximal (12 nM) [3 H]DAMGO binding was unchanged in the spinal cord of $G\alpha_o$ +/- mice when compared with wild type littermate controls ($t(4)=1.186$, $p=0.301$).

G protein activation in $G\alpha_o$ transgenic mouse brain and spinal cord

To examine the importance of $G\alpha_o$ for μ opioid receptor function, the [35 S]GTP γ S binding assay was utilized to evaluate the first component of μ opioid signaling, namely, G protein activation, in membranes from either whole brain or spinal cord of $G\alpha_o$ transgenic mice (Figure 2.5; Table 2.3). In whole brain, basal levels of [35 S]GTP γ S incorporation (Table 2.3) were significantly reduced in $G\alpha_o$ +/- and $G\alpha_o$ -/- mice, compared with wild type littermates ($F(2,5)=20.06$, $p=0.004$), suggesting that $G\alpha_o$ is responsible for some, but not all, basal G protein activity. The μ selective agonist DAMGO produced a dose-dependent stimulation of [35 S]GTP γ S binding that was reduced in $G\alpha_o$ +/- and $G\alpha_o$ -/- mice when compared with wild type controls (Figure 2.5a). There was a significant concentration \times genotype interaction for this response ($F(14,39)=6.700$, $p<0.001$), including main effects of both concentration ($F(7,39)=43.38$, $p<0.001$) and genotype ($F(2,39)=72.02$, $p<0.001$). Maximal DAMGO-stimulated binding (E_{max}) (Table 2.3) was decreased in $G\alpha_o$ transgenic mice in a genotype-dependent manner ($F(2,5)=64.69$, $p<0.001$); this reduction in maximal stimulation was without a change in potency (EC_{50}) between wild type and $G\alpha_o$ +/- mice ($t(2)=1.307$, $p=0.321$). Morphine also produced a dose-dependent stimulation of [35 S]GTP γ S binding that was significantly decreased in $G\alpha_o$ +/- and $G\alpha_o$ -/- mice when compared with wild type littermates (Figure 2.5b). There were significant main effects of both concentration and genotype, as well as a significant concentration \times genotype interaction (concentration: $F(7,39)=15.79$, $p<0.001$; genotype: $F(2,39)=51.45$, $p<0.001$; concentration \times genotype: $F(14,39)=3.468$, $p=0.001$).

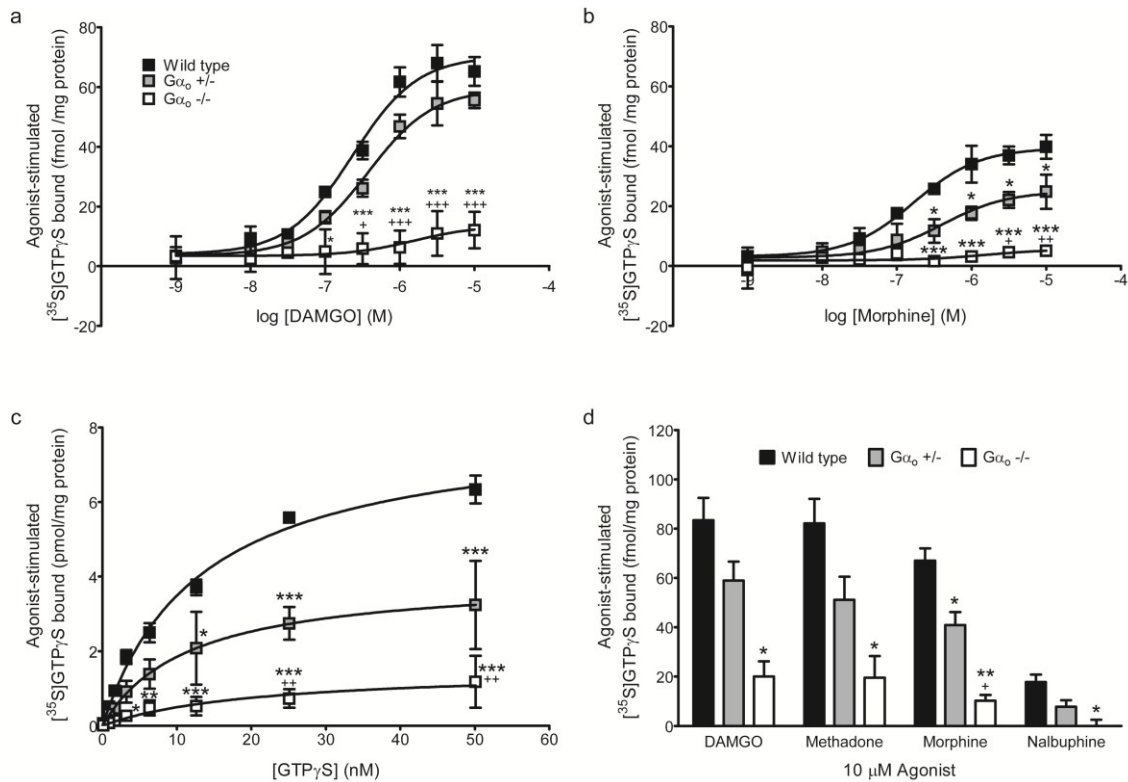


Figure 2.5: Ability of opioid agonists to stimulate G protein activity in whole brain homogenates from $G\alpha_o$ transgenic mice.

Agonist-stimulated [35 S]GTP γ S (0.1 nM) binding was measured in the presence of various concentrations of the opioid agonists (a) DAMGO or (b) morphine, (c) in the presence of 10 μ M DAMGO plus increasing concentrations of unlabeled GTP γ S, and (d) in the presence of 10 μ M DAMGO, methadone, morphine or nalbuphine in membrane homogenates from whole brain of wild type, $G\alpha_o$ +/- and $G\alpha_o$ -/- mice. Non-specific binding was evaluated in the presence of unlabeled GTP γ S (10 μ M). Data are plotted as agonist-stimulated [35 S]GTP γ S binding, defined as the increase in [35 S]GTP γ S incorporation in the presence of agonist over that of basal (measured in the absence of agonist), and represent the mean \pm SEM ($n=2-3$ performed in at least duplicate). Legend in (a) also applies to panels (b) and (c). Symbols indicate a statistical difference versus wild type (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) or $G\alpha_o$ +/- (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$) by Bonferroni's post-test.

Table 2.3: Properties of agonist-stimulated [³⁵S]GTPγS binding in membranes from whole brain of wild type and Gα_o transgenic mice.

Tissue	Genotype	Basal [³⁵ S]GTPγS Binding (fmol/mg protein)	Agonist-Stimulated [³⁵ S]GTPγS Binding				DAMGO-Stimulated [³⁵ S]GTPγS Saturation Binding	
			DAMGO		Morphine		<i>B</i> _{max} (pmol/mg protein)	<i>K</i> _D (nM)
			<i>E</i> _{max} (fmol/mg protein)	<i>EC</i> ₅₀ (nM)	<i>E</i> _{max} (fmol/mg protein)	<i>EC</i> ₅₀ (nM)		
Whole brain	Wild type	62.2 ± 2.5	71.3 ± 3.2	287 ± 91	39.4 ± 4.5	165 ± 14	8.31 ± 1.01	14.2 ± 4.0
	Gα _o +/-	49.1 ± 5.8	59.4 ± 4.0	382 ± 23	25.6 ± 3.9	350 ± 76	4.26 ± 1.31	16.5 ± 6.7
	Gα _o -/-	22.2 ± 0.3** ⁺	13.1 ± 2.3*** ⁺⁺⁺	NC	5.2 ± 1.0**	NC	1.18 ± 0.53*	7.1 ± 4.4
Spinal cord	Wild type	59.0 ± 10.9	46.4 ± 5.4	ND	36.3 ± 4.0	ND	ND	ND
	Gα _o +/-	49.0 ± 11.1	35.0 ± 6.7 [#]	ND	19.5 ± 3.1 ^{##}	ND	ND	ND

NC, not calculated; ND, not determined. Agonist-stimulated [³⁵S]GTPγS binding is defined as the increase in [³⁵S]GTPγS incorporation in the presence of agonist, over that of basal (measured in the absence of agonist). Data represent the mean ± SEM (*n*=2-3 performed in duplicate). Symbols indicate a statistical difference *versus* wild type whole brain (**p*<0.05, ***p*<0.01, ****p*<0.001) or Gα_o +/- whole brain (⁺*p*<0.05, ⁺⁺⁺*p*<0.001) by Bonferroni's post-test or *versus* wild type spinal cord by Students' paired t-test ([#]*p*<0.05, ^{##}*p*<0.01).

The E_{\max} for morphine (Table 2.3) was reduced as a function of genotype ($F(2,5)=16.24$, $p=0.007$), and was accompanied by a non-significant trend toward a reduction in the EC_{50} value for $G\alpha_o$ +/- mice, compared with wild type littermates ($t(4)=2.407$, $p=0.074$).

Given that DAMGO-stimulated [35 S]GTP γ S incorporation was significantly attenuated, saturation analysis of DAMGO-stimulated binding was performed (Figure 2.5c; Table 2.3) to measure the maximal number of G proteins (B_{\max}) activated by agonist-occupied μ receptors and the ability of agonist to induce formation of GTP-bound $G\alpha$ (K_D) (Traynor and Nahorski, 1995; Selley et al., 1997b). In membranes from whole brain, DAMGO stimulated [35 S]GTP γ S incorporation was increased as a function of increasing concentration of GTP γ S, but was significantly reduced in $G\alpha_o$ +/- and $G\alpha_o$ -/- mice when compared with wild type controls (Figure 2.5c). There were significant main effects of both concentration and genotype, as well as a significant concentration \times genotype interaction (concentration: $F(7,44)=37.50$, $p<0.001$; genotype: $F(2,44)=79.22$, $p<0.001$; concentration \times genotype: $F(14,44)=7.482$, $p<0.001$). This reduction was manifested as a decrease in B_{\max} for GTP γ S binding ($F(2,5)=9.359$, $p=0.020$), without an accompanying change in the K_D for GTP γ S (Table 2.3) ($F(2,5)=0.6608$, $p=0.556$).

[35 S]GTP γ S binding stimulated by a maximal concentration of DAMGO, morphine, methadone or nalbuphine was evaluated in whole brain homogenates from $G\alpha_o$ transgenic mice (Figure 2.5d). In wild type mice, the opioid agonists tested elicited maximal [35 S]GTP γ S stimulation according to the rank order of efficacy DAMGO = methadone > morphine >> nalbuphine. When compared with wild type controls, $G\alpha_o$ +/- and $G\alpha_o$ -/- mice exhibited a reduction in G protein stimulation across all opioid agonists tested, including: DAMGO (wild type: 83.4 ± 9.1 fmol/mg; $G\alpha_o$ +/-: 59.0 ± 7.7 fmol/mg; $G\alpha_o$ -/-: 20.1 ± 6.1 fmol/mg; $F(2,5)=12.98$, $p=0.011$), methadone (wild type: 82.2 ± 9.9 fmol/mg; $G\alpha_o$ +/-: 51.2 ± 9.3 fmol/mg; $G\alpha_o$ -/-: 19.6 ± 8.7 fmol/mg; $F(2,5)=9.407$, $p=0.020$), morphine (wild type: 67.1 ± 5.0 fmol/mg; $G\alpha_o$ +/-: 40.9 ± 5.3 fmol/mg; $G\alpha_o$ -/-: 10.3 ± 2.3 fmol/mg; $F(2,5)=29.93$, $p=0.002$) and nalbuphine (wild type: 17.8 ± 3.0 fmol/mg; $G\alpha_o$ +/-: 7.7 ± 2.6 fmol/mg; $G\alpha_o$ -/-: 0.01 ± 2.49 fmol/mg; $F(2,5)=8.770$, $p=0.035$).

In spinal cord homogenates, basal levels of [35 S]GTP γ S incorporation (Table 2.3) were not different between $G\alpha_o$ +/- mice and their wild type littermates ($t(4)=0.6431$,

$p=0.555$), suggesting that $G\alpha_o$ is not as important for basal G protein activity in the spinal cord. DAMGO-stimulated binding (Figure 2.6; Table 2.3) was significantly reduced in $G\alpha_o$ +/- mice when compared with wild type controls ($t(2)=7.072$, $p=0.019$). Similarly, morphine-stimulated [35 S]GTP γ S binding in spinal cord (Figure 2.6; Table 2.3) showed a decrease in $G\alpha_o$ +/- mice, as compared with wild type littermates ($t(2)=17.54$, $p=0.003$). Western blot analysis of $G\alpha_o$ expression in spinal cord membranes confirmed that there was a significant reduction in $G\alpha_o$ protein levels in these samples, as compared with the loading control tubulin (Figure 2.6, *inset*).

Discussion

This study shows that reduction in the expression of the inhibitory $G\alpha$ isoform, $G\alpha_o$, attenuates μ agonist-mediated antinociception in mice at both the supraspinal and the spinal level. However, whether a genotype-dependent difference was seen depended upon the efficacy of the agonist and the strength of the noxious stimulus; a greater effect of the $G\alpha_o$ +/- genotype was manifested in the presence of the partial agonist nalbuphine or against a higher temperature stimulus. In contrast, there were no differences observed in the antinociceptive response to morphine in mice that were null for either $G\alpha_{i2}$ or $G\alpha_{i3}$, compared with their respective wild type littermates, at either the supraspinal or the spinal level. Furthermore, the loss of $G\alpha_o$ protein in $G\alpha_o$ -/- mice resulted in a decrease in $G\beta$ and $G\gamma$ expression, a reduction in the number of high-affinity μ opioid receptor binding sites, and consequently, attenuation of μ agonist-stimulated [35 S]GTP γ S binding. Together, these results provide strong evidence that μ opioid receptor coupling to $G\alpha_o$ is important for opioid antinociception.

μ Agonist-mediated antinociception

In wild type mice, there was no difference in the potency of morphine observed at the higher hot plate temperature of 55°C when compared with 52°C, and morphine remained fully effective at both temperatures. However, this effect of temperature was exaggerated in $G\alpha_o$ +/- mice such that a larger shift in the potency of morphine was realized at the higher hot plate temperature, and even at 100 mg/kg, full antinociception was not attained. This suggests a reduced efficiency of antinociceptive processing in the $G\alpha_o$ +/- mice, leading to a higher agonist efficacy requirement.

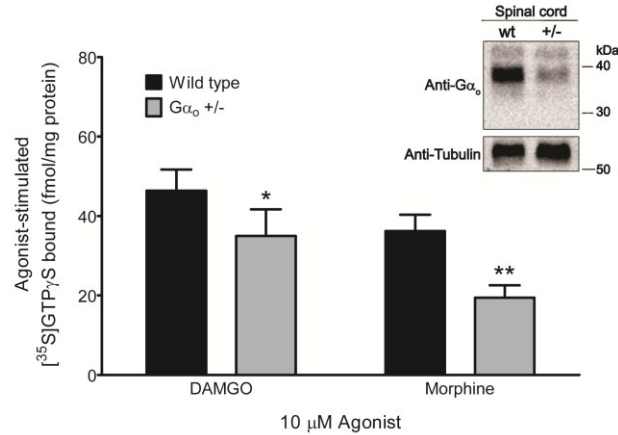


Figure 2.6: DAMGO- and morphine-stimulated G protein activity in spinal cord homogenates from *Gα_o* transgenic mice.

[³⁵S]GTPγS (0.1 nM) incorporation stimulated by 10 μM DAMGO or morphine was evaluated in membrane homogenates from spinal cord of wild type and *Gα_o*^{+/-} mice. Non-specific binding was evaluated in the presence of unlabeled GTPγS (10 μM). Data are plotted as agonist-stimulated [³⁵S]GTPγS binding, defined as the increase in [³⁵S]GTPγS binding in the presence of agonist over that of basal (measured in the absence of agonist), and represent the mean ± SEM (*n*=3 performed in quadruplicate). Asterisks indicate a statistical difference *versus* wild type by Students' paired t-test (**p*<0.05, ***p*<0.01). *Inset*, representative Western blot in spinal cord membranes showing reduced *Gα_o* protein expression in *Gα_o*^{+/-} mice (+/-) when compared with wild type (wt) controls; membranes were probed for tubulin as a loading control.

In confirmation of this, methadone, which has higher efficacy than morphine (Adams et al., 1990; Peckham and Traynor, 2006; McPherson et al., 2010), showed a smaller genotype difference. These findings confirm a role for $G\alpha_o$ in opioid agonist-mediated supraspinal antinociception against a thermal stimulus, but also indicate that in the $G\alpha_o$ +/- mice, sufficient $G\alpha_o$ protein remains to give a robust response and/or that other $G\alpha_{i/o}$ proteins are involved in the response. However, this latter suggestion is less likely given the absence of a difference between $G\alpha_{i2}$ or $G\alpha_{i3}$ null mice and their wild type littermates and the lack of compensatory changes in the expression of other $G\alpha_{i/o}$ proteins in $G\alpha_o$ null mice.

Surprisingly, in light of findings in the hot plate test, but in agreement with previous ODN studies (Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Standifer et al., 1996; Sanchez-Blazquez et al., 2001), there was not a genotype-dependent difference in the ability of systemic morphine to produce antinociception between wild type and $G\alpha_o$ +/- mice using the tail withdrawal test. However, there was a profound shift in the potency of the partial agonist nalbuphine, which has lower efficacy than morphine (Dykstra et al., 1997; Selley et al., 1998). This suggests, as with the hot plate test, that the relationship between the strength of the noxious stimulus and the efficacy of the ligand determines if a genotype difference is observed. These findings imply that blockade of spinal nociception, as measured in the tail withdrawal test, requires less agonist efficacy. As a result, even with a large reduction in $G\alpha_o$ protein, the system is still able to function efficiently.

Previous studies have shown that ODN knockdown of $G\alpha$ subunits inhibits antinociception in an agonist-specific manner, suggesting that different agonists may cause the μ receptor to signal through different $G\alpha$ proteins. For example, antinociception induced by the partial agonist buprenorphine in the warm-water tail withdrawal test was significantly reduced after administration of antisense ODNs targeting $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o2}$, $G\alpha_z$ or $G\alpha_q$, whereas morphine antinociception was only attenuated in the presence of ODNs targeting $G\alpha_{i2}$ or $G\alpha_z$ (Sanchez-Blazquez et al., 2001). However, in our study, morphine antinociception in the tail withdrawal test was not altered upon loss of $G\alpha_o$, $G\alpha_{i2}$ or $G\alpha_{i3}$. Our findings indicate this may be due to differences in relative agonist efficacy, which suggests that there is a $G\alpha_o$ protein reserve for full agonists such that

even a significant knockdown of $G\alpha_o$ does not necessarily alter the ability of morphine to elicit antinociception, whereas a partial agonist, such as nalbuphine, is more susceptible. Indeed, Standifer et al. (1996) reported a reduction in morphine antinociception in the radiant-heat tail flick assay in mice exhibiting >60% knockdown of $G\alpha_o$. On the other hand, the reason(s) why knockdown of $G\alpha_{i2}$ and other $G\alpha$ subunits affected antinociception in a ligand-dependent manner in previous studies is not clear, but may be due to differences in the route of administration (central *versus* peripheral) or the approach used (ODN *versus* constitutive knockdown). For example, in our constitutive knockdown, although no compensatory changes in $G\alpha_{i/o}$ protein expression were observed, other developmental changes may have occurred to substitute for the loss of $G\alpha_o$ specifically.

μ Receptor-dependent G protein activation

Loss of $G\alpha_o$, as determined by Western blot, was accompanied by a reduction in both $G\beta$ and $G\gamma$ subunits. Valenzuela et al. (1997) observed a similar decrease in $G\beta$ protein in ventricular membranes from a separately generated $G\alpha_o$ $-/-$ mouse. This reduction in $G\beta\gamma$ is likely due to the instability of these subunits in the absence of sufficient concentrations of $G\alpha$ protein (Hwang et al., 2005). A mechanism of regulated $G\alpha$ and $G\beta\gamma$ expression would prevent the accumulation of free $G\beta\gamma$ dimers that are functionally competent in the absence of receptor agonist (Jiang et al., 1998). Reductions in free $G\beta$ and $G\gamma$ levels were not observed in brains from mice lacking either $G\alpha_{i2}$ or $G\alpha_{i3}$ (data not shown), presumably due to the lower expression levels of these $G\alpha$ proteins.

This decrease in $G\alpha_o$ and accompanying $G\beta$ and $G\gamma$ subunits, in addition to reducing the antinociceptive response, also reduced the ability of μ agonists to stimulate [35 S]GTP γ S incorporation in whole brain or spinal cord homogenates. Indeed, DAMGO- and morphine-stimulated binding of 0.1 nM [35 S]GTP γ S were abolished in whole brain homogenates from $G\alpha_o$ $-/-$ mice, confirming the importance of $G\alpha_o$ for μ opioid receptor signaling (Jiang et al., 1998; Jiang et al., 2001). The reduction in $G\alpha_o$ and cognate $G\beta$ and $G\gamma$ subunits also resulted in a decrease in high-affinity μ receptor binding sites, but not total μ receptor sites, suggesting a reduction in heterotrimeric G protein coupling.

However, high-affinity μ opioid receptor binding was still present in the complete absence of $G\alpha_o$, which could indicate that other $G\alpha_{i/o}$ subunits are taking the place of $G\alpha_o$ and providing a functional compensation, even though there were no obvious increases in the levels of these isoforms. Indeed, analysis of DAMGO-stimulated [35 S]GTP γ S saturation binding revealed a $G\alpha$ protein to high-affinity μ receptor ratio ($G\alpha$: μ receptor) of approximately 34:1 in wild type mice, compared with 24:1 in $G\alpha_o$ +/- mice and 10:1 in $G\alpha_o$ -/- mice. These results suggest that, in the brain, $G\alpha$ proteins other than $G\alpha_o$ are able to form complexes with μ opioid receptors. Such complexes might also help to translocate μ receptors to the cell surface, as with δ opioid receptor/ $G\alpha_{i2}$ complexes that are preassembled in secretory vesicles before delivery to the plasma membrane (Zhao et al.). However, G protein was not required for δ opioid receptor translocation; if this was also true for μ receptors, it would explain the high level of low-affinity μ opioid receptors present in the $G\alpha_o$ -/- mice.

In spinal cord homogenates, both total and high-affinity μ opioid receptor numbers are considerably less than in whole brain of wild type mice. Furthermore, there was no change in μ receptor expression observed in spinal cord tissue from $G\alpha_o$ +/- mice. This could be because of an overabundance of $G\alpha_o$ compared with μ opioid receptors in the spinal cord. It is unlikely that other $G\alpha$ subunits are making a bigger contribution in the spinal cord given that there is no difference in morphine antinociception in the tail withdrawal test between $G\alpha_{i2}$ or $G\alpha_{i3}$ null mice and their wild type littermates. Similarly, differences between supraspinal and spinal antinociceptive circuitry have been demonstrated in a $G\alpha_z$ -deficient mouse (Hendry et al., 2000), although the mechanisms underlying these supraspinal *versus* spinal differences were not further characterized. Together, these findings suggest that μ opioid receptor signaling in the spinal cord may be more efficient, such that full behavioral responses can be achieved at much lower μ receptor expression and/or upon activation of a smaller fraction of the total pool of G proteins.

Concluding remarks

The present results using $G\alpha_o$ +/- mice demonstrate that $G\alpha_o$ plays an important role in opioid antinociception. Moreover, changes observed in opioid antinociception in

$G\alpha_o$ +/- mice were paralleled by similar alterations in opioid-dependent signaling at the cellular level. This conclusion is further supported by the recent work of Kest et al. (2009), who showed that $G\alpha_o$ expression modulates opioid dependence by targeted knockdown in mice of $G\alpha_o$ mRNA, which reduced the expression of withdrawal after chronic heroin or morphine. However, despite the strong evidence linking $G\alpha_o$ to opioid antinociception, these findings cannot be taken as absolute proof that μ opioid receptor coupling to $G\alpha_o$ is required for morphine analgesia. $G\alpha_o$ is important for the signaling and activity of many neurotransmitter receptors in the central nervous system (reviewed in Jiang and Bajpayee, 2009). Thus, it is possible that non-opioid pathways are compromised in the $G\alpha_o$ +/- mice and contribute to the altered antinociceptive responses (Connor and Christie, 1999). These and other questions related to the consequences of regional knockdown of $G\alpha_o$ will be addressed in future studies. Nevertheless, the finding that in addition to antinociception, both high-affinity μ receptor expression and μ agonist-stimulated G protein activity are reduced strongly supports the notion that the $G\alpha_o$ - μ receptor complex plays a key role in opioid antinociception.

Chapter 3

Differential Control of Opioid Antinociception to Thermal Stimuli in a Knock-In Mouse Expressing RGS-Insensitive $G\alpha_o$ Protein

Summary

RGS proteins classically function as negative modulators of GPCR signaling. *In vitro*, RGS proteins have been shown to inhibit signaling by agonists at the μ opioid receptor, including morphine. The goal of the present study was to evaluate the contribution of endogenous RGS proteins to the antinociceptive effects of morphine and other opioid agonists. To do this, a knock-in mouse that expresses an RGSi mutant $G\alpha_o$ protein, $G\alpha_o^{G184S}$ ($G\alpha_o$ RGSi), was evaluated for morphine or methadone antinociception in response to noxious thermal stimuli. Mice expressing $G\alpha_o$ RGSi subunits exhibited a naltrexone-sensitive enhancement of baseline nociception in both the hot plate and warm-water tail withdrawal tests. In the hot plate test, a measure of supraspinal nociception, morphine antinociception was increased, and this was associated with an increased ability of opioids to inhibit presynaptic GABA neurotransmission in the PAG. In contrast, antinociception produced by either morphine or methadone was reduced in the tail withdrawal test, a measure of spinal nociception. In whole brain and spinal cord homogenates from mice expressing $G\alpha_o$ RGSi subunits, there was a small loss of $G\alpha_o$ expression and an accompanying decrease in basal G protein activity. Overall, this work strongly supports a role for RGS proteins as negative regulators of opioid supraspinal antinociception. Further, these studies also reveal a potential novel function of RGS proteins as positive regulators of opioid spinal antinociceptive pathways.

Introduction

Morphine produces analgesia by activating the μ opioid receptor, a member of the GPCR superfamily. μ Opioid receptor stimulation results in the activation of heterotrimeric $G_{i/o}$ proteins composed of a $G\alpha_{i/o}$ subunit and a $G\beta\gamma$ heterodimer.

Signaling is terminated *via* the intrinsic GTPase activity of the $G\alpha_{i/o}$ subunit, and this process is enhanced by RGS proteins. RGS proteins are GAPs and therefore reduce $G\alpha_{i/o}$ -mediated signaling duration and intensity (De Vries et al., 2000; Ross and Wilkie, 2000; Hollinger and Hepler, 2002). Consequently, RGS proteins have been proposed as drug targets for several disease states, including both pain and addiction (Neubig and Siderovski, 2002; Traynor and Neubig, 2005).

There are 20 RGS proteins with GAP activity. These are divided into several families based on the structure of the RH domain that binds $G\alpha$ and is responsible for the classical GAP function (Figure 1.4). RGS proteins have been demonstrated to negatively regulate signaling through several GPCRs *in vitro*, including μ opioid receptors (Potenza et al., 1999; Clark et al., 2003; Clark and Traynor, 2004; Psifogeorgou et al., 2007). Studies evaluating the contribution of individual RGS proteins to opioid effects *in vivo* have generally utilized knockdown or gene knockout strategies in mice (for examples, see Garzon et al., 2003; Zachariou et al., 2003b; Garzon et al., 2004; Garzon et al., 2005; Grillet et al., 2005; Han et al., 2010). However, the phenotypic effect(s) of eliminating a single RGS protein are often reported to be quite small (Grillet et al., 2005), which could be due to developmental compensations and/or redundancy within the RGS family.

The aim of the present study was to test the hypothesis that endogenous RGS proteins negatively regulate opioid antinociception *via* interaction with $G\alpha_o$ subunits using a novel knock-in mouse that expresses the $G\alpha_o$ RGSi subunits (Goldenstein et al., 2009). The relationship between RGS proteins and $G\alpha_o$ is of particular interest in light of our previous work demonstrating that $G\alpha_o$ plays a significant role in opioid antinociception (Lamberts et al., 2011; see also Chapter 2 of this thesis). For these studies, $G\alpha_o$ RGSi heterozygous knock-in mice ($G\alpha_o$ +/GS) were compared with wild type littermates, as homozygous knock-in mice ($G\alpha_o$ GS/GS) are not viable (Goldenstein et al., 2009; Kehrl et al., 2012). Morphine or methadone antinociception was evaluated in $G\alpha_o$ +/GS mice using two different noxious thermal stimuli: the hot plate test for supraspinal nociception and the warm-water tail withdrawal test for spinal nociception. In addition, opioid modulation of GABA synaptic transmission was monitored in PAG neurons. Loss of RGS activity toward $G\alpha_o$ resulted in prolonged baseline latencies in

both nociceptive tests due to an enhancement of endogenous opioid peptide signaling. Moreover, there was an enhanced potency of morphine to elicit antinociception in the hot plate test and to inhibit GABAergic transmission in the PAG in $G\alpha_o$ +/GS mice, all pointing to negative regulation of μ opioid receptor signaling by RGS proteins. In contrast, a paradoxical decrease in antinociception was observed in the tail withdrawal test.

Methods

Transgenic mice

Knock-in mice expressing $G\alpha_o$ RGSi subunits were generated as previously described (Fu et al., 2004; Fu et al., 2006; Huang et al., 2006; Goldenstein et al., 2009) and were maintained for 6 generations on a 129S1/SvImJ (129S1) background. $G\alpha_o$ +/GS and wild type littermates were obtained at the expected Mendelian frequency for wild type and $G\alpha_o$ +/GS crosses (data not shown). Experiments were performed using male and female mice aged 10 – 25 weeks and weighing 20 – 25 g. Mice were group-housed by sex with unlimited access to food and water. Lights were maintained on a 12-h light/dark cycle (lights on at 7:00), and all testing was performed during the light phase. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health and all experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Antinociceptive tests

Supraspinal antinociception was evaluated in the hot plate test and spinal antinociception was measured in the warm-water tail withdrawal assay using a cumulative dosing procedure as previously described (Lamberts et al., 2011). Briefly, mice were administered saline followed by 3-4 increasing doses of morphine or methadone in 30 min intervals, and latency was evaluated 30 min following each injection (i.p.). To evaluate the role of endogenous opioid peptides in baseline nociception, latency was determined 30 min after the injection of the opioid antagonist naltrexone (10 mg/kg, i.p.).

For the hot plate test, mice were placed on a hot plate analgesia meter (Columbus Instruments, Columbus, OH) maintained at $52.0 \pm 0.2^{\circ}\text{C}$ and the latency to lick forepaw(s) or jump was measured with a cutoff time of 60 s to prevent tissue damage. For the tail withdrawal test, mice were lightly restrained and the distal tip of the mouse's tail was placed in a water bath (Fisher Scientific, Waltham, MA) maintained at $50.0 \pm 0.5^{\circ}\text{C}$. The latency to tail flick was measured with a cutoff time of 20 s.

Membrane preparation

Mice were killed by cervical dislocation and whole brain tissue (minus cerebellum) or thoracic and lumbar spinal cord was removed and immediately chilled in ice-cold 50 mM Tris, pH 7.4 (Tris buffer). Homogenates were prepared as previously described (Lester and Traynor, 2006) and final membrane pellets were resuspended in Tris buffer and stored at -80°C until use, unless otherwise indicated. Protein content was determined by the method of Bradford (Bradford, 1976).

Western blot analysis of G proteins

Whole brain or spinal cord homogenates (20 μg protein) were mixed with sample buffer (63 mM Tris, pH 6.8, with 2% SDS, 10% glycerol, 0.008% bromophenol blue and 50 mM dithiothreitol) and separated by SDS-PAGE on polyacrylamide gels. Proteins were transferred to nitrocellulose (Pierce, Rockford, IL) and probed with rabbit polyclonal anti-G α_o (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Samples were also probed with mouse monoclonal anti- α -tubulin (1:1000; Sigma-Aldrich, St. Louis, MO) as a loading control. Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:10,000) and immunoreactivity was detected by enhanced chemiluminescence in an EpiChem3 Benchtop Darkroom (UVP, Upland, CA). Band densities were quantified using Image J software (<http://rsbweb.nih.gov/ij/index.html>).

Receptor binding assays

To evaluate total opioid receptor and total μ receptor expression, homogenates from whole brain (100 μg protein) or spinal cord (100-200 μg protein, freshly prepared) were incubated in Tris buffer with the radiolabeled opioid antagonist [^3H]diprenorphine (4 nM) in the absence or presence the μ -selective antagonist CTAP (300 nM) to define μ

opioid receptors. To measure high-affinity μ receptor expression, homogenates from whole brain (100 μg protein) were incubated in Tris buffer with increasing concentrations of the radiolabeled μ -selective agonist [^3H]DAMGO (0.24 – 44 nM). Homogenates from spinal cord (100-200 μg protein, freshly prepared) were incubated in Tris buffer with 12 nM [^3H]DAMGO. All binding reactions were incubated for 60 min at 25°C. Non-specific binding was evaluated in the presence of the opioid antagonist naloxone (10 μM). Reactions were stopped by rapid filtration through GF/C filtermats (Whatman, Kent, UK) using a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD). Bound radioactivity was determined by liquid scintillation counting using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

[^3S]GTP γS binding assays

To measure G protein activity, the incorporation of a slowly-hydrolyzed GTP analog, [^3S]GTP γS , into activated $\text{G}\alpha$ subunits was monitored *ex vivo*. Homogenates from whole brain (10 μg protein) or spinal cord (25-50 μg protein, freshly prepared) were pre-incubated in [^3S]GTP γS binding buffer (50 mM Tris, 5 mM MgCl_2 , 100 mM NaCl and 1 mM EDTA, pH 7.4, with 2 mM dithiothreitol, 100 μM GDP and 0.4 U/mL adenosine deaminase) for 10 min at 25°C with or without opioid agonist (DAMGO, morphine or methadone). Reactions were started by the addition of 0.1 nM [^3S]GTP γS , followed by incubation for 90 min at 25°C. Non-specific binding was evaluated in the presence of 10 μM unlabeled GTP γS . Binding reactions were stopped by rapid filtration and bound radioactivity was measured by liquid scintillation counting, as above.

Electrophysiology

Mice were deeply anesthetized with isoflurane and brains were rapidly removed and placed in ice-cold cutting buffer (75 mM NaCl, 2.5 mM KCl, 0.1 mM CaCl_2 , 6 mM MgSO_4 , 1.2 mM NaH_2PO_4 , 25 mM NaHCO_3 , 2.5 mM D-glucose and 50 mM sucrose). Coronal sections (~230 μm) containing the PAG were sliced in cutting buffer oxygenated with 95% O_2 and 5% CO_2 . Slices were then maintained at 35°C in oxygenated artificial cerebrospinal fluid (126 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl_2 , 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 21.4 mM NaHCO_3 , and 11.1 mM D-dextrose, pH 7.4, at 300-310 mOsm) until experimentation.

Whole-cell patch-clamp recordings were made from visually identified PAG neurons. Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) on a two-stage puller (Narishige, Tokyo, JAPAN). Pipettes had a resistance of 2-4 M Ω and intracellular solutions contained 130 mM CsCl, 5.4 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 1.1 mM EGTA, 30 mM D-dextrose, 4 mM Mg-ATP and 1 mM Na-GTP, pH 7.3, at 280-290 mOsm. Whole-cell series resistance was compensated ~80%. Evoked GABA-mediated inhibitory post-synaptic currents (eIPSCs) were elicited with a bipolar stimulating electrode placed ~200-300 μ m distally from the recorded cell at a holding potential of -70 mV in the presence of the AMPA receptor antagonist NBQX (5 μ M). Stimulation pulses (2 ms) were delivered at 0.05 Hz. Currents were collected at 2 kHz and digitized at 5 kHz using an Axopatch 200B amplifier controlled by Axograph Data Acquisition software (Axograph X, Sydney, AUS). During each experiment, a voltage step of -10 mV from the holding potential was applied periodically to monitor cell capacitance and access resistance. Recordings in which access resistance or capacitance changed by >15% during the experiment were excluded from data analysis.

Drugs

For behavioral experiments, all drugs were diluted in sterile water. Morphine sulfate was from RTI (Research Triangle Park, NC), Naltrexone hydrochloride was from Endo Pharmaceuticals (Newark, DE), and L-methadone hydrochloride was from Eli Lilly and Company (Indianapolis, IN). [³H]diprenorphine, [³H]DAMGO and [³⁵S]GTP γ S were purchased from PerkinElmer. Adenosine deaminase was obtained from Calbiochem (San Diego, CA). DAMGO, CTAP, naloxone, ME, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Data analysis

All data were analyzed using GraphPad Prism 5 (San Diego, CA). Differences between genotypes were evaluated using Students' unpaired *t*-test or two-way ANOVA with Bonferroni's post-tests, where appropriate. For all statistical tests, significance was set at *p*<0.05 and was adjusted for multiple comparisons if necessary. Initial statistical analysis revealed a lack of sex \times genotype interaction for any measure, so data from both male and female mice were pooled for final genotype comparisons. ED₅₀ was calculated

by fitting the compiled antinociception data to an agonist *versus* response curve (Hill slope=1), B_{\max} and K_D were derived by fitting each radioligand binding experiment to a one-site saturation binding curve (Hill slope=1), and EC_{50} was calculated by fitting individual [35 S]GTP γ S binding experiments to an agonist *versus* response curve (Hill slope=1). All data are reported as the mean \pm SEM, except ED_{50} values which are expressed as the mean (95% CI).

Results

G α_o +/GS mice demonstrate enhanced morphine antinociception in the hot plate test

$G\alpha_o$ has previously been shown to play an important role in opioid antinociception (Lamberts et al., 2011). To determine whether antinociception mediated by $G\alpha_o$ is modulated by interactions with RGS proteins, $G\alpha_o$ +/GS mice were evaluated for opioid supraspinal antinociception using the 52°C hot plate test (Figure 3.1). In the absence of agonist, baseline hot plate latency was significantly prolonged in $G\alpha_o$ +/GS mice, when compared with wild type controls ($p < 0.01$; Figure 3.1a). To evaluate whether the increase in baseline hot plate latency was due to enhanced opioidergic tone, a separate group of mice was pre-treated with the opioid antagonist naltrexone (10 mg/kg, i.p.) prior to determination of hot plate latency (Figure 3.1a). Pre-treatment with naltrexone blocked the increase in baseline hot plate latency in $G\alpha_o$ +/GS mice ($p < 0.01$) but had no effect in wild type controls ($p > 0.05$). Two-way ANOVA revealed significant effects of both genotype ($F(1,66)=5.8$, $p=0.019$) and treatment ($F(1,66)=8.4$, $p=0.005$), with a non-significant genotype \times treatment interaction ($F(1,66)=2.9$, $p=0.094$).

Morphine evoked a dose-dependent increase in hot plate latency that was significantly enhanced (~2-fold) in $G\alpha_o$ +/GS mice, when compared with wild type controls (Figure 3.1b). In wild type mice, the potency (ED_{50}) of morphine was 2.71 (2.10 – 3.49) mg/kg compared with 1.46 (1.11 – 1.93) mg/kg in $G\alpha_o$ +/GS mice. There were significant effects of both dose ($F(4,71)=79$, $p < 0.001$) and genotype ($F(1,71)=7.7$, $p=0.007$), while the dose \times genotype interaction was not significant ($F(4,71)=2.0$, $p=0.100$).

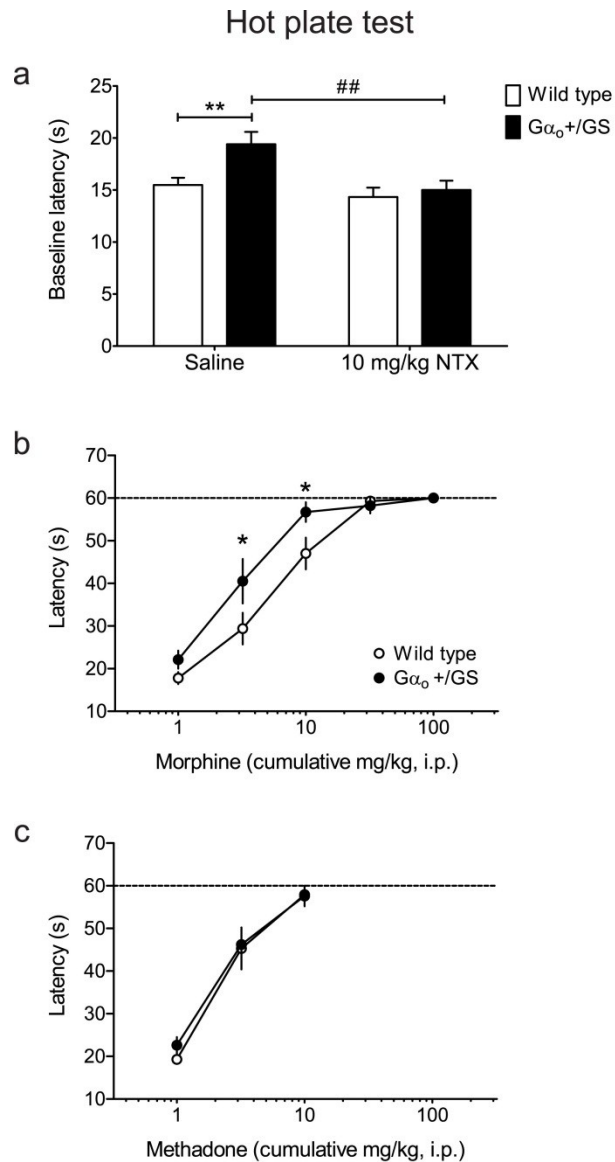


Figure 3.1: Baseline nociception and opioid antinociception in the 52°C hot plate test in wild type and $G\alpha_o +/GS$ mice.

(a) Baseline hot plate latency was evaluated 30 min following saline (wild type, $n=24$; $G\alpha_o +/GS$, $n=18$) or naltrexone (NTX; wild type, $n=15$; $G\alpha_o +/GS$, $n=13$). $**p<0.01$ compared with saline-treated wild type mice, $^{##}p<0.01$ compared with saline-treated $G\alpha_o +/GS$ mice by Bonferroni's post-test. (b,c) Opioid supraspinal antinociception was evaluated as hot plate latency 30 min following increasing cumulative doses of (b) morphine (wild type, $n=6-11$; $G\alpha_o +/GS$, $n=6-9$) or (c) methadone (wild type, $n=12$; $G\alpha_o +/GS$, $n=9$). $*p<0.05$ compared with wild type mice at the corresponding dose by Bonferroni's post-test. Legend in panel (b) also describes panel (c). Dotted lines indicate the test cutoff time. All data are plotted as the mean \pm SEM.

In contrast, there was no change in the antinociception produced by methadone in $G\alpha_o$ +/-GS mice, when compared with wild type littermates (Figure 3.1c). The ED_{50} values for methadone were 1.41 (1.01 – 1.96) mg/kg and 1.24 (0.91 – 1.69) mg/kg for wild type and $G\alpha_o$ +/-GS mice, respectively. Statistical analysis revealed a significant effect of dose ($F(2,57)=74, p<0.001$), but neither a significant effect of genotype ($F(1,57)=0.27, p=0.608$) nor a significant dose \times genotype interaction ($F(2,57)=0.18, p=0.839$).

Opioid antinociception is reduced in $G\alpha_o$ +/-GS mice in the tail withdrawal test

To evaluate whether the enhancement of morphine antinociception in $G\alpha_o$ +/-GS mice was specific to supraspinal pathways, antinociception was also evaluated in the 50°C warm-water tail withdrawal test (Figure 3.2). The tail withdrawal test is thought to measure primarily spinal nociception and involves modulation of a simple spinal reflex (Irwin et al., 1951). At baseline, tail flick latency was slightly prolonged in $G\alpha_o$ +/-GS mice, when compared with wild type littermates ($p<0.05$; Figure 3.2a). Similar to observations in the hot plate test, pre-treatment with naltrexone (10 mg/kg, i.p.) reversed the increase in tail flick latency in $G\alpha_o$ +/-GS mice ($p<0.05$), but did not affect tail flick latency in wild type animals ($p>0.05$; Figure 3.2a). There was a significant genotype \times treatment interaction ($F(1,77)=5.2, p=0.026$), although the main effects of either genotype ($F(1,77)=0.63, p=0.428$) or treatment ($F(1,77)=1.4, p=0.236$) were not significant.

Increasing doses of morphine produced an increase in tail flick latency that was significantly reduced (~3-fold) in $G\alpha_o$ +/-GS mice compared with wild type littermates (Figure 3.2b), with ED_{50} values of 3.08 (2.49 – 3.82) mg/kg and 1.11 (0.92 – 1.33) mg/kg, respectively. There were significant effects of both dose ($F(3,76)=180, p<0.001$) and genotype ($F(1,76)=66, p<0.001$), as well as a significant dose \times genotype interaction ($F(3,76)=10, p<0.001$).

Like morphine, methadone was also less potent (~2-fold) in $G\alpha_o$ +/-GS mice, when compared with wild type controls (Figure 3.2c), with ED_{50} values of 0.27 (0.22 – 0.34) mg/kg and 0.12 (0.09 – 0.15) mg/kg, respectively. There were significant effects of both dose ($F(3,78)=77, p<0.001$) and genotype ($F(1,78)=23, p<0.001$), as well as a significant dose \times genotype interaction ($F(3,78)=3.1, p=0.031$).

Warm-water tail withdrawal test

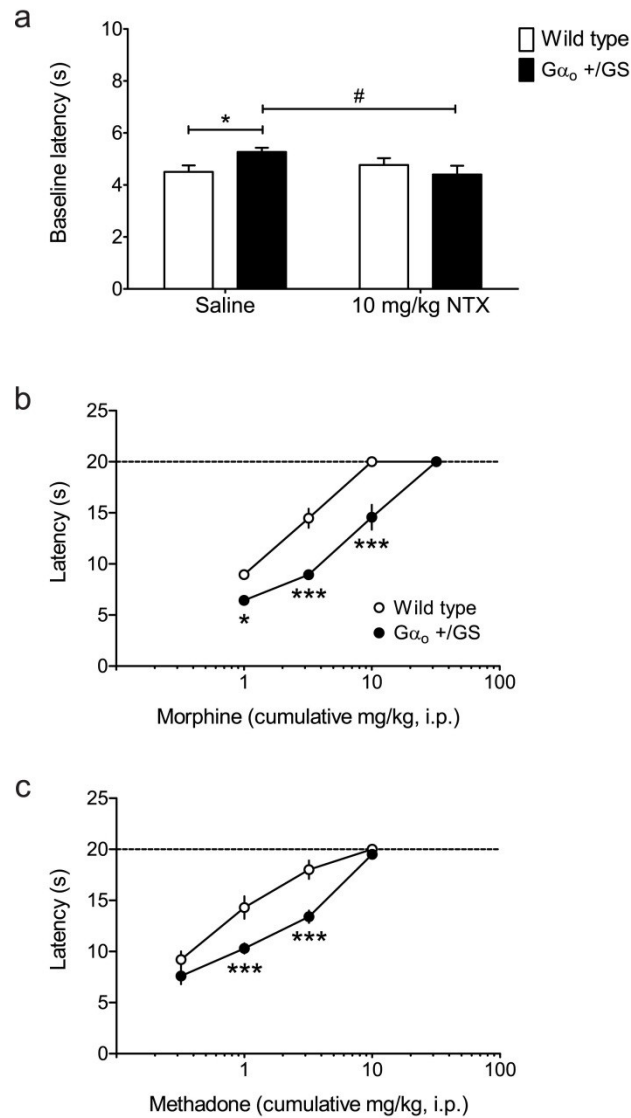


Figure 3.2: Baseline nociception and opioid antinociception in the 50°C warm water tail withdrawal test in wild type and $G\alpha_0$ +/GS mice.

(a) Baseline tail flick latency was evaluated 30 min following saline (wild type, $n=21$; $G\alpha_0$ +/GS, $n=24$) or naltrexone (NTX; wild type, $n=23$; $G\alpha_0$ +/GS, $n=13$). * $p<0.05$ compared with saline-treated wild type mice, # $p<0.05$ compared with saline-treated $G\alpha_0$ +/GS mice by Bonferroni's post-test. (b,c) Opioid spinal antinociception was evaluated as tail flick latency 30 min following increasing doses of (b) morphine (wild type, $n=11$; $G\alpha_0$ +/GS, $n=10$) or (c) methadone (wild type, $n=6-10$; $G\alpha_0$ +/GS, $n=8-14$). * $p<0.05$, *** $p<0.001$ compared with wild type mice at the corresponding dose by Bonferroni's post-test. Legend in panel (b) also describes panel (c). Dotted lines indicate the test cutoff time. All data are plotted as the mean \pm SEM.

Opioid inhibition of GABAergic transmission is potentiated in PAG neurons from $G\alpha_o$ +/GS mice

One of the mechanisms by which opioids produce antinociception is by removing tonic GABA inhibition (*i.e.* by GABA disinhibition) of descending antinociceptive neurons that emanate from the PAG (Moreau and Fields, 1986; Reichling et al., 1988). This effect can be measured by evaluating the ability of opioids to inhibit electrically-evoked GABAergic eIPSCs in slices containing the PAG (Vaughan and Christie, 1997; Vaughan et al., 1997). To determine the role of RGS proteins in opioid-mediated GABA disinhibition, slices containing the PAG were isolated from wild type and $G\alpha_o$ +/GS mice and the ability of either morphine or ME to inhibit eIPSCs was measured using whole-cell voltage-clamp electrophysiology (Figure 3.3).

Superfusion of morphine inhibited the amplitude of GABA eIPSCs in both wild type and $G\alpha_o$ +/GS mice, but the inhibition elicited by a submaximal concentration of morphine (5 μ M) was enhanced in slices from $G\alpha_o$ +/GS mice ($p < 0.05$; Figure 3.3a). There were significant main effects of both concentration ($F(1,18)=16$, $p < 0.001$) and genotype ($F(1,18)=11$, $p=0.003$), although the concentration \times genotype interaction was not significant ($F(1,18)=0.82$, $p=0.377$). Similarly, application of ME at a concentration of either 300 nM or 10 μ M resulted in a greater inhibition of eIPSCs in slices from $G\alpha_o$ +/GS mice ($p < 0.05$), when compared with slices from wild type littermates (Figure 3.3b). There were significant effects of both concentration ($F(1,8)=36$, $p < 0.001$) and genotype ($F(1,8)=21$, $p=0.002$), while the concentration \times genotype interaction was not significant ($F(1,8)=0.00$, $p=0.989$).

$G\alpha_o$ +/GS mice exhibit a loss of $G\alpha_o$ expression in brain and spinal cord

To determine whether the knock-in mutation affected G protein levels, whole brain or spinal cord homogenates from $G\alpha_o$ +/GS mice were subjected to Western blot analysis of G protein expression (Figure 3.4). Quantification of Western blot images revealed that in $G\alpha_o$ +/GS mice, total $G\alpha_o$ protein expression was significantly reduced (~25-35%) in both whole brain ($t(14)=2.2$, $p=0.048$; Figure 3.4a) and spinal cord ($t(12)=2.2$, $p=0.049$; Figure 3.4b) when compared with wild type controls.

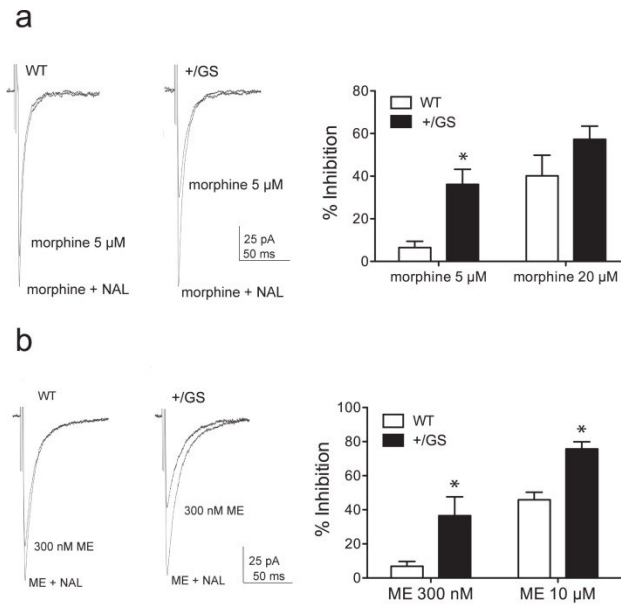


Figure 3.3: Opioid inhibition of GABA-mediated eIPSCs in slices containing the PAG from wild type (WT) and $G\alpha_o$ +/GS (+/GS) mice.

Inhibition of GABA eIPSCs by either (a) morphine ($n=5-6$) or (b) ME ($n=3$) is shown as averaged traces before and after application of naloxone (NAL, 1 μ M; *left*) and as compiled % inhibition of GABA eIPSC amplitude (*right*). * $p<0.05$ compared with wild type by Bonferroni's post-test. Data are plotted as the mean \pm SEM.

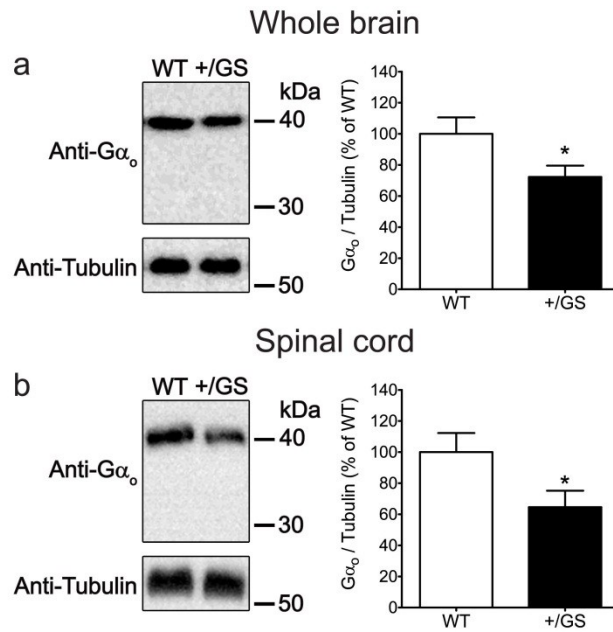


Figure 3.4: $G\alpha_o$ protein expression in whole brain or spinal cord homogenates from wild type (WT) and $G\alpha_o$ +/GS (+/GS) mice.

Homogenates from (a) whole brain ($n=8$) or (b) spinal cord ($n=7$) were separated by SDS-PAGE, transferred to nitrocellulose, and probed for the expression of $G\alpha_o$ using tubulin as a loading control. $G\alpha_o$ band densities were quantified in Image J, normalized to tubulin band densities, and data are plotted as a percent of WT (mean \pm SEM).

* $p<0.05$ compared with wild type by Students' t -test.

In contrast, the expression of several other G protein subunits, including $G\alpha_z$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\beta_{1-4}$ and $G\gamma_2$, was unchanged in either whole brain or spinal cord from $G\alpha_o$ +/GS mice (data not shown).

It was previously demonstrated that loss of $G\alpha_o$ protein in mice results in reduced high-affinity μ opioid receptor expression, with no change in total μ receptor number (Lamberts et al., 2011). To evaluate whether the reduction in $G\alpha_o$ expression in $G\alpha_o$ +/GS mice affected high-affinity μ receptor levels, whole brain or spinal cord homogenates were subjected to radioligand binding analysis using the μ -selective agonist [3 H]DAMGO (Table 3.1). In homogenates from whole brain, saturation binding experiments revealed no difference in maximal [3 H]DAMGO binding (B_{max}) between genotypes ($t(11)=0.73$, $p=0.479$; Table 3.1). Furthermore, there were no differences in [3 H]DAMGO binding affinity (K_D) between $G\alpha_o$ +/GS mice and wild type controls ($t(11)=0.54$, $p=0.600$; Table 3.1). Similarly, there were no changes in high-affinity μ opioid receptor expression in spinal cord homogenates from $G\alpha_o$ +/GS mice, as measured by [3 H]DAMGO binding at a maximal concentration (12 nM; $t(6)=0.43$, $p=0.683$; Table 3.1). Total opioid receptor expression (μ , δ and κ opioid receptors), as measured by the non-selective antagonist [3 H]diprenorphine (4 nM), was not different in either whole brain ($t(9)=0.57$, $p=0.582$) or spinal cord ($t(6)=0.56$, $p=0.596$) of $G\alpha_o$ +/GS mice compared with wild type littermates (Table 3.1). μ Receptor expression was isolated from the total pool of opioid receptors using the μ -selective antagonist CTAP (300 nM). Neither whole brain ($t(9)=0.56$, $p=0.590$) nor spinal cord expression of μ receptors ($t(6)=0.73$, $p=0.495$) was altered in $G\alpha_o$ +/GS mice, in comparison with wild type controls (Table 3.1).

To determine whether the loss of $G\alpha_o$ protein in $G\alpha_o$ +/GS mice was associated with a reduction in G protein activation, opioid agonist-stimulated G protein activity was evaluated in whole brain or spinal cord homogenates using the [35 S]GTP γ S binding assay (Figure 3.5, Table 3.2). In whole brain, basal [35 S]GTP γ S binding was significantly lower in $G\alpha_o$ +/GS mice, when compared with wild type littermates ($t(17)=3.5$, $p=0.003$; Table 3.2). However, [35 S]GTP γ S incorporation stimulated by the μ -selective agonist DAMGO was unchanged in whole brain from $G\alpha_o$ +/GS mice (Figure 3.5a, *top*).

Table 3.1: Agonist and antagonist radioligand binding in whole brain or spinal cord homogenates from wild type and $G\alpha_o$ +/-GS mice

Tissue	Genotype	$[^3\text{H}]\text{DAMGO}$ binding		$[^3\text{H}]\text{Diprenorphine}$ binding	
		B_{max} (fmol/mg protein)	K_D (nM)	Total (fmol/mg protein)	μ Receptors (fmol/mg protein)
Whole brain	Wild type	219 ± 23	3.3 ± 0.3	357 ± 56	190 ± 33
	$G\alpha_o$ +/-GS	198 ± 18	3.0 ± 0.5	312 ± 56	166 ± 29
Spinal cord	Wild type	123 ± 26	ND	184 ± 21	98 ± 16
	$G\alpha_o$ +/-GS	109 ± 22	ND	202 ± 24	116 ± 19

ND, not determined. μ Opioid receptor expression was measured by evaluating the amount of bound $[^3\text{H}]\text{diprenorphine}$ displaced by the μ -selective antagonist CTAP (300 nM). Data represent the mean ± SEM (Whole brain: $n=5-7$; Spinal cord: $n=4$). Each sample was assayed in duplicate.

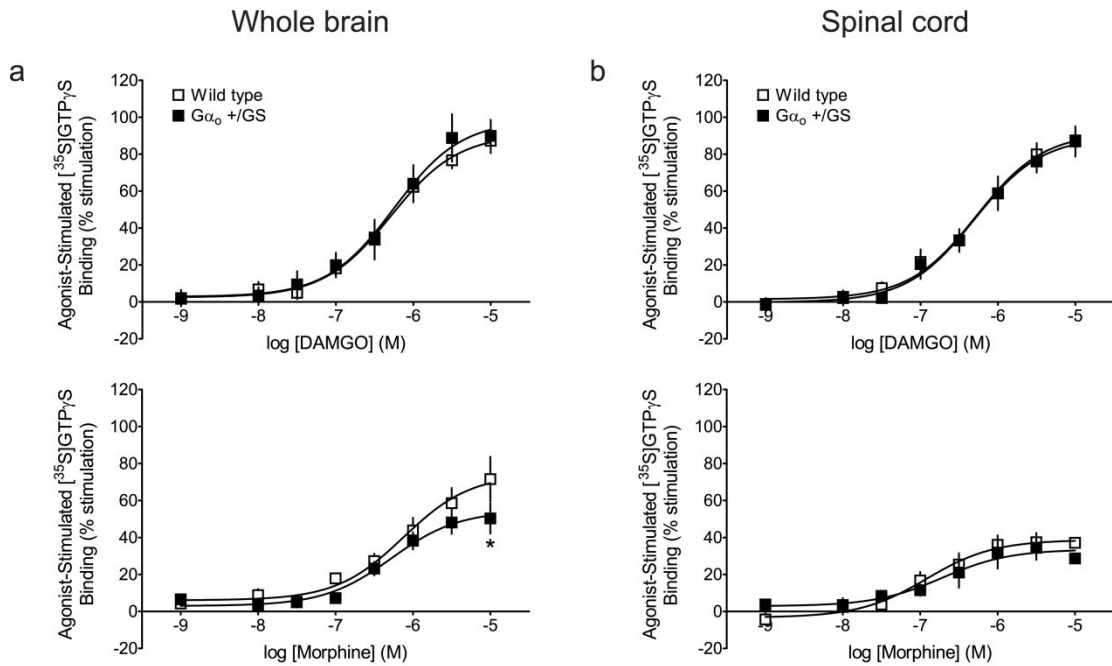


Figure 3.5: Agonist-stimulated G protein activity in whole brain or spinal cord homogenates from wild type and $G\alpha_0$ +/GS mice.

$[^3\text{S}]\text{GTP}\gamma\text{S}$ binding was measured in (a) whole brain ($n=8-9$) and (b) spinal cord ($n=3$) in the presence of increasing concentrations of DAMGO (*top*) or morphine (*bottom*).

* $p < 0.05$ compared with wild type mice at the corresponding concentration by Bonferroni's post-test. Legend in top panel also describes bottom panel. For all experiments, non-specific binding was determined using $10 \mu\text{M}$ $\text{GTP}\gamma\text{S}$. Agonist-stimulated $[^3\text{S}]\text{GTP}\gamma\text{S}$ binding is shown as % stimulation, where % stimulation = $[(\text{Drug binding} - \text{Basal binding}) / \text{Basal binding}] \times 100$. All data are plotted as the mean \pm SEM.

Table 3.2: Basal and agonist-stimulated [³⁵S]GTPγS binding in membranes from whole brain or spinal cord of wild type and Gα_o +/-GS mice.

Tissue	Genotype	Basal [³⁵ S]GTPγS binding (fmol/mg protein)	Agonist-stimulated [³⁵ S]GTPγS binding	
			<i>DAMGO</i> <i>EC</i> ₅₀ (nM)	<i>Morphine</i> <i>EC</i> ₅₀ (nM)
Whole brain	Wild type	65.9 ± 2.2	524 ± 39	806 ± 210
	Gα _o +/-GS	46.5 ± 4.9**	722 ± 116	526 ± 98
Spinal cord	Wild type	66.8 ± 7.6	547 ± 103	174 ± 88
	Gα _o +/-GS	45.3 ± 4.6*	524 ± 121	332 ± 113

p*<0.05 compared with wild type spinal cord, *p*<0.01 compared with wild type whole brain by Students' *t*-test. Data represent the mean ± SEM (Whole brain: *n*=8-9, Basal *n*=10; Spinal cord: *n*=3, Basal *n*=5). Each sample was assayed in duplicate.

Statistical analysis of DAMGO concentration-response curves obtained in whole brain homogenates from wild type and $G\alpha_o$ +/-GS mice revealed a significant effect of concentration ($F(7,128)=53, p<0.001$), while there was neither a significant effect of genotype ($F(1,128)=0.45, p=0.503$) nor a significant concentration \times genotype interaction ($F(7,128)=0.22, p=0.980$). There was also no change in DAMGO potency (EC_{50}) between $G\alpha_o$ +/-GS mice and wild type littermates ($t(16)=1.6, p=0.126$; Table 3.2). In contrast, morphine-stimulated G protein activation was attenuated in whole brain homogenates from $G\alpha_o$ +/-GS mice compared with wild type controls (Figure 3.5a, *bottom*). Analysis of the morphine concentration-response in whole brain homogenates from wild type and $G\alpha_o$ +/-GS mice demonstrated significant effects of both concentration ($F(7,112)=36, p<0.001$) and genotype ($F(1,112)=6.6, p=0.012$), although the concentration \times genotype interaction was not significant ($F(7,112)=0.92, p=0.493$). However, there was no difference in the EC_{50} for morphine between $G\alpha_o$ +/-GS and wild type mice ($t(14)=1.2, p=0.247$; Table 3.2). G protein activation was also measured in whole brain homogenates using a saturating concentration of methadone (10 μ M). [35 S]GTP γ S incorporation stimulated by methadone was unchanged in whole brain from $G\alpha_o$ +/-GS mice (% stim: $64.2 \pm 11, n=4$), when compared with wild type controls (% stim: 69.0 ± 15 fmol/mg, $n=3$; $t(5)=0.27, p=0.801$).

In the spinal cord, there was also a reduction in basal [35 S]GTP γ S incorporation in $G\alpha_o$ +/-GS mice ($t(8)=2.4, p=0.042$; Table 3.2). DAMGO stimulation of G protein activation was not different between wild type and $G\alpha_o$ +/-GS spinal cord (Figure 3.5b, *top*). There was a significant main effect of concentration ($F(7,32)=88, p<0.001$), although the effect of genotype ($F(1,32)=0.25, p=0.623$), and the concentration \times genotype interaction were not significant ($F(7,32)=0.08, p=0.999$). Moreover, there was no difference in DAMGO EC_{50} between genotypes in this tissue ($t(4)=0.14, p=0.892$; Table 3.2). Morphine-stimulated G protein activity was also unchanged in spinal cord from $G\alpha_o$ +/-GS mice in comparison with wild type littermates (Figure 3.5b, *bottom*). There was a significant effect of concentration ($F(7,32)=21, p<0.001$), while there was no significant effect of genotype ($F(1,32)=0.37, p=0.545$) and no significant concentration \times genotype interaction ($F(7,32)=0.72, p=0.658$). Moreover, morphine EC_{50} was not altered

in spinal cord homogenates from $G\alpha_o$ +/GS mice as compared with wild type littermates ($t(4)=1.1$, $p=0.332$; Table 3.2).

Discussion

These studies show that endogenously-expressed RGS proteins regulate opioid antinociception by acting at $G\alpha_o$. Mice expressing $G\alpha_o$ RGSi subunits demonstrated an opioid-dependent increase in baseline responsiveness to two different thermal stimuli: the hot plate test, a measure of supraspinal nociception (Heinricher and Morgan, 1999), and the warm-water tail withdrawal test, which primarily involves spinal nociceptive pathways (Irwin et al., 1951; Cesselin et al., 1999). Furthermore, these mice exhibited an enhancement of morphine-mediated antinociception in the hot plate test, as well as a potentiation of morphine or ME inhibition of presynaptic GABA transmission in the PAG. Together, these data confirm the hypothesis that RGS proteins negatively regulate μ opioid receptor signaling and antinociception. In contrast, there was no effect of the loss of RGS regulation on methadone antinociception in the hot plate test and an unexpected reduction in morphine and methadone antinociception in the tail withdrawal test. Overall, the results demonstrate that although RGS proteins negatively regulate μ opioid receptor signaling *in vivo*, they differentially alter opioid-mediated antinociception depending upon the agonist and nociceptive pathway(s) involved.

Pretreatment of wild type mice with naltrexone did not affect baseline latency in either the hot plate or the tail withdrawal test, indicating that endogenous opioid peptide tone is insufficient to cause an antinociceptive response. In contrast, $G\alpha_o$ +/GS mice exhibited a naltrexone-sensitive increase in baseline latency in both the hot plate and tail withdrawal tests, compared with their wild-type littermates. This is likely due to enhanced μ receptor signaling in response to endogenous opioid peptides only in mice expressing $G\alpha_o$ RGSi subunits.

Removal of negative regulation of $G\alpha_o$ by RGS proteins also resulted in enhanced morphine-mediated antinociception in the hot plate test, indicating that RGS proteins function as negative regulators of morphine supraspinal antinociception. In support of this, and in line with the role of RGS proteins as negative regulators of signaling, there was a robust potentiation of opioid (morphine or ME) inhibition of GABAergic

neurotransmission in PAG from $G\alpha_o$ +/-GS mice. The ability of opioids to inhibit presynaptic GABA neurotransmission in the PAG is thought to underlie the production of antinociception (Moreau and Fields, 1986; Reichling et al., 1988).

In contrast to the hot plate test, morphine antinociception as measured in the tail withdrawal test was significantly reduced in $G\alpha_o$ +/-GS mice. Although a reduction in $G\alpha_o$ protein was observed in the spinal cord, it is unlikely that $G\alpha_o$ levels are a limiting factor for morphine spinal antinociception, given that a >50% loss of $G\alpha_o$ protein did not affect morphine antinociception in the tail withdrawal test (Lamberts et al., 2011). Thus, it appears that the reduction in morphine spinal antinociception in $G\alpha_o$ +/-GS mice is a direct consequence of the inability of $G\alpha_o$ RGSi subunits to bind RGS proteins, indicating that RGS proteins act as positive regulators of opioid antinociception in this test. The reasons for this are not immediately obvious given that there was a naloxone-sensitive increase in baseline nociception in $G\alpha_o$ +/-GS mice, which implicates negative regulation of endogenous opioid peptide signaling by RGS proteins that was confirmed by the electrophysiological measurements in the PAG. On the other hand, a contribution from altered endogenous opioid peptide release in $G\alpha_o$ +/-GS mice cannot be discounted.

Another possible explanation is that the endogenous enkephalins are discretely released at specific synapses, whereas the systemically-administered morphine acts at many spinal and supraspinal sites and so may recruit opposing transmitter systems that are also subject to regulation by RGS proteins. Alternatively, μ opioid receptor function may be differentially regulated by RGS proteins in diverse neuronal systems due to variations in the μ receptor environment, for example the presence of particular accessory and/or scaffolding proteins may lead to differential regulation between spatially or temporally distinct signaling pathways such that they enhance some responses while simultaneously inhibiting others (Clark et al., 2003; Zhong et al., 2003). In any case, our findings in $G\alpha_o$ +/-GS mice are reminiscent of observations made in RGS9 knockout mice, in which morphine supraspinal antinociception was enhanced (Zachariou et al., 2003b), while morphine spinal antinociception was reduced (Papachatzaki et al., 2011). These authors showed that RGS9 was required for the opioid peptide DAMGO to cause hyperpolarization in lamina II dorsal horn neurons, and therefore they suggest that

RGS9-2 performs a scaffolding role. However, our results in $G\alpha_o$ +/-GS mice indicate that loss of RGS GAP activity alone is sufficient to observe this phenomenon. The reason for the difference in responses between endogenous opioid peptides and morphine in the tail withdrawal test could then be explained by a predominantly central site (*i.e.* PAG) for opioid peptide action and a predominantly spinal action of systemically-administered morphine.

There were also agonist-specific differences in the two antinociceptive tests. In the hot plate test, methadone did not produce different responses between $G\alpha_o$ +/-GS mice and their wild type littermates. In contrast, in the tail withdrawal test methadone antinociception was shifted to a lower potency, although the effect was less than that seen with morphine. There are other reports that RGS proteins can act as either positive or negative regulators of opioid antinociception, depending upon the agonist tested. For example, knockout of RGS9 has been shown to enhance morphine antinociception but inhibit methadone or fentanyl antinociception in the hot plate test (Psifogeorgou et al., 2011), while in the tail withdrawal test knockout of RGS4 did not alter morphine-mediated antinociception but did inhibit fentanyl and methadone antinociception (Han et al., 2010). At least for RGS9-2, this effect has been ascribed to the formation of complexes containing RGS9-2 and μ opioid receptors in association with different $G\alpha$ subunits, depending on the μ agonist. However, our current results suggest that GAP activity alone might be responsible for the agonist-specific results. In particular, the differences observed between morphine and fentanyl or methadone could be attributed to the higher efficacy of the latter compounds, as compared with morphine (Adams et al., 1990; Peckham and Traynor, 2006; McPherson et al., 2010), since RGS proteins are much less effective in modulating full *versus* partial agonists (Clark et al., 2003; Clark et al., 2008).

An important caveat to our findings is that mice expressing $G\alpha_o$ RGSi subunits exhibited baseline alterations in G protein expression and activity. Specifically, in whole brain and spinal cord homogenates from $G\alpha_o$ +/-GS mice, there was a 25-35% decrease in total $G\alpha_o$ protein expression, which was consistent with the observed reduction in basal [³⁵S]GTP γ S binding. This loss of $G\alpha_o$ protein is likely a compensatory response to the enhanced signaling activity of $G\alpha_o$ RGSi subunits. Alternatively, there may be altered

expression of the $G\alpha_o$ RGSi mutant allele that contains a non-genomic insertion in exon 5 of *Gnao1* (Fu et al., 2004; Goldenstein et al., 2009). However, the reduction in $G\alpha_o$ protein in these mice was not sufficient to affect the expression of μ opioid receptors in whole brain or spinal cord, and it had only a small effect on the maximum stimulation of [³⁵S]GTP γ S by the partial agonist morphine in whole brain, but not spinal cord. Thus, the 25-35% reduction in $G\alpha_o$ expression in $G\alpha_o$ +/GS mice is unlikely to contribute to the behavioral differences observed in this study. Indeed, the effects observed in the PAG and on the antinociceptive behavior of both morphine and endogenous opioid peptides are likely to be an underestimate of the degree of RGS modulation of μ receptor-mediated signaling and behavior, given that heterozygous mice have only one allele of *Gnao1* that expresses $G\alpha_o$ RGSi.

In conclusion, the current studies utilize a novel knock-in mouse model to demonstrate a role for RGS proteins in opioid antinociception mediated specifically by $G\alpha_o$. The results demonstrate that endogenous RGS GAP activity negatively regulates (1) antinociceptive responses to endogenous enkephalins, (2) morphine antinociception in the hot plate test, and (3) opioid inhibition of GABAergic transmission in the PAG. In contrast, these studies revealed a potential role of RGS proteins as positive regulators of morphine and methadone antinociception in the tail withdrawal assay. Thus, the present work provides evidence that endogenous RGS proteins are able to differentially regulate diverse nociceptive and antinociceptive pathways that are activated by a single nociceptive modality. Although the importance of the interaction between RGS proteins and $G\alpha_o$ subunits for μ opioid receptor function remains to be fully elucidated, this interface could represent a novel target for the development of more effective pain therapeutics and/or new treatments for drug addiction. For example, the fact that $G\alpha_o$ +/GS mice show reduced responsiveness to a noxious stimulus suggests that inhibition of RGS activity alone could afford an antinociceptive effect.

Chapter 4

Role of $G\alpha_o$ in Morphine Tolerance and Dependence: Studies in 129S6 Mice

Summary

Morphine is a powerful pain reliever, although the long-term use of morphine and other opioids is limited by the development of adaptations such as analgesic tolerance and physical dependence. Morphine produces its behavioral effects by activating the μ opioid receptor, which couples to inhibitory ($G\alpha_{i/o}$ -containing) heterotrimeric G proteins. Recent evidence suggests that the antinociceptive effects of morphine in mice are mediated by $G\alpha_o$. However, the role of $G\alpha_o$ in the development of morphine tolerance and dependence is currently not known. To evaluate the contribution of $G\alpha_o$ to adaptations associated with chronic morphine use, $G\alpha_o$ +/- mice were repeatedly administered morphine over a short (acute) or long (chronic) timescale and then examined for tolerance using the 52°C hot plate as the nociceptive stimulus and for dependence by evaluating the severity of opioid antagonist-precipitated withdrawal. Wild type littermates on the same genetic background as $G\alpha_o$ +/- mice (129S6) were evaluated in parallel as controls. Following either acute or chronic morphine treatment, all 129S6 mice developed antinociceptive tolerance and physical dependence, regardless of genotype. However, $G\alpha_o$ +/- mice developed tolerance more rapidly and displayed more severe antagonist-precipitated withdrawal than did wild type littermates following chronic morphine treatment. Morphine tolerance was not associated with changes in μ opioid receptor function in brain homogenates from either wild type or $G\alpha_o$ +/- mice. Thus, $G\alpha_o$ protein appears to offer some protection against morphine tolerance and dependence.

Introduction

Morphine is an extremely effective analgesic drug. Unfortunately, chronic use of morphine and other opioids results in the development of behavioral adaptations such as

tolerance and dependence, which reduces the effectiveness of these drugs over the long-term. In humans, morphine analgesic tolerance is characterized by a decrease in analgesic efficacy such that higher doses of morphine (in some cases more than 10-fold) are required for the continued therapeutic management of pain (Buntin-Mushock et al., 2005). Further, repeated use of morphine results in physical dependence, which is characterized by an unpleasant, though non-life threatening withdrawal syndrome upon abrupt discontinuation of treatment (Farrell, 1994). Despite all that is known regarding morphine tolerance and dependence, the inherent complexity of these adaptations requires that they be further studied before better analgesic treatments that are devoid of such adaptations can be proposed.

Morphine elicits the majority of its behavioral effects, including physical dependence, by activating the μ opioid receptor (Matthes et al., 1996), a member of the GPCR superfamily of transmembrane receptors. GPCRs are characterized by their coupling to heterotrimeric G proteins composed of a $G\alpha$ subunit bound to a $G\beta\gamma$ heterodimer. At the cellular level, acute stimulation of μ receptors by morphine results in the activation of $G\alpha_{i/o}$ -containing G proteins that inhibit the activity of AC enzymes, activate GIRK channels, inhibit Ca^{2+} channels, and stimulate MAPK phosphorylation, among other pathways (Law et al., 2000). μ Receptor coupling to $G\alpha_{i/o}$ proteins, and in particular $G\alpha_o$, is important for the production of μ agonist-mediated antinociception in mice (Lamberts et al., 2011).

Chronic/continuous morphine exposure has been associated with the uncoupling of several μ opioid receptor-dependent, $G\alpha_{i/o}$ -mediated signaling pathways in both mice and rats (Eitan et al., 2003; Bagley et al., 2005a; Fyfe et al., 2010). This loss of μ receptor signaling following chronic morphine treatment is thought to result from either a reduction in the expression of $G\alpha_{i/o}$ subunits (Selley et al., 1997a; Yoburn et al., 2003) and/or a decrease in the interaction between μ receptors and $G\alpha_{i/o}$ proteins (Sim et al., 1996; Elliott et al., 1997; Maher et al., 2001; Wang et al., 2005; Sim-Selley et al., 2007; Smith et al., 2007).

On the other hand, μ opioid receptor uncoupling from $G\alpha_{i/o}$ proteins is not always observed following chronic morphine treatment (Contet et al., 2008; Madia et al., 2012),

and in fact several studies have reported that chronic morphine induces the upregulation of $G\alpha_{i/o}$ protein expression in various brain regions (Nestler et al., 1989; Terwilliger et al., 1991; Fabian et al., 2002; Wang et al., 2004). There is also evidence of enhanced μ receptor-dependent signaling *via* $G\alpha_{i/o}$ following chronic morphine treatment (Ingram et al., 2008). Moreover, both knockdown and overexpression of $G\alpha_{i2}$ have been shown to ameliorate morphine antinociceptive tolerance in mice (Sanchez-Blazquez and Garzon, 1994; Garzon and Sanchez-Blazquez, 2001; Yoburn et al., 2003). Thus, although $G\alpha_{i/o}$ activation represents a critical first step in the initiation of μ opioid receptor signaling, the role of this early phase of μ receptor activity in the adaptations associated with chronic morphine use remains controversial.

It has recently been demonstrated that mice with a 50% reduction in $G\alpha_o$ protein ($G\alpha_o$ +/- mice) exhibit a significant decrease in morphine-mediated antinociception (Lamberts et al., 2011). Furthermore, it has been hypothesized that genetic variation in the expression level of the $G\alpha_o$ gene, *Gnao1*, determines the severity of morphine dependence (Kest et al., 2009). Here, to determine whether $G\alpha_o$ plays a role in the adaptations that accompany chronic morphine treatment, the development of morphine tolerance and dependence were evaluated in $G\alpha_o$ +/- mice alongside wild type 129S6 littermates as controls. Furthermore, biochemical endpoints were examined in brain homogenates from mice treated chronically with morphine to determine whether morphine tolerance was associated with changes in μ opioid receptor number and G protein activation in brain regions that are thought to play a role in the development of morphine antinociceptive tolerance (Morgan et al., 2005).

Methods

Transgenic mice

$G\alpha_o$ +/- mice were generated on a pure 129S6 background as described (Duan et al., 2007; Lamberts et al., 2011). Mice were group-housed in a facility where lights were maintained on a 12 h light/dark cycle (lights on at 07:00) and had unlimited access to food and water. Opioid-naïve mice between 10 and 20 weeks of age were utilized for all experiments and all testing was performed between 07:00 and 19:00. Male mice were used for all behavioral testing; however, due to a limited availability of transgenic mice,

both male and female mice were used in the biochemical studies. All protocols were approved by the University of Michigan Committee on the Use and Care of Animals and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Behavior

Hot plate test

For all tolerance studies, morphine antinociception was evaluated using the 52°C hot plate test. Hot plate responses were measured by placing mice on a hot plate analgesia meter maintained at $52.0 \pm 0.2^\circ\text{C}$ (Columbus Instruments, Columbus, OH), and the latency to lick forepaw(s) or jump was measured. The cutoff latency was set at 60 s to prevent tissue damage. Data are plotted as % MPE, where % MPE = (Drug latency – Baseline latency) / (Cutoff latency – Baseline latency).

Acute morphine treatment

Acute antinociceptive tolerance was induced in male wild type and $G\alpha_o$ +/- mice through administration of a single injection of morphine (128 mg/kg, i.p.; Table 4.1). After 6 h, hot plate latencies were recorded immediately prior to a 10 mg/kg morphine (i.p.) challenge to determine baseline latency and again 30 min after mice received the challenge. Control mice were administered saline (i.p.) 6 h prior to the 10 mg/kg morphine challenge.

To measure acute physical dependence, wild type and $G\alpha_o$ +/- mice that received both the morphine treatment (128 mg/kg, i.p.) and the morphine challenge (10 mg/kg, i.p.) were injected 1-2 h later with the general opioid antagonist naltrexone (10 mg/kg, subcutaneous; s.c.) to precipitate morphine withdrawal (Table 4.1). Withdrawal behaviors were then counted for 30 min as described in below.

Chronic morphine treatment

Chronic antinociceptive tolerance was elicited in male wild type and $G\alpha_o$ +/- mice during an 8 day morphine treatment scheme, as follows (Table 4.2). On Day 1, morphine antinociception was evaluated using a cumulative dosing paradigm.

Table 4.1: Acute morphine treatment paradigm.

Acute Treatment Group	Morphine Dose (mg/kg, i.p.)		
	<i>0 h</i>	<i>6 h</i>	<i>7-8 h</i>
Saline	Saline	10 ^a	–
128 mg/kg Morphine	128	10 ^a	NTX ^b (10 mg/kg, s.c.)

^a Hot plate latency (52°C) was measured 30 min following morphine. ^b Withdrawal behaviors were counted for 30 min immediately following naltrexone (NTX).

Table 4.2: Chronic morphine treatment paradigm.

Chronic Treatment Group	Morphine Dose (mg/kg, i.p.)				
	Day 1	Days 2 – 7		Day 8	
	AM	AM	PM	AM	PM
<i>Behavior</i>					
Saline	10-100 ^a (cumulative)	Saline ^b	Saline	10-100 ^a (cumulative)	–
56 mg/kg Morphine	10-100 ^a (cumulative)	56 ^b	56	32-320 ^a (cumulative)	NTX ^c (10 mg/kg, s.c.)
128 mg/kg Morphine	10-100 ^a (cumulative)	128 ^b	128	32-320 ^a (cumulative)	NTX ^c (10 mg/kg, s.c.)
<i>Biochemistry</i>					
Saline	Saline	Saline	Saline	Collect tissue	–
128 mg/kg Morphine	10-100 (cumulative)	128	128	Collect tissue	–

^a Hot plate latency (52°C) was measured 30 min following each cumulative injection of morphine. ^b Hot plate latency (52°C) was measured daily 30 min following each injection of saline or morphine. ^c Withdrawal behaviors were counted for 30 min immediately following naltrexone (NTX).

Briefly, mice were given two injections of saline (i.p.) to determine baseline latency, followed by three cumulative doses of morphine (10-100 mg/kg, i.p.) in 30 min intervals, and hot plate latencies were recorded 30 min after each injection. On Day 2, mice received two injections of either 56 or 128 mg/kg morphine (i.p.) separated by ~12 h. Control mice received two injections of saline (i.p.) according to the same schedule. Twice-daily injections continued for a total of 6 days, ending on Day 7. Hot plate latencies were recorded immediately prior to each morning injection of saline or morphine to determine baseline latency and again 30 min after mice received the injection. On Day 8, morphine antinociception was re-evaluated in the hot plate test using cumulative dosing (10-100 or 32-320 mg/kg morphine, i.p.), as above.

To measure physical dependence, wild type and $G\alpha_o$ +/- mice that received twice-daily injections of either 56 or 128 mg/kg morphine were injected with naltrexone (10 mg/kg, s.c.) 2-4 h after the final cumulative morphine dose on Day 8 to precipitate morphine withdrawal (Table 4.2). Withdrawal behaviors were then counted for 30 min as described in Section 2.2.4.

Morphine withdrawal scoring

Withdrawal behaviors were scored essentially as described (Divin et al., 2008), with slight modifications. Immediately following injection of naltrexone (10 mg/kg, s.c.), mice were placed individually in Plexiglas boxes and withdrawal behaviors were observed for 30 min. The number of occurrences of jumping, wet dog shakes, and paw tremors was recorded in 5 min intervals. The presence of ptosis, chewing, and diarrhea was scored during each 5 min interval, as follows: intervals during which the behavior was absent were given a score of 0, whereas intervals in which the behavior was present were given a score of 1, for a maximum possible score of 6. The % occurrence was then calculated for these scored behaviors, where % occurrence = (Observed score / Maximum score) \times 100. Mice were also weighed immediately preceding and immediately following the 30 min observation period, and weight loss was calculated as % decrease in body weight (g). Global withdrawal scores were calculated by assigning each withdrawal behavior a weighting factor (jumping \times 0.8, wet dog shakes \times 1, paw tremors \times 0.35, ptosis \times 1.5, chewing \times 1.5, diarrhea \times 1.5) and summing the resultant values (Maldonado et al., 1992; Berrendero et al., 2003).

Biochemistry

Chronic morphine treatment and tissue collection

For biochemistry studies, male and female wild type and $G\alpha_o$ +/- mice were treated chronically with morphine and brain tissue was removed and stored for future analysis (Table 4.2). On Day 1, mice were treated with increasing cumulative doses of morphine (10-100 mg/kg, i.p.). Mice were then treated twice-daily for 6 days (Days 2-7) with 128 mg/kg morphine (i.p.). Control mice received an equivalent number of saline injections (i.p.) on Day 1, followed by twice-daily injections of saline (i.p.) on Days 2-7. On the morning of Day 8, mice were killed and a midbrain section containing the PAG and a hindbrain section containing the RVM were dissected and rapidly frozen in isopentane. Brain tissue was then stored at -80°C until use.

Membrane preparation

Brain tissue was brought to 4°C in ice-cold 50 mM Tris, pH 7.4, and homogenates were prepared as previously described (Lester and Traynor, 2006). Final membrane pellets were resuspended in Tris buffer and assayed immediately. Protein content was determined using the BCA protein assay (ThermoFisher Scientific, Waltham, MA).

Agonist-stimulated [^{35}S]GTP γS binding assay

Incorporation of the GTP analog [^{35}S]GTP γS into activated $G\alpha$ subunits was monitored *in vitro* (Traynor and Nahorski, 1995). Brain homogenates (10 μg protein) were incubated for 2 h at 25°C in [^{35}S]GTP γS binding buffer (50 mM Tris, 5 mM MgCl_2 , 100 mM NaCl and 1 mM EDTA, pH 7.4, with 2 mM dithiothreitol, 100 μM GDP and 0.4 U/mL adenosine deaminase) with 0.1 nM [^{35}S]GTP γS and a maximal concentration (10 μM) of either DAMGO or morphine. Non-specific binding was evaluated in the presence of 10 μM unlabeled GTP γS . Reactions were stopped by rapid filtration through GF/C filtermats (Whatman, Kent, UK) using a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD), and bound radioactivity was determined by liquid scintillation counting using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

Radioligand binding assays

Total opioid receptor expression was evaluated by incubating brain homogenates (50 μg protein) in Tris buffer with a saturating concentration (4 ± 0.4 nM) of the

radiolabeled opioid antagonist [³H]diprenorphine. μ Opioid receptor expression was defined using the μ -selective antagonist CTAP (300 nM). To measure high-affinity μ receptor expression, brain homogenates (50 μ g protein) were incubated in Tris buffer with a saturating concentration (12 ± 1.2 nM) of the radiolabeled μ -selective agonist [³H]DAMGO. All binding reactions were incubated for 60 min at 25°C. Non-specific binding was evaluated in the presence of the opioid antagonist naloxone (10 μ M). Binding reactions were stopped by rapid filtration and bound radioactivity was measured by liquid scintillation counting, as above.

Materials

For behavioral experiments, all drugs were diluted in sterile water. Morphine sulfate was from RTI (Research Triangle Park, NC) and naltrexone hydrochloride was from Endo Pharmaceuticals (Newark, DE). Radiochemicals including [³H]diprenorphine, [³H]DAMGO, and [³⁵S]GTP γ S, were purchased from PerkinElmer. Adenosine deaminase was obtained from Calbiochem (San Diego, CA). DAMGO, CTAP, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Data analysis

All data were analyzed using GraphPad Prism 6 (San Diego, CA). Differences between groups were evaluated using unpaired *t*-tests or two-way ANOVA with Bonferroni's post-tests, where appropriate. For all statistical tests, significance was set at $p < 0.05$. ED₅₀ (potency) was calculated by fitting the compiled antinociception data to an agonist *versus* response curve (Hill slope=1), and data are reported as the mean (95% CI). All other values are reported as the mean \pm SEM.

Results

Acute morphine tolerance and dependence are unaffected by loss of G α_o protein

Wild type and G α_o +/- mice were first subjected to a short (acute) tolerance paradigm (Table 4.1). In wild type mice that had received 128 mg/kg morphine (i.p.) 6 h earlier, acute challenge with 10 mg/kg morphine (i.p.) resulted in a significant decrease in antinociception in the 52°C hot plate test ($p < 0.01$) compared with wild type mice that had received saline (Figure 4.1a).

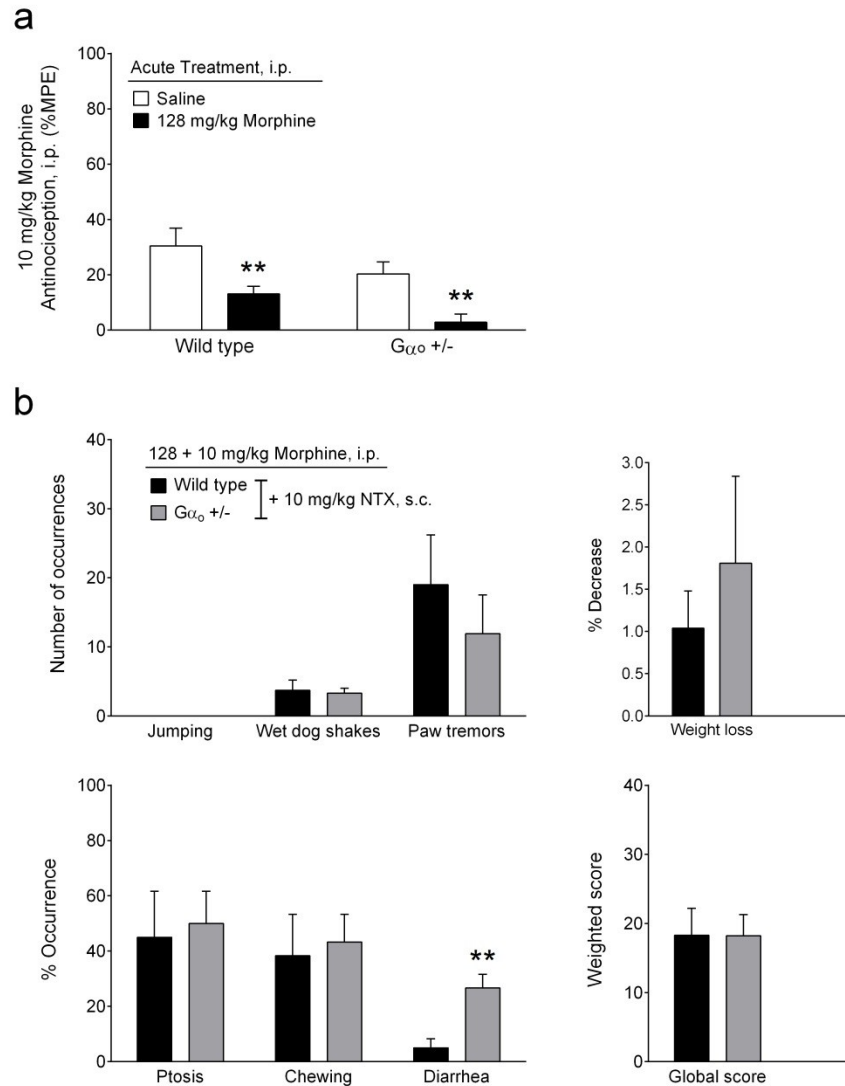


Figure 4.1: Acute morphine tolerance and dependence.

(a) Antinociception was measured in the 52°C hot plate test 30 min following a challenge injection of morphine (10 mg/kg) in wild type and $G\alpha_o$ +/- mice that were treated with either saline ($n=6-7$) or 128 mg/kg morphine ($n=10$) 6 h prior (see Materials and Methods). $**p<0.01$ compared with saline treatment by Bonferroni's post-test. (b) Naltrexone (NTX; 10 mg/kg)-precipitated withdrawal in wild type ($n=6$) and $G\alpha_o$ +/- mice ($n=7$) that were treated with 128 mg/kg morphine and challenged with 10mg/kg morphine 6 h later (see Materials and Methods). Withdrawal behaviors were scored for a period of 30 min (see Materials and Methods). $**p<0.01$ compared with wild type by Students' t -test. All data are plotted as the mean \pm SEM.

Similarly, morphine-treated $G\alpha_o$ +/- mice exhibited a significant reduction in morphine antinociception ($p < 0.01$), as compared with saline-treated $G\alpha_o$ +/- mice (Figure 4.1a). Thus, a single morphine injection was capable of producing acute tolerance to a challenge dose of morphine given 6 h later in both wild type and $G\alpha_o$ +/- mice, although acute morphine antinociception was overall lower in the $G\alpha_o$ +/- mice (main effect of treatment: $F(1,29)=19.8$, $p < 0.001$; main effect of genotype: $F(1,29)=6.7$, $p = 0.015$). The treatment \times genotype interaction was not significant ($F(1,29)=0.00$, $p = 0.987$).

Following the morphine challenge (1-2 h), mice that had been previously treated with morphine were injected with the general opioid antagonist naltrexone (10 mg/kg, s.c.) to precipitate the morphine withdrawal syndrome; withdrawal signs were scored for 30 min (Table 4.1). Preliminary studies demonstrated that the number of naltrexone-elicited behaviors was significantly greater following acute morphine treatment than following acute saline injection for both wild type and $G\alpha_o$ +/- mice (data not shown). Neither wild type nor $G\alpha_o$ +/- mice exhibited any jumping behavior when withdrawal was precipitated following acute morphine treatment (Figure 4.1b, *Jumping*). Of the additional withdrawal signs scored, only diarrhea was different between genotypes, with $G\alpha_o$ +/- mice exhibiting significantly more diarrhea during acute morphine withdrawal than wild type littermates ($p < 0.01$; Figure 4.1b, *Diarrhea*). In contrast, there were no genotype differences observed for other withdrawal signs ($p > 0.05$; Figure 1b), and the overall withdrawal syndrome was equivalent between wild type and $G\alpha_o$ +/- mice ($p > 0.05$; Figure 4.1b, *Global score*).

Chronic morphine tolerance develops more rapidly in $G\alpha_o$ +/- mice

Mice were randomly assigned to receive repeated injections of saline, 56 mg/kg/injection morphine or 128 mg/kg/injection morphine (i.p.). In preliminary studies, 56 and 128 mg/kg morphine corresponded to the 80% effective dose (ED_{80}) dose for antinociception in the 52°C hot plate test in wild type and $G\alpha_o$ +/- mice, respectively (data not shown). Twice-daily saline or morphine treatment began on Day 2 and continued for 6 days (until Day 7). Hot plate latency was checked daily following each morning injection (Figure 4.2).

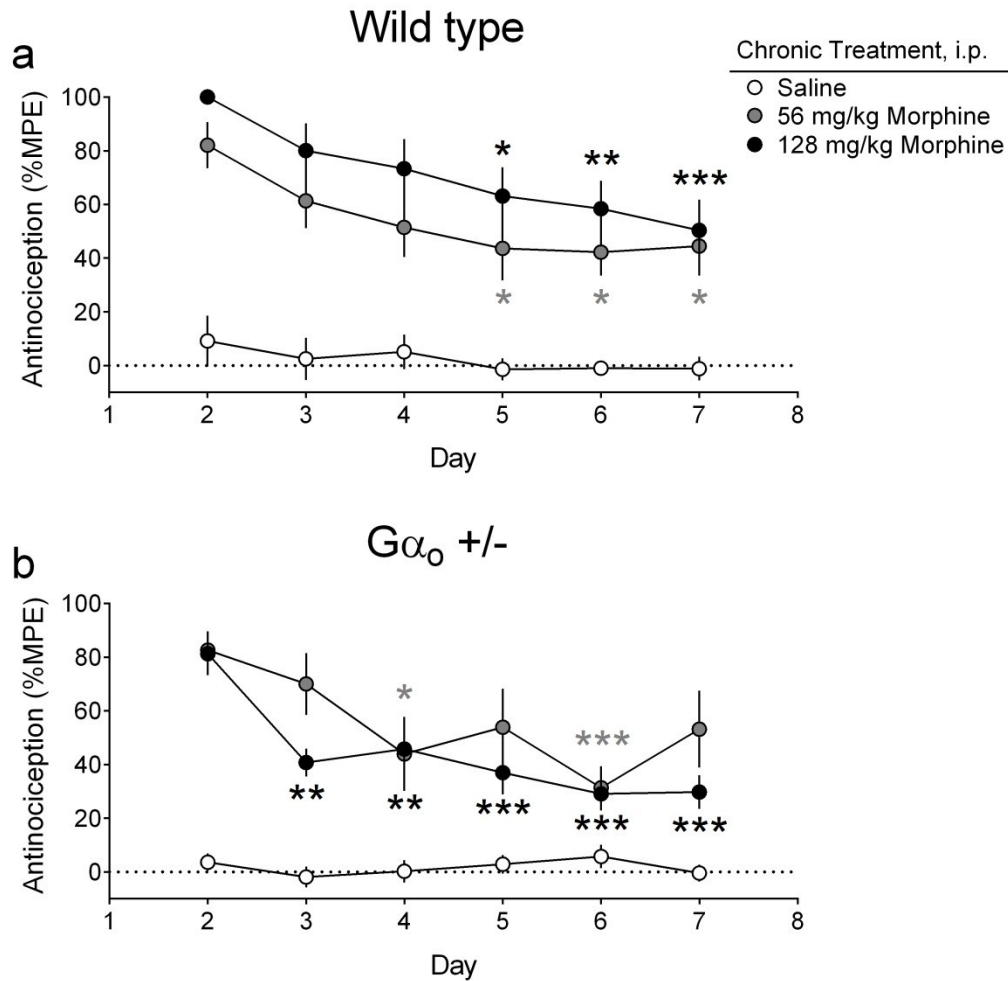


Figure 4.2: Chronic morphine tolerance – daily responsiveness.

Daily hot plate responsiveness in (a) wild type and (b) $G\alpha_0$ +/- mice treated twice-daily with saline, 56 mg/kg morphine or 128 mg/kg morphine (see Materials and Methods). Antinociception was measured in the 52°C hot plate test 30 min following each morning injection of morphine. * p <0.05, ** p <0.01, *** p <0.001 compared with antinociception on Day 2 within the same chronic treatment group (grey symbols, 56 mg/kg morphine; black symbols, 128 mg/kg morphine) by Bonferroni's post-test. See Table 4.3 for numbers of subjects per group. All data are plotted as the mean \pm SEM.

In wild type mice, saline treatment did not alter hot plate latency during the 6 day treatment paradigm ($p < 0.05$ compared with Day 2; Figure 4.2a). In contrast, treatment of wild type mice with either 56 or 128 mg/kg morphine resulted in a significant reduction in antinociception beginning on Day 5 ($p < 0.05$ compared with Day 2; Figure 4.2a). Two-way ANOVA analysis revealed significant main effects of treatment ($F(2,138)=83$, $p < 0.001$) and day ($F(5,138)=4.9$, $p < 0.001$), while the treatment \times day interaction was not significant ($F(10,138)=0.65$, $p=0.764$).

In $G\alpha_o$ +/- mice, hot plate latency did not change over 6 days of saline treatment ($p > 0.05$ compared with Day 2; Figure 4.2b). $G\alpha_o$ +/- mice exhibited a variable response to treatment with 56 mg/kg morphine, with significant decreases in antinociception only being observed on Day 4 ($p < 0.05$ compared with Day 2) and Day 6 ($p < 0.001$ compared with Day 2; Figure 4.2b). However, treatment of $G\alpha_o$ +/- mice with 128 mg/kg morphine resulted in a consistent reduction in antinociception beginning as early as Day 3 ($p < 0.01$ compared with Day 2; Figure 4.2b). Statistical analysis by two-way ANOVA revealed significant main effects of both treatment ($F(2,132)=64$, $p < 0.001$) and day ($F(5,132)=5.5$, $p < 0.001$), as well as a significant treatment \times day interaction ($F(10,132)=2.0$, $p=0.037$).

Morphine-dose response curves were established in all mice on Day 1 and again on Day 8 following twice-daily treatment with saline, 56 mg/kg morphine or 128 mg/kg morphine (Figure 4.3). Morphine potency was determined by calculating the ED_{50} , and potencies measured on Day 8 were compared with the initial potency measured on Day 1 (Table 4.3). Treatment with saline did not elicit a change in morphine potency in wild type or $G\alpha_o$ +/- mice (Figure 4.3, Table 4.3). In wild type mice, administration of either 56 or 128 mg/kg morphine produced a significant decrease in morphine potency, indicative of the development of tolerance (Figure 4.3a, Table 4.3). In $G\alpha_o$ +/- mice treated with 56 mg/kg morphine, there was no change in morphine potency between Day 1 and Day 8 (Figure 4.3b, Table 4.3). On the other hand, administration of 128 mg/kg morphine to $G\alpha_o$ +/- mice resulted in a significant decrease in morphine potency (Figure 4.3b, Table 4.3).

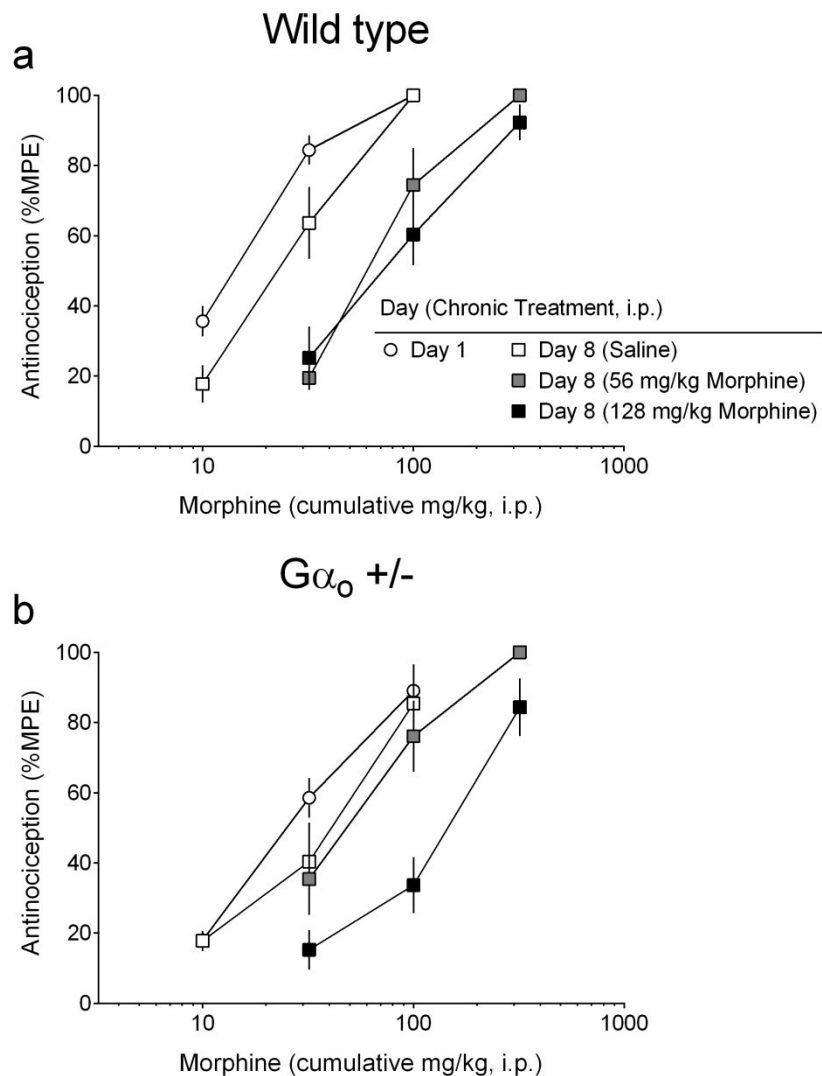


Figure 4.3: Chronic morphine tolerance – dose-response.

Morphine dose-response curves in (a) wild type and (b) $G\alpha_o$ +/- mice treated twice-daily with saline, 56 mg/kg morphine or 128 mg/kg morphine (see Materials and Methods). Morphine antinociception was evaluated in the 52°C hot plate test using a cumulative dosing procedure in all mice on Day 1 and again in the same mice on Day 8 following chronic administration of saline, 56 mg/kg morphine or 128 mg/kg morphine. See Table 4.3 for numbers of subjects per group. All data are plotted as the mean \pm SEM.

Table 4.3: Morphine ED₅₀ in the 52°C hot plate test in wild type and Gα_o +/- mice treated chronically with morphine.

Day / Chronic Treatment ^a	Wild type			Gα _o +/-		
	Morphine ED ₅₀ (mg/kg)	n	Tolerance Ratio ^b	Morphine ED ₅₀ (mg/kg)	n	Tolerance Ratio ^b
Day 1	11.1 (8.70 – 14.2)	26		25.1 (19.7 – 32.0)	25	
Day 8 / Saline	20.8 (12.9 – 33.5)	7	1.9	35.9 (20.9 – 61.7)	7	1.4
Day 8 / 56 mg/kg Morphine	54.3 (34.3 - 86.0)*	9	5.1	40.3 (23.8 - 68.1)	8	1.7
Day 8 / 128 mg/kg Morphine	66.9 (42.6 - 105)*	10	6.2	133 (84.9 - 207)*	10	5.6

^a Chronic treatment refers to the stated agent being administered twice-daily (i.p.) for 6 days (see Materials and Methods). ^b Tolerance Ratio = Day 8 ED₅₀ / Day 1 ED₅₀. Data are presented as the mean (95% CI). * Significantly different from Day 1 ED₅₀ as determined by non-overlapping 95% CIs.

Withdrawal from chronic morphine is more severe in $G\alpha_o$ +/- mice

Immediately following the re-evaluation of morphine potency on Day 8 (2-4 h), mice were injected with naltrexone (10 mg/kg, s.c.) to precipitate withdrawal, and withdrawal signs were counted during the next 30 min (Table 4.2). In initial testing, significantly more naltrexone-elicited behaviors were observed following chronic morphine treatment than following an equivalent number of saline injections in both wild type and $G\alpha_o$ +/- mice (data not shown). Compared with wild type littermates, $G\alpha_o$ +/- mice exhibited a significantly greater number of jumps (main effect of genotype: $F(1,33)=7.4$, $p=0.010$; main effect of treatment: $F(1,33)=0.03$, $p=0.858$; genotype \times treatment interaction: $F(1,33)=1.3$, $p=0.262$; Figure 4.4, *Jumping*) and wet dog shakes (main effect of genotype: $F(1,33)=4.7$, $p=0.038$; main effect of treatment: $F(1,33)=1.4$, $p=0.246$; genotype \times treatment interaction: $F(1,33)=1.4$, $p=0.246$; Figure 4.4, *Wet dog shakes*). In particular, $G\alpha_o$ +/- mice treated with 56 mg/kg morphine jumped over 4 times more often than did wild type mice treated with the same dose of morphine ($p<0.05$; Figure 4.4, *Jumping*). In contrast, there were no differences between genotypes for any of the other counted or scored withdrawal signs (main effect of genotype, $p>0.05$; Figure 4.4). Overall, naltrexone-precipitated morphine withdrawal was more severe in $G\alpha_o$ +/- mice in comparison with wild type controls (main effect of genotype: $F(1,33)=7.9$, $p=0.008$; main effect of treatment: $F(1,33)=4.1$, $p=0.052$; genotype \times treatment interaction: $F(1,33)=0.73$, $p=0.398$), especially within the 56 mg/kg morphine treatment group ($p<0.05$; Figure 4.4, *Global score*).

Chronic morphine treatment is not associated with changes at the level of μ receptors

To evaluate if morphine tolerance and dependence were associated with changes at the receptor level in wild type and $G\alpha_o$ +/- mice, μ opioid function was measured in brain homogenates prepared from mice treated chronically with either morphine or saline (Table 4.2). After chronic treatment with saline (i.p.) or 128 mg/kg morphine (i.p.), a midbrain section containing the PAG and a hindbrain section containing the RVM were removed. Membranes prepared from these sections were subjected to radioligand binding and [35 S]GTP γ S binding analyses to measure μ receptor expression (Table 4.4) and μ receptor activity (Figure 4.5), respectively.

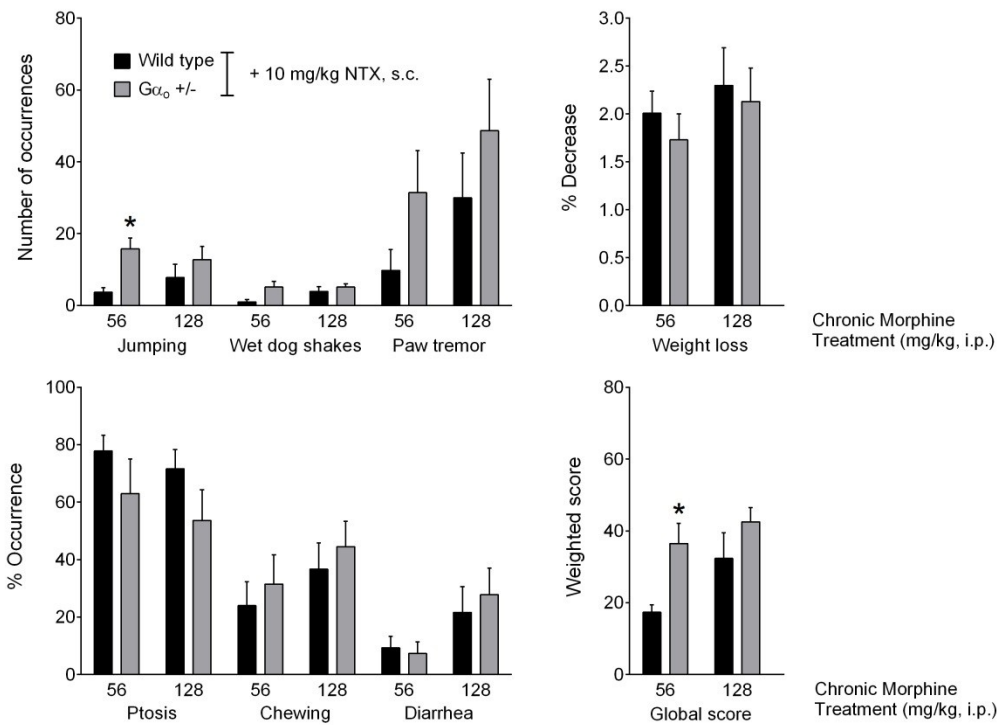


Figure 4.4: Chronic morphine dependence.

Naltrexone (NTX; 10 mg/kg)-precipitated withdrawal in wild type ($n=9-10$) and $G\alpha_o$ +/- mice ($n=9$) that were treated twice-daily with either 56 mg/kg morphine or 128 mg/kg morphine (see Materials and Methods). Withdrawal behaviors were scored for a period of 30 min (see Materials and Methods). * $p<0.05$ compared with wild type mice that received the same chronic treatment by Students' t -test. All data are plotted as the mean \pm SEM.

Table 4.4: Opioid receptor expression in brain homogenates from wild type and $G\alpha_o$ +/- mice treated chronically with morphine.

Brain Region	Chronic Treatment ^a	4 nM [³ H]Diprenorphine binding (fmol/mg protein)				12 nM [³ H]DAMGO binding (fmol/mg protein)	
		<i>Wild type</i>		<i>Gα_o +/-</i>		<i>Wild type</i>	<i>Gα_o +/-</i>
		<i>Total</i>	<i>μ Receptors</i>	<i>Total</i>	<i>μ Receptors</i>		
Midbrain	Saline	487 ± 36	355 ± 22	432 ± 103	306 ± 77	336 ± 36	267 ± 62
	128 mg/kg Morphine	456 ± 45	298 ± 42	436 ± 7	306 ± 9	326 ± 50	362 ± 27
Hindbrain	Saline	336 ± 16	199 ± 7	381 ± 56	274 ± 66	209 ± 12	238 ± 37
	128 mg/kg Morphine	393 ± 35	262 ± 27	349 ± 21	245 ± 25	222 ± 24	223 ± 15

^a Chronic treatment refers to the stated agent being given twice-daily (i.p.) for 6 days (see Materials and Methods). Data are presented as the mean ± SEM ($n=3-4$ performed in triplicate).

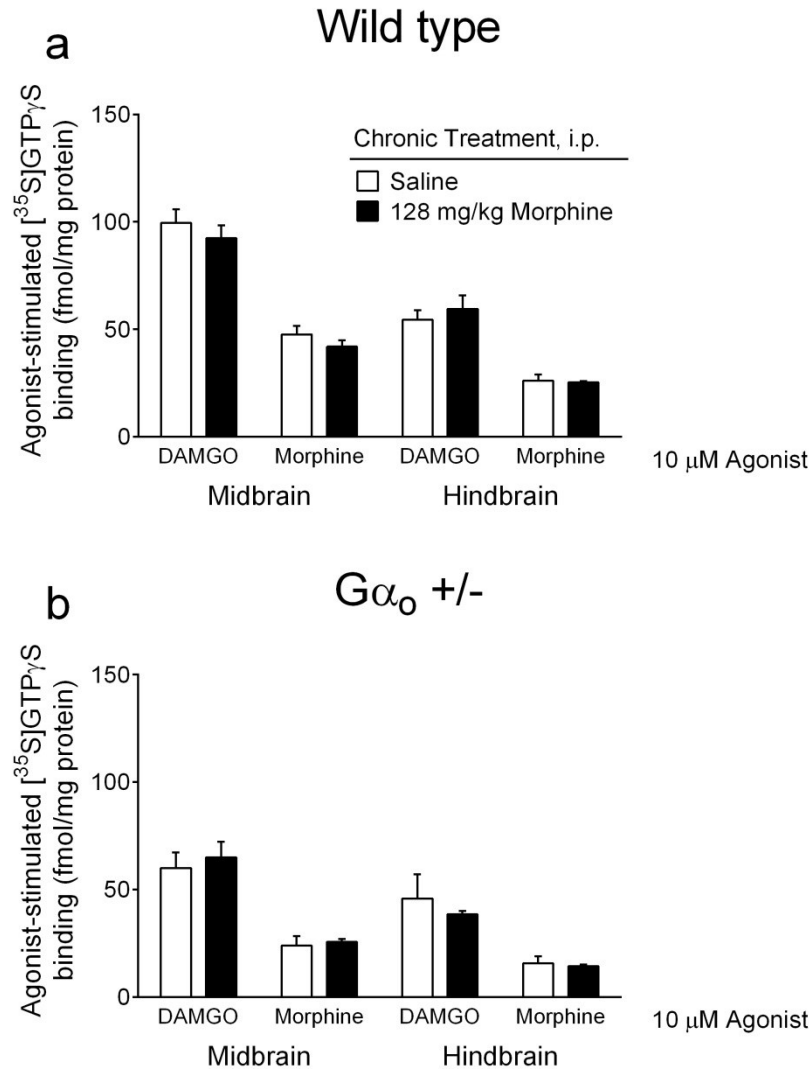


Figure 4.5: μ Opioid-stimulated G protein activity.

Midbrain and hindbrain sections were collected from (a) wild type ($n=3-4$) and (b) $G\alpha_o$ +/- mice ($n=3-4$) following twice-daily treatment with saline or 128 mg/kg morphine, and membranes prepared from these sections were evaluated for 10 μ M DAMGO- or 10 μ M morphine-stimulated [35 S]GTP γ S incorporation (see Materials and Methods). Legend in panel (a) also describes panel (b). Agonist-stimulated [35 S]GTP γ S binding was calculated by subtracting basal binding from binding that occurred in the presence of agonist. Basal [35 S]GTP γ S binding was as follows (fmol/mg protein): wild type midbrain (saline, 56 ± 6 ; morphine, 60 ± 3); wild type hindbrain (saline, 42 ± 2 ; morphine, 45 ± 2); $G\alpha_o$ +/- midbrain (saline, 38 ± 6 ; morphine, 38 ± 1); $G\alpha_o$ +/- hindbrain (saline, 35 ± 4 ; morphine, 34 ± 1). All data are presented as the mean \pm SEM.

To measure total opioid receptor expression, brain membranes from wild type and $G\alpha_o$ +/- mice were incubated with a saturating concentration (4nM) of [3 H]diprenorphine, which binds μ as well as δ and κ opioid receptors. Total μ receptor number was measured by displacing [3 H]diprenorphine with the μ -selective antagonist CTAP (300 nM). There was no effect of morphine treatment on total opioid receptor or total μ receptor expression in either midbrain or hindbrain homogenates from wild type and $G\alpha_o$ +/- mice ($p>0.05$; Table 4.4). Furthermore, there was no effect of chronic morphine treatment on high-affinity μ opioid receptor expression in either midbrain or hindbrain from wild type and $G\alpha_o$ +/- mice, as measured by 12 nM [3 H]DAMGO binding ($p>0.05$; Table 4.4).

In wild type mice, maximal DAMGO or morphine-stimulated G protein activation was unchanged following chronic morphine treatment in either midbrain or hindbrain homogenates ($p>0.05$; Figure 4.5a). Similarly, chronic morphine treatment did not affect opioid agonist-stimulated G protein activity in either midbrain or hindbrain of $G\alpha_o$ +/- mice ($p>0.05$; Figure 4.5b).

Discussion

Together, these studies demonstrate that both wild type and $G\alpha_o$ +/- mice on a 129S6 background developed antinociceptive tolerance and physical dependence following either acute or chronic morphine treatment. While there were no appreciable differences between genotypes in the development of these adaptations following acute morphine treatment, $G\alpha_o$ +/- mice appeared to develop tolerance to chronic morphine more quickly than their wild type littermates, as measured using the 52°C hot plate test. In addition, naltrexone-precipitated withdrawal following long-term morphine administration was more severe in $G\alpha_o$ +/- mice. Overall, these studies provide evidence that $G\alpha_o$ protein is protective against morphine tolerance and dependence.

The transgenic mice utilized in these studies were generated on a pure 129S6 background, and these mice developed antinociceptive tolerance to morphine in the 52°C hot plate test following either single or repeated drug administration. Our findings contrast with evidence in the literature that 129S6 mice do not develop chronic tolerance to morphine in either the tail flick or the hot plate test. This lack of morphine tolerance in 129S6 mice is thought to be due to a defect in the NMDA receptor (Kolesnikov et al.,

1998; Nitsche et al., 2002) or GM1 ganglioside (Crain and Shen, 2000). In contrast, Bryant et al. (2006) were able to demonstrate chronic morphine tolerance in 129S6 mice in both the hot plate and tail flick tests by giving once-daily injections of 10 – 40 mg/kg morphine (s.c.) for 6 days followed by a single challenge injection of 7.5 mg/kg morphine (s.c.). These authors concluded that the ability to observe morphine tolerance in 129S6 mice depends upon both the nociceptive test and the dosing regimen employed.

Indeed, a major difference between the current study and previous reports in which chronic morphine tolerance was not observed in 129S6 mice is the dose of morphine used. In particular, mice in our study received approximately ED₈₀ doses, either 56 or 128 mg/kg morphine (i.p.), twice-daily for 6 days. In contrast, mice in previous studies received 75 mg morphine (s.c. pellet) over 3 or more days (Kolesnikov et al., 1998; Nitsche et al., 2002), 2.5 mg/kg morphine (i.p.) once-daily for 5 days (Kolesnikov et al., 1998), or 3 mg/kg morphine (s.c.) once-daily for 5 days (Crain and Shen, 2000). Therefore, significantly higher doses of morphine are required to elicit antinociceptive tolerance in 129S6 mice as compared with more “tolerance-sensitive” strains, such as CD-1 or C57BL/6 (Kest et al., 2002a). Moreover, the severity of withdrawal exhibited by 129S6 mice in our hands was similar to that reported for 129P3/J (129P3) mice and is much lower than the extent of withdrawal observed in other strains (Kest et al., 2002b). Thus, 129S6 mice may possess certain genetic modifiers that have a dampening effect on the development of morphine tolerance and dependence, making them more resistant to these chronic adaptations.

Although the 129S6 mice in this study developed significant tolerance to chronic morphine treatment, these behavioral changes were not associated with alterations in μ opioid receptor expression or activity in a midbrain section containing the PAG or a hindbrain section containing the RVM. These brain areas are important for both morphine antinociception (Yaksh et al., 1988) and the development of morphine tolerance (Morgan et al., 2005), and previous reports have shown that μ receptor coupling to G $\alpha_{i/o}$ proteins is reduced in these regions when rats (Sim et al., 1996; Wang et al., 2005; Smith et al., 2007) or mice (Sim-Selley et al., 2007) are treated chronically with morphine. In contrast, other studies have not found any changes in μ opioid receptor activation of G proteins in mouse brain or spinal cord following chronic morphine

treatment (Contet et al., 2008; Madia et al., 2012). However, Sim-Selley and colleagues (2007) have shown that the degree of the reduction in μ agonist-stimulated [35 S]GTP γ S binding is highly dependent on the severity of the morphine treatment regime. This may be particularly true in 129S6 mice, which express high levels of μ receptors and so may possess higher receptor reserve. Moreover, gross dissection of brain regions coupled with homogenization in the present study could mask alterations in μ opioid receptor signaling that occurred within specific neuronal populations (Morgan et al., 2003; Sim-Selley et al., 2007) and/or subcellular locations (Fabian et al., 2002; Madia et al., 2012). Our results also do not rule out the possibility that there are changes downstream of G protein activation (Eitan et al., 2003; Bagley et al., 2005a; Fyfe et al., 2010). Nevertheless, it is unlikely that changes in μ receptor coupling to G proteins alone can fully account for behavioral tolerance to morphine in our model (Gintzler and Chakrabarti, 2006; Christie, 2008).

Wild type 129S6 mice treated with 56 mg/kg morphine developed significant antinociceptive tolerance in the 52°C hot plate test. In contrast, although hot plate latency was decreased over time in $G\alpha_o$ +/- mice receiving twice-daily injections of 56 mg/kg morphine, there was not a significant rightward shift in the morphine dose-effect curve following this treatment paradigm. On the other hand, a similar level of tolerance (~6-fold) was observed for both wild type and $G\alpha_o$ +/- mice treated with 128 mg/kg/ injection morphine. Whereas morphine responsiveness in wild type mice treated with 128 mg/kg morphine was significantly attenuated beginning on Day 5, $G\alpha_o$ +/- mice treated with the same dose of morphine exhibited a decrease in antinociception as early as the second day of injections (Day 3). It is unclear why tolerance would develop more quickly in $G\alpha_o$ +/- mice since antinociception is reduced in these animals (Lamberts et al., 2011). However, with the 50% reduction in $G\alpha_o$ protein in $G\alpha_o$ +/- mice, signaling systems are less efficient and therefore have a decreased receptor reserve. This could make $G\alpha_o$ +/- mice more susceptible to the development of morphine tolerance (Morgan and Christie, 2011), which is in agreement with the current findings.

There is the additional complication that $G\alpha_o$ +/- mice express reduced $G\alpha_o$ protein throughout development. Given that chronic morphine tolerance is characterized

by the presence of adaptations at the cellular, synaptic, and neuronal network levels (Williams et al., 2001; Christie, 2008), $G\alpha_o$ +/- mice may possess an unknown developmental compensation that causes these adaptations to occur more quickly. One initial adaptation to chronic morphine is desensitization, whereby μ receptor coupling to $G\alpha_{i/o}$ is reduced during the continued presence of drug *via* receptor phosphorylation and recruitment of arrestin (Gainetdinov et al., 2004). However, acute antinociceptive tolerance is thought to more closely mimic initial μ opioid receptor desensitization (Williams et al., 2001; Christie, 2008), and this was not different between wild type and $G\alpha_o$ +/- mice. This suggests that the mechanism by which chronic morphine tolerance develops more quickly in $G\alpha_o$ +/- mice does not involve differential desensitization.

Compared with wild type littermates, $G\alpha_o$ +/- mice also demonstrated enhanced physical dependence, as measured by the severity of naltrexone-precipitated withdrawal following chronic treatment with morphine. Since dependence is presumably a homeostatic response to increased signaling, this is opposite to the finding that might have been expected. Moreover, previous studies have shown that knockdown of $G\alpha_o$ expression reduces the severity of antagonist-precipitated withdrawal from chronic morphine (Sanchez-Blazquez and Garzon, 1994; Kest et al., 2009). On the other hand, there are several “anti-withdrawal” systems in the brain that, when activated, serve to dampen the severity of morphine withdrawal. Several of these systems, including the neuropeptides N/OFQ (Kest et al., 2001) and galanin (Zachariou et al., 2003a), activate $G\alpha_{i/o}$ -coupled receptors to oppose morphine withdrawal. In $G\alpha_o$ +/- mice, these systems may have reduced activity such that “pro-withdrawal” systems dominate, thereby leading to a worsening of morphine physical dependence. Future studies can address the mechanism of the enhancement in morphine dependence in $G\alpha_o$ +/- mice by evaluating various systems that modulate the severity of morphine withdrawal (Valeri et al., 1989; Kest et al., 1996; Kest et al., 2001; McNally and Akil, 2002; Georgescu et al., 2003; Zachariou et al., 2003a; Bagley et al., 2005b; Hao et al., 2011).

In conclusion, $G\alpha_o$ +/- mice, which express ~50% less $G\alpha_o$ protein, exhibited more rapid tolerance and more severe naltrexone-precipitated withdrawal following chronic morphine treatment, suggesting that $G\alpha_o$ signaling offers some protection against

morphine antinociceptive tolerance and physical dependence. The differences observed were quantitatively minor, which is likely due to the fact that $G\alpha_o$ +/- mice only express one null allele of *Gnao1*. Nevertheless, the finding that 129S6 mice developed morphine tolerance and dependence when administered high doses of morphine indicates that the 129S6 strain of mice and knockdown of $G\alpha_o$ may be useful models for evaluating characteristics that diminish or enhance morphine tolerance and dependence, respectively. The identification of factors that counteract morphine tolerance and dependence could help uncover novel targets for the development of opioid analgesics that are devoid of these adaptations.

Chapter 5

General Discussion

Summary and significance

The studies described in this thesis have addressed the role of the $G\alpha$ subunit $G\alpha_o$ in both μ opioid receptor signaling and opioid-generated behavioral responses. In particular, I have examined the requirement for $G\alpha_o$ expression using a strain of transgenic mice that constitutively lacks $G\alpha_o$ protein. Experiments using these mice demonstrated that $G\alpha_o$ protein couples to the μ opioid receptor *in vivo* and is important for the production of opioid antinociception. These studies also showed that $G\alpha_o$ is involved in pathways that mediate morphine antinociceptive tolerance and physical dependence, where it serves to counteract the development of these adaptations.

Additionally, I have evaluated the importance of $G\alpha_o$ regulation by RGS proteins using a second strain of transgenic mice that constitutively expresses $G\alpha_o$ RGSi subunits. Studies in $G\alpha_o$ +/GS mice demonstrated that endogenous RGS protein activity at $G\alpha_o$ negatively regulates opioid supraspinal antinociception as well as the ability of opioids to modulate GABAergic neurotransmission in the PAG. However, the same study also revealed a potential novel role of RGS proteins as positive regulators of opioid spinal antinociception.

Overall, my work demonstrates that $G\alpha_o$ protein plays an important role in opioid antinociception, and that RGS proteins regulate this behavior by directly interacting with $G\alpha_o$ subunits and acting as GAPs. These findings are significant in that they highlight the RGS: $G\alpha_o$ interface as a potential target for the development of safer analgesics. Specifically, I hypothesize that enhancing $G\alpha_o$ activity by blocking RGS regulation could be viable strategy for enhancing opioid analgesia without increasing unwanted side effects. This hypothesis was directly validated by the studies in $G\alpha_o$ +/GS mice, which showed that blocking the interaction between endogenous RGS proteins and $G\alpha_o$

increases opioid supraspinal antinociception in the hot plate test. The idea that blocking RGS regulation might not concurrently increase unwanted side effects was indirectly supported by experiments in $G\alpha_o$ +/- mice demonstrating that $G\alpha_o$ protein is protective against morphine tolerance and dependence. Thus, it can be hypothesized by extension that mice with increased $G\alpha_o$ activity, such as $G\alpha_o$ +/-GS mice, would exhibit reduced tolerance and dependence. Nevertheless, future studies are necessary to confirm that blocking RGS regulation of $G\alpha_o$ either reduces or does not alter tolerance and dependence to opioids.

Finally, it has been proposed that combining an RGS inhibitor with an opioid partial agonist would preferentially enhance analgesic effects without increasing side effects since partial agonists generally have a reduced side effect profile (Clark et al., 2008). My work also supports this hypothesis, because the phenotypic effect of expressing $G\alpha_o$ RGSi subunits was more substantial in the presence of a partial agonist (i.e. morphine) than a full agonist (i.e. methadone). Therefore, future preclinical studies should evaluate the combination of an RGS inhibitor and an opioid partial agonist as a mechanism for producing effective analgesia with reduced side effects.

Future directions

Although my studies in $G\alpha_o$ knockout and $G\alpha_o$ RGSi mice have provided new evidence regarding the importance of $G\alpha_o$ for opioid signaling and behavior, there are some caveats to be considered. Additionally, my work has raised several unanswered questions that should be the subject of further inquiry. The following sections will discuss these caveats and remaining questions as they relate to the study of either $G\alpha_{i/o}$ subunits or RGS proteins, or to the use of the various experimental models. In addition, I will also provide suggestions for how to address such issues in future work.

Studies in $G\alpha_o$ knockout mice

Role of specific $G\alpha_{i/o}$ proteins in opioid-induced behaviors

My work has shown that $G\alpha_o$ is a vital intracellular mediator of opioid antinociception. However, one of the remaining concerns related to these behavioral studies in $G\alpha_o$ +/- mice is whether or not $G\alpha_o$ is the only $G\alpha_{i/o}$ isoform involved in

opioid antinociception. Using $G\alpha_o$ +/- mice, I demonstrated that $G\alpha_o$ mediates both spinal and supraspinal opioid antinociception using the tail withdrawal and hot plate tests, respectively. In agreement with previous studies (Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Sanchez-Blazquez et al., 2001), I found that a reduction in $G\alpha_o$ protein expression did not alter morphine antinociception in the tail withdrawal test. However, evaluation of spinal antinociception elicited by the opioid partial agonist nalbuphine in $G\alpha_o$ +/- mice revealed that $G\alpha_o$ does mediate this behavior, but that signaling systems are more efficient such that a 50% reduction in $G\alpha_o$ protein was not sufficient to affect responses produced by an efficacious agonist such as morphine. Thus, my findings help explain why previous studies using antisense ODN knockdown of individual $G\alpha$ subunits may not have uncovered a role for $G\alpha_o$ in morphine spinal antinociception until >60% local knockdown of $G\alpha_o$ levels was achieved (Standifer et al., 1996).

On the other hand, there remains a discrepancy between those previous studies that demonstrated a role for $G\alpha_{i2}$ in morphine spinal antinociception (Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Sanchez-Blazquez et al., 2001) and the present work, which found no differences in morphine antinociception between wild type and $G\alpha_{i2}$ -/- mice. My studies do not necessarily rule out that other $G\alpha_{i/o}$ subtypes contribute to opioid antinociception; rather, they simply indicate that the $G\alpha_o$ subunit is an important mediator of these behaviors. It is possible that other $G\alpha_{i/o}$ subunits play a role or have brain region-specific functions, and this could be evaluated using either mice with site-specific knockdown of $G\alpha_{i/o}$ subunits by RNA interference (RNAi) (Kuhn et al., 2007) or conditional $G\alpha_{i/o}$ null mice with inducible and/or local knockout of specific $G\alpha_{i/o}$ isoforms (Matthaei, 2007; Castrop, 2010). The use of conditional transgenic mice will be considered later on in this section.

In addition, although my work demonstrates that $G\alpha_o$ plays a protective role in pathways that mediate morphine tolerance and dependence, it does not confirm whether $G\alpha_o$ is the only or even the main $G\alpha_{i/o}$ subunit involved. Given that knockout of $G\alpha_z$ has also been shown to cause more rapid morphine tolerance in the hot plate test (Hendry et al., 2000; Leck et al., 2004), it is possible that these two $G\alpha_{i/o}$ subtypes work in concert to counteract adaptations that lead to tolerance or have region-specific roles. Therefore,

future studies should examine the development of morphine antinociceptive tolerance and physical dependence in transgenic mice null for individual $G\alpha_{i/o}$ subunits or mice with RNAi-mediated knockdown of $G\alpha_{i/o}$ proteins.

Lastly, the role of $G\alpha_{i/o}$ subunits in other μ opioid receptor-mediated behaviors, such as respiratory depression, constipation, or locomotor activation, was not evaluated in the present work. Previous studies have shown that antisense ODN knockdown of $G\alpha_{i2}$ did not affect morphine-induced constipation (Raffa et al., 1996), and therefore this raises the possibility that $G\alpha_o$, or another $G\alpha_{i/o}$ subtype other than $G\alpha_{i2}$, is responsible for this effect of morphine. Similarly, although changes in G protein activity have been shown to occur in a model of morphine reward (Narita et al., 2003; Vigano et al., 2003), it is unknown which $G\alpha_{i/o}$ subunit, if any, is responsible. To this end, future studies using isoform-specific $G\alpha_{i/o}$ knockout mice should be used to determine the contribution of individual $G\alpha_{i/o}$ subunits to the various behavioral effects of morphine and other opioids.

Site of $G\alpha_o$ control of opioid antinociception

My demonstration that μ opioid receptor coupling to G proteins is reduced in $G\alpha_o$ knockout mice supports the idea that $G\alpha_o$ plays a key role in the initiation of μ opioid receptor signaling that eventually leads to antinociception. However, these studies were performed in homogenates prepared from whole brain or spinal cord, and therefore they do not provide any information about which regions or neuronal populations are involved. One way to evaluate which part of the nociceptive pathway is important for the production of opioid antinociception mediated specifically by $G\alpha_o$ is to use electrophysiological techniques to measure opioid modulation of synaptic transmission in various regions from $G\alpha_o$ knockout mice. In $G\alpha_o$ +/GS mice, opioid inhibition of GABA neurotransmission was enhanced in the PAG, so it is likely that the opposite result would be observed in PAG from $G\alpha_o$ knockout mice. Nevertheless, opioids produce antinociception by acting at several sites along the neuraxis (Cesselin et al., 1999; Heinricher and Morgan, 1999; see also Chapter 1 of this thesis), and therefore any evaluation of changes in opioid signaling should also include other regions, such as the RVM and the spinal cord dorsal horn.

Studies in $G\alpha_o$ RGSi mice

Role of individual RGS proteins in opioid antinociception

My work demonstrated, using $G\alpha_o$ +/-GS mice, that endogenous RGS proteins modulate opioid antinociception by acting as GAPs at $G\alpha_o$. These studies are novel in that they show for the first time that RGS proteins exert these effects *via* direct GAP activity at $G\alpha_o$ and not through some other function, such as scaffolding. Nonetheless, one caveat of the $G\alpha_o$ RGSi model is that it cannot determine which RGS isoform(s) are involved in this regulation. A clue as to which RGS protein is involved comes from my finding that the direction of this RGS regulation was qualitatively different, with negative regulation being observed in the hot plate test and positive regulation occurring in the tail withdrawal test. These results mirror previous studies in which RGS9-2 positively or negatively regulated morphine antinociception in the same manner, depending on the nociceptive test employed (Zachariou et al., 2003b; Papachatzaki et al., 2011). However, Zachariou and colleagues ascribed these effects to a scaffolding role of the various protein-protein binding domains on RGS9-2, whereas my results suggest that GAP activity alone is sufficient to achieve positive or negative RGS regulation. Nonetheless, RGS9-2 is a likely candidate contributing to opioid antinociception mediated by $G\alpha_o$. On the other hand, several other RGS proteins have been implicated in μ opioid receptor signaling *in vitro*, including RGS4, RGS8 and RGS19 (Wang et al., 2009; Talbot et al., 2010; Wang and Traynor, 2012), and these could be important in brain regions where RGS9-2 is not expressed or is present at low levels.

One way to test the hypothesis that RGS9-2, but not other RGS subtypes, modulates opioid antinociception is to use RNAi strategies in mice to knock down individual RGS isoforms in specific brain regions (Kuhn et al., 2007). An alternate approach is to use small molecule RGS inhibitors. Small molecules that target various aspects of RGS function are being developed (Roman and Traynor, 2011), and significant progress has been made toward the identification and characterization of high potency inhibitors of RGS4 (Blazer et al., 2011). Alternatively, the regulation of RGS proteins is currently being explored as a way to modify RGS function, for example by altering protein degradation (Sjögren and Neubig, 2010). The use of pharmacological manipulation of RGS proteins is a therapeutically-relevant method, and studies

examining the effects of RGS inhibition (or activation) on opioid antinociception would provide important information for drug discovery programs that target the RGS:G α interface for the treatment of pain (Neubig and Siderovski, 2002).

Although these studies provide evidence that the RGS:G α_o interface is a viable target for the development of improved pain therapeutics, the specificity of RGS:G α_o complexes for opioid antinociception over other opioid and non-opioid behaviors has not been fully addressed here. Thus, future work is needed to determine how targeting this interface alters signaling of other neuronal GPCRs that couple to G α_o , such as dopamine, serotonin, adrenergic and muscarinic receptors (Jiang and Bajpayee, 2009).

RGS function in spinal and supraspinal pathways

My studies in G α_o +/-GS mice also raise several additional questions that will be important to examine in future work. In particular, it is still unclear as to why the direction of RGS regulation of opioid antinociception was opposite between the hot plate test (supraspinal) and the tail withdrawal test (spinal). Moreover, it is peculiar that baseline spinal nociception (endogenous opioid peptide-mediated) and spinal antinociception (opioid agonist-mediated) were differentially regulated by RGS proteins despite being measured in the same nociceptive test, the tail withdrawal test. An understanding of the mechanisms underlying these observations will require several parallel and complementary approaches.

First, opioids in the present study were administered systemically and so will activate several spinal and supraspinal pathways simultaneously (Cesselin et al., 1999; Heinricher and Morgan, 1999). Thus, one way to begin teasing apart the seemingly contradictory findings is to better characterize the specific site(s) of opioid action in G α_o +/-GS mice. For example, hot plate and tail withdrawal responses could be evaluated following either i.c.v. or intrathecal administration of morphine. Furthermore, the contribution of ascending and descending nociceptive pathways to each nociceptive response should be tested by specifically ablating either ascending or descending projections. This can be accomplished by either manually destroying specific brain areas or applying various neurotoxins (Heinricher and Morgan, 1999).

Secondly, the differences observed between opioid spinal and supraspinal antinociception and/or between spinal nociception and antinociception could involve

differences in the consequences of RGS GAP activity between nociceptive pathways. Specifically, my results can be explained if RGS proteins inhibit μ opioid receptor signaling in supraspinal networks yet enhance μ opioid receptor activity in spinal circuits. This may depend upon which signaling pathways are activated by μ opioid receptors in different populations of neurons, because there is evidence to suggest that RGS proteins inhibit signaling to AC and MAPK yet promote intracellular Ca^{2+} responses (Clark et al., 2011). Thus, if Ca^{2+} -dependent pathways are preferentially activated in neurons of the spinal cord, this could explain the observed qualitative difference between opioid spinal and supraspinal antinociception.

Indeed, there are known differences in the signaling mechanisms that are activated by μ opioid receptors between spinal and supraspinal pathways, and these could be responsible for the behavioral observations in $\text{G}\alpha_o$ +/-GS mice. For example, presynaptic μ receptors in the PAG activate a voltage-sensitive potassium channel *via* phospholipase A2 (Vaughan et al., 1997), while μ receptors in the spinal cord do not appear to use this mechanism (Heinke et al., 2011). In contrast, postsynaptic μ receptors in both the PAG and spinal cord activate GIRK channels and inhibit voltage-gated calcium channels (Chieng and Christie, 1994a; Connor et al., 1999; Heinke et al., 2011). I have already demonstrated that endogenous RGS proteins negatively regulate opioid presynaptic inhibition of GABAergic transmission in PAG neurons. However, the role of RGS proteins in the postsynaptic effects of μ opioid receptors in the brain and spinal cord is not known. Thus, μ opioid receptor coupling to these effectors should be evaluated in both supraspinal and spinal neurons from $\text{G}\alpha_o$ +/-GS mice using electrophysiology and other similar spatially- and temporally-resolved methods.

Furthermore, as opioid antinociception is known to be mediated in part by the inhibition of pronociceptive neuropeptide release in the spinal cord (Cesselin et al., 1999; see also Chapter 1 of this thesis), it is possible that removal of RGS GAP activity actually diminishes this effect rather than producing the expected enhancement. A decrease in the ability of opioids to inhibit spinal neuropeptide release from primary afferent nociceptors would partially explain why I observed a loss of opioid antinociception in $\text{G}\alpha_o$ +/-GS mice in the tail flick test. Moreover, opioids have been shown in some cases to facilitate

neuropeptide release, such as the enhanced spinal release of cholecystinin mediated by morphine acting at δ opioid receptors (Benoliel et al., 1994; Gustafsson et al., 2001). If this effect of morphine was enhanced by removal of RGS activity at $G\alpha_o$, that too would explain my results in $G\alpha_o$ +/-GS mice. Therefore, the release of neuropeptides such as substance P and cholecystinin should be measured in the spinal cord of $G\alpha_o$ +/-GS mice.

Third, the behavioral observations in $G\alpha_o$ +/-GS mice could be due to adaptations within other neurotransmitter receptor systems. This is because the $G\alpha_o$ RGSi mutation will increase the lifetime of active $G\alpha_o$ -GTP and $\beta\gamma$ subunits in any GPCR pathway in which $G\alpha_o$ functions. Therefore, it is possible that the apparent positive regulatory function of RGS proteins in spinal pathways is due to a lack of RGS activity within pronociceptive receptor systems that signal *via* $G\alpha_o$, such as NOP (Mogil and Pasternak, 2001). Indeed, NOP activity in the PAG has been shown to inhibit opioid spinal antinociception in rats (Scoto et al., 2007), and alterations within this system could underlie the reduction in opioid spinal antinociception observed in $G\alpha_o$ +/-GS mice. In addition, both adrenergic and serotonergic systems are involved in descending antinociceptive pathways (Millan, 2002), and changes within either of these pathways could have also contributed to my results in $G\alpha_o$ +/-GS mice. To address these possibilities, electrophysiological experiments as well as neurotransmitter release studies should be performed in the brain and spinal cord to evaluate NOP, adrenergic, and serotonergic signaling.

Lastly, in addition to blocking RGS GAP activity at $G\alpha_o$, the $G\alpha_o$ RGSi mutation will prevent $G\alpha_o$ binding to RGS proteins from the RA and RL families that have an RH domain but exhibit low or no GAP activity (Hollinger and Hepler, 2002). In wild type mice, these RGS proteins may either sequester $G\alpha_o$, prolonging the lifetime of active $\beta\gamma$ subunits, or link $G\alpha_o$ to diverse signaling pathways. In either case, loss of RH domain binding to $G\alpha_o$, as in $G\alpha_o$ +/-GS mice, would reduce μ opioid receptor signaling. To evaluate this mechanism, RGS proteins of the RA and RL families could be locally knocked down in the spinal cord prior to evaluating opioid antinociception in the tail withdrawal test.

Use of nociceptive tests

My studies have evaluated opioid antinociception in both $G\alpha_o$ +/- and $G\alpha_o$ +/-GS mice using only two noxious stimuli, the tail withdrawal test and the hot plate test, both of which are thermal stimulus modalities (Le Bars et al., 2001; Barrot, 2012). Thus, another unknown is whether or not $G\alpha_o$ subunits mediate opioid antinociception to other noxious stimuli, such as chemical or mechanical. Although opioids are used to treat a wide variety of pain states, not all stimulus modalities are equally blocked by opioid analgesic drugs; this can be observed both clinically (Kindler et al., 2011) and experimentally (Scherrer et al., 2009). Therefore, from a therapeutic perspective, it will be important to understand the role of interactions between μ opioid receptors, $G\alpha_o$ subunits, and RGS proteins across several noxious tests. For instance, $G\alpha_o$ +/- and $G\alpha_o$ +/-GS mice could be tested for morphine antinociception against a chemical stimulus, such as in the acetic acid stretching assay, or against a mechanical insult, such as in the paw pressure test (Le Bars et al., 2001; Barrot, 2012).

In addition, chronic pain states such as inflammatory or neuropathic pain are more clinically-relevant, and therefore the translational value of these studies would be enhanced by evaluating these types of sustained nociceptive stimuli. Models of both inflammatory and neuropathic pain produce allodynia (perceiving a previously innocuous stimulus as being noxious) and hyperalgesia (increased sensitivity to a noxious stimulus), which can be measured using standard thermal and mechanical nociceptive tests (Barrot, 2012). In the case of inflammatory pain, rodents are usually injected in the paw with an immunogenic agent such as Freund's adjuvant or carrageenan, following which allodynia and hyperalgesia will be present for several hours. Experimental neuropathic pain in rodents is generally induced by ligating the sciatic nerve, which results in lasting allodynia and hyperalgesia, depending on the severity of the ligation. Evaluation of these pain states in $G\alpha_o$ +/- and $G\alpha_o$ +/-GS mice would provide valuable information regarding the role of G protein signaling in chronic pain and could potentially uncover novel targets for the treatment of these debilitating conditions (Rosenblum et al., 2008).

Use of transgenic mouse models

The crux of my thesis is the use of two transgenic mouse models, one with targeted knockout of *Gnao1* ($G\alpha_o$ knockout mice) and the other with targeted knock-in of an RGSi mutant *Gnao1* allele ($G\alpha_o$ RGSi mice). Though these models have provided novel information regarding the role of $G\alpha_o$ protein and its regulation in opioid-mediated signaling and behavior, their use was associated with two important concerns. First, both strains of transgenic mice were constitutive, meaning that expression of the transgene was initiated at birth and occurred in all cell types. There are several potential issues with transgenic mouse models that utilize constitutive transgene expression, and these will be discussed below. Second, the transgenic mice used in the current studies were developed on two different 129 sub-strains, and there may be confounds related to these background genotypes.

Constitutive transgene expression

Although constitutive transgenic models have been useful for the past few decades, there are several caveats to consider when using constitutive transgenic mice that could potentially complicate the genotype-to-phenotype association (Matthaei, 2007; Castrop, 2010). First, depending on the gene that is being manipulated, constitutive transgene expression could possibly lead to developmental problems, or even embryonic lethality, if the gene is important for normal growth (Matthaei, 2007; Castrop, 2010). This is clearly the case for both the $G\alpha_o$ knockout and the $G\alpha_o$ RGSi strain utilized in the present studies. Specifically, only a few $G\alpha_o$ $-/-$ mice were obtained for biochemical analysis, and $G\alpha_o$ GS/GS were not viable (Goldenstein et al., 2009). Though neither strain was embryonic lethal, these findings clearly demonstrate the function and regulation of $G\alpha_o$ protein is important for mouse development. Indeed, two independently-generated strains of $G\alpha_o$ knockout mice were reported to exhibit multiple signaling defects that could potentially underlie the shortened life-span of $G\alpha_o$ $-/-$ mice. For example, $G\alpha_o$ $-/-$ mice had altered Ca^{2+} current regulation in DRG neurons (Jiang et al., 1998) and reduced muscarinic regulation of Ca^{2+} channels in the heart (Valenzuela et al., 1997). Moreover, the same strain of $G\alpha_o$ knockout mice used in the current study has previously been shown to exhibit decreased muscarinic regulation of heart rate (Duan et

al., 2007). Therefore, it is likely that normal heart and/or neuronal development is altered in $G\alpha_o$ $-/-$ mice such that few of these animals survive until weaning. It is unclear why $G\alpha_o$ GS/GS mice are not viable, although the mechanism in this case may also involve the heart. The viability of $G\alpha_o$ GS/GS mice is the subject of another thesis in this department (Kehrl et al., 2012).

Another issue associated with the use of constitutive transgenic mice is the potential for compensatory or developmental changes in genes or signaling pathways beyond the gene of interest (Matthaei, 2007; Castrop, 2010). Though my studies demonstrated that other $G\alpha_{i/o}$ subtypes were not altered in either $G\alpha_o$ knockout or $G\alpha_o$ $+/GS$ mice, this represents only a fraction of the number of genes that could be changed. Since $G\alpha_o$ is important for signaling at several neurotransmitter receptors (Brown and Sihra, 2008; Jiang and Bajpayee, 2009), it is possible that receptors other than the μ opioid receptor could be affected in these mice. Indeed, in $G\alpha_o$ $-/-$ mice, the activity of several GPCRs, including dopamine, muscarinic and serotonin receptors, has been shown to be reduced (Valenzuela et al., 1997; Jiang et al., 2001; Duan et al., 2007), in addition to the observed decrease in μ opioid receptor signaling (Jiang et al., 1998; Jiang et al., 2001; see also Chapter 2 of this thesis). Moreover, responses to adrenergic agonists are enhanced in hippocampus from $G\alpha_o$ $+/GS$ mice (Goldenstein et al., 2009). Thus, a complete evaluation of both $G\alpha_o$ knockout and $G\alpha_o$ RGSi mice should include an examination of signaling and behavior mediated by other GPCRs to either confirm or refute the presence of such compensatory changes.

To overcome these specific limitations related to the use of constitutive transgene expression, there exist several strategies for developing transgenic mice with more temporal and/or spatial control over the expression of the transgene (Matthaei, 2007; Castrop, 2010). Such models are termed “conditional” because the expression – or lack of expression – of a particular gene is dependent upon specifically designed constraints. In particular, tissue-specific transgene expression can be obtained using the phage-derived *Cre/loxP* system, which utilizes tissue-specific expression of Cre recombinase to locally delete a gene of interest that has been flanked by *loxP* sites (Gu et al., 1994). Additional manipulations, such as the use of a tamoxifen-inducible Cre/estrogen receptor construct,

can add temporal control to the classic *Cre/loxP* system (Sohal et al., 2001). In addition, local and inducible transgene expression can be achieved with the use of a tetracycline system, which is able to control gene transcription in response to the presence of tetracycline (or doxycycline) (Kistner et al., 1996). Ideally, the development of conditional $G\alpha_o$ knockout and $G\alpha_o$ RGSi mice using one of these response systems would overcome the aforementioned limitations of constitutive transgenic strategies while also allowing for the examination of the role of $G\alpha_o$ in specific neuronal cell types.

Genetics of background strain

A further concern related to the use of transgenic mouse models is the choice of background strain on which the genetically-modified mice are developed. Specifically, it is possible that modifier genes unique to the background strain could affect the observed phenotype independently of the transgene (Castrop, 2010). This is especially true for studies of nociception in mice, because widely different nociceptive phenotypes are apparent among commonly used background strains, including C57BL/6 and 129 (Lariviere et al., 2001; Leo et al., 2008). Thus, the results I obtained in $G\alpha_o$ knockout and $G\alpha_o$ +/GS mice, which were developed on pure 129S6 and 129S1 backgrounds, respectively, may be unique to these background strains. In fact, I demonstrated here that the 129S6 mouse strain is relatively resistant to morphine tolerance and dependence, and therefore it is possible that different results would have been obtained using mice on a pure C57BL/6 background, as these mice are more sensitive to the development of morphine tolerance and dependence (Kest et al., 2002a; Kest et al., 2002b). On the other hand, 129S1 mice have been shown to exhibit impaired corticolimbic circuit function, and since this network is involved in nociceptive processing it could have impacted the observed phenotype in $G\alpha_o$ +/GS mice (Hefner et al., 2008).

The current recommendation for avoiding confounds due to the choice of background genotype is to utilize an F₁ hybrid cross between two different congenic mutant lines, for example 50% C57BL/6 and 50% 129P3 (Silva et al., 1997). This strategy should eliminate some of the non-specific effects due to background genotype, and transgenic phenotypes are likely to be more comparable across hybrids than across inbred strains. Therefore, another potential improvement to the current studies would be

to analyze opioid signaling and behavior in $G\alpha_o$ knockout and $G\alpha_o$ +/-GS mice generated from F₁ hybrid crosses.

Overall conclusions

In sum, the work outlined in this thesis accomplished the initial goal of better understanding the role of the $G\alpha$ subunit $G\alpha_o$ in the behavioral effects of morphine and other opioids acting at the μ receptor. My studies underscore the importance of the $G\alpha_o$ subunit for normal growth and development, which is not surprising given that it is the most abundant $G\alpha_{i/o}$ subtype expressed in the brain (Gierschik et al., 1986) and is also enriched in the heart (Valenzuela et al., 1997). In addition, I demonstrated for the first time that RGS regulation of $G\alpha_o$ signaling is mediated *via* direct interaction of endogenous RGS proteins with the $G\alpha_o$ subunit. This work is relevant to future drug development strategies that target the RGS: $G\alpha_o$ interface as a mechanism of enhancing the analgesic effects of opioids without increasing their deleterious side effects.

References

- Adams JU, Paronis CA and Holtzman SG (1990) Assessment of relative intrinsic activity of μ -opioid analgesics *in vivo* by using β -funaltrexamine. *J Pharmacol Exp Ther* **255**:1027-1032.
- Bagley EE, Chieng BC, Christie MJ and Connor M (2005a) Opioid tolerance in periaqueductal gray neurons isolated from mice chronically treated with morphine. *Br J Pharmacol* **146**:68-76.
- Bagley EE, Gerke MB, Vaughan CW, Hack SP and Christie MJ (2005b) GABA transporter currents activated by protein kinase A excite midbrain neurons during opioid withdrawal. *Neuron* **45**:433-445.
- Bailey CP and Connor M (2005) Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* **5**:60-68.
- Barrot M (2012) Tests and models of nociception and pain in rodents. *Neuroscience* **211**:39-50.
- Benoliel JJ, Collin E, Mauborgne A, Bourgoin S, Legrand JC, Hamon M and Cesselin F (1994) μ and δ Opioid receptors mediate opposite modulations by morphine of the spinal release of cholecystinin-like material. *Brain Res* **653**:81-91.
- Berrendero F, Castane A, Ledent C, Parmentier M, Maldonado R and Valverde O (2003) Increase of morphine withdrawal in mice lacking A_{2A} receptors and no changes in CB_1/A_{2A} double knockout mice. *Eur J Neurosci* **17**:315-324.
- Besse D, Lombard MC, Zajac JM, Roques BP and Besson JM (1990) Pre- and postsynaptic distribution of μ , δ and κ opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord. *Brain Res* **521**:15-22.
- Blazer LL, Zhang H, Casey EM, Husbands SM and Neubig RR (2011) A nanomolar-potency small molecule inhibitor of regulator of G-protein signaling proteins. *Biochemistry* **50**:3181-3192.
- Bokoch GM, Katada T, Northup JK, Hewlett EL and Gilman AG (1983) Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J Biol Chem* **258**:2072-2075.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.

- Brown DA and Sihra TS (2008) Presynaptic signaling by heterotrimeric G-proteins. *Handb Exp Pharmacol*:207-260.
- Bryant CD, Roberts KW, Byun JS, Fanselow MS and Evans CJ (2006) Morphine analgesic tolerance in 129P3/J and 129S6/SvEv mice. *Pharmacol Biochem Behav* **85**:769-779.
- Buntin-Mushock C, Phillip L, Moriyama K and Palmer PP (2005) Age-dependent opioid escalation in chronic pain patients. *Anesth Analg* **100**:1740-1745.
- Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, Low M and Grandy DK (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a μ , δ or κ opioid receptor type. *FEBS Lett* **347**:284-288.
- Carter BD and Medzihradsky F (1993) G_o mediates the coupling of the μ opioid receptor to adenylyl cyclase in cloned neural cells and brain. *Proc Natl Acad Sci U S A* **90**:4062-4066.
- Castrop H (2010) Genetically modified mice-successes and failures of a widely used technology. *Pflugers Arch* **459**:557-567.
- Cesselin F, Benoliel JJ, Bourgoin S, Collin E, Phol M and Hamon M (1999) Spinal mechanisms of opioid analgesia, in *Opioids in pain control : basic and clinical aspects* (Stein C ed) pp 70-95, Cambridge University Press, Cambridge, UK.
- Chakrabarti S, Prather PL, Yu L, Law PY and Loh HH (1995) Expression of the μ -opioid receptor in CHO cells: ability of μ -opioid ligands to promote alpha-azidoanilido[32 P]GTP labeling of multiple G protein α subunits. *J Neurochem* **64**:2534-2543.
- Chalecka-Franaszek E, Weems HB, Crowder AT, Cox BM and Cote TE (2000) Immunoprecipitation of high-affinity, guanine nucleotide-sensitive, solubilized μ -opioid receptors from rat brain: coimmunoprecipitation of the G proteins $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i3}$. *J Neurochem* **74**:1068-1078.
- Chan JS, Chiu TT and Wong YH (1995) Activation of type II adenylyl cyclase by the cloned μ -opioid receptor: coupling to multiple G proteins. *J Neurochem* **65**:2682-2689.
- Chieng B and Christie MJ (1994a) Hyperpolarization by opioids acting on m-receptors of a sub-population of rat periaqueductal gray neurones *in vitro*. *Br J Pharmacol* **113**:121-128.
- Chieng B and Christie MJ (1994b) Inhibition by opioids acting on μ -receptors of GABAergic and glutamatergic postsynaptic potentials in single rat periaqueductal gray neurones *in vitro*. *Br J Pharmacol* **113**:303-309.

- Christie MJ (2008) Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *Br J Pharmacol* **154**:384-396.
- Chung KY, Rasmussen SG, Liu T, Li S, DeVree BT, Chae PS, Calinski D, Kobilka BK, Woods VL, Jr. and Sunahara RK (2011) Conformational changes in the G protein G_s induced by the β_2 adrenergic receptor. *Nature* **477**:611-615.
- Clark MJ, Furman CA, Gilson TD and Traynor JR (2006) Comparison of the relative efficacy and potency of μ -opioid agonists to activate G $\alpha_{i/o}$ proteins containing a pertussis toxin-insensitive mutation. *J Pharmacol Exp Ther* **317**:858.
- Clark MJ, Harrison C, Zhong H, Neubig RR and Traynor JR (2003) Endogenous RGS protein action modulates μ -opioid signaling through G α_o . Effects on adenylyl cyclase, extracellular signal-regulated kinases, and intracellular calcium pathways. *J Biol Chem* **278**:9418-9425.
- Clark MJ, Lamberts JT and Traynor J (2011) *G $\alpha_{i/o}$ -mediated release of internal calcium stores in C6 glioma cells stably expressing the μ opioid receptor: positive modulation by endogenous RGS proteins*. Unpublished manuscript. University of Michigan, Ann Arbor, MI.
- Clark MJ, Linderman JJ and Traynor JR (2008) Endogenous regulators of G protein signaling differentially modulate full and partial μ -opioid agonists at adenylyl cyclase as predicted by a collision coupling model. *Mol Pharmacol* **73**:1538-1548.
- Clark MJ, Neubig RR and Traynor JR (2004) Endogenous regulator of G protein signaling proteins suppress G α_o -dependent, μ -opioid agonist-mediated adenylyl cyclase supersensitization. *J Pharmacol Exp Ther* **310**:215-222.
- Clark MJ and Traynor JR (2004) Assays for G-protein-coupled receptor signaling using RGS-insensitive G α subunits. *Methods Enzymol* **389**:155-169.
- Clark MJ and Traynor JR (2005) Endogenous regulator of G protein signaling proteins reduce μ -opioid receptor desensitization and down-regulation and adenylyl cyclase tolerance in C6 cells. *J Pharmacol Exp Ther* **312**:809-815.
- Clark MJ and Traynor JR (2006) Mediation of adenylyl cyclase sensitization by PTX-insensitive G α_{oA} , G α_{i1} , G α_{i2} or G α_{i3} . *J Neurochem* **99**:1494-1504.
- Cochin J and Kornetsky C (1964) Development and loss of tolerance to morphine in the rat after single and multiple injections. *J Pharmacol Exp Ther* **145**:1-10.
- Codina J, Hildebrandt J, Iyengar R, Birnbaumer L, Sekura RD and Manclark CR (1983) Pertussis toxin substrate, the putative N_i component of adenylyl cyclases, is an α β heterodimer regulated by guanine nucleotide and magnesium. *Proc Natl Acad Sci U S A* **80**:4276-4280.

- Connor M and Christie MD (1999) Opioid receptor signalling mechanisms. *Clin Exp Pharmacol Physiol* **26**:493-499.
- Connor M, Schuller A, Pintar JE and Christie MJ (1999) μ -Opioid receptor modulation of calcium channel current in periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. *Br J Pharmacol* **126**:1553-1558.
- Contet C, Filliol D, Matifas A and Kieffer BL (2008) Morphine-induced analgesic tolerance, locomotor sensitization and physical dependence do not require modification of μ opioid receptor, cdk5 and adenylate cyclase activity. *Neuropharmacology* **54**:475-486.
- Crain SM and Shen K (2000) Enhanced analgesic potency and reduced tolerance of morphine in 129/SvEv mice: evidence for a deficiency in GM1 ganglioside-regulated excitatory opioid receptor functions. *Brain Res* **856**:227-235.
- D'Amour FE and Smith DL (1941) A method for determining loss of pain sensation. *J Pharmacol Exp Ther* **72**:74-79.
- De Vries L, Zheng B, Fischer T, Elenko E and Farquhar MG (2000) The regulator of G protein signaling family. *Annu Rev Pharmacol Toxicol* **40**:235-271.
- Dhawan BN, Cesselin F, Raghbir R, Reisine T, Bradley PB, Portoghese PS and Hamon M (1996) International Union of Pharmacology. XII. Classification of opioid receptors. *J Pharmacol Exp Ther* **48**:567-592.
- DiBello PR, Garrison TR, Apanovitch DM, Hoffman G, Shuey DJ, Mason K, Cockett MI and Dohlman HG (1998) Selective uncoupling of RGS action by a single point mutation in the G protein α -subunit. *J Biol Chem* **273**:5780-5784.
- Divin MF, Holden Ko MC and Traynor JR (2008) Comparison of the opioid receptor antagonist properties of naltrexone and 6 β -naltrexol in morphine-naive and morphine-dependent mice. *Eur J Pharmacol* **583**:48-55.
- Duan SZ, Christie M, Milstone DS and Mortensen RM (2007) G_o but not G_{i2} or G_{i3} is required for muscarinic regulation of heart rate and heart rate variability in mice. *Biochem Biophys Res Commun* **357**:139-143.
- Dykstra LA, Preston KL and Bigelow GE (1997) Discriminative stimulus and subjective effects of opioids with μ and κ activity: data from laboratory animals and human subjects. *Psychopharmacology (Berl)* **130**:14-27.
- Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, Keith D, Jr., Polakiewicz R and Evans CJ (2003) Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. *J Neurosci* **23**:8360-8369.

- Elliott J, Guo L and Traynor JR (1997) Tolerance to μ -opioid agonists in human neuroblastoma SH-SY5Y cells as determined by changes in guanosine-5'-O-(3-[³⁵S]-thio)triphosphate binding. *Br J Pharmacol* **121**:1422-1428.
- Evans CJ, Keith DE, Morrison H, Magendzo K and Edwards RH (1992) Cloning of a δ opioid receptor by functional expression. *Science* **258**:1952-1955.
- Fabian G, Bozo B, Szikszay M, Horvath G, Coscia CJ and Szucs M (2002) Chronic morphine-induced changes in μ -opioid receptors and G proteins of different subcellular loci in rat brain. *J Pharmacol Exp Ther* **302**:774-780.
- Fang FG, Fields HL and Lee NM (1986) Action at the μ receptor is sufficient to explain the supraspinal analgesic effect of opiates. *J Pharmacol Exp Ther* **238**:1039-1044.
- Fang FG, Haws CM, Drasner K, Williamson A and Fields HL (1989) Opioid peptides (DAGO-enkephalin, dynorphin A(1-13), BAM 22P) microinjected into the rat brainstem: comparison of their antinociceptive effect and their effect on neuronal firing in the rostral ventromedial medulla. *Brain Res* **501**:116-128.
- Farrell M (1994) Opiate withdrawal. *Addiction* **89**:1471-1475.
- Fields H (2004) State-dependent opioid control of pain. *Nat Rev Neurosci* **5**:565-575.
- Fong HK, Yoshimoto KK, Eversole-Cire P and Simon MI (1988) Identification of a GTP-binding protein α subunit that lacks an apparent ADP-ribosylation site for pertussis toxin. *Proc Natl Acad Sci U S A* **85**:3066-3070.
- Fu Y, Huang X, Zhong H, Mortensen RM, D'Alecy LG and Neubig RR (2006) Endogenous RGS proteins and G α subtypes differentially control muscarinic and adenosine-mediated chronotropic effects. *Circ Res* **98**:659-666.
- Fu Y, Zhong H, Nanamori M, Mortensen RM, Huang X, Lan K and Neubig RR (2004) RGS-insensitive G-protein mutations to study the role of endogenous RGS proteins. *Methods Enzymol* **389**:229-243.
- Fyfe LW, Cleary DR, Macey TA, Morgan MM and Ingram SL (2010) Tolerance to the antinociceptive effect of morphine in the absence of short-term presynaptic desensitization in rat periaqueductal gray neurons. *J Pharmacol Exp Ther* **335**:674-680.
- Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ and Caron MG (2004) Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci* **27**:107-144.
- Garnier M, Zaratini PF, Ficalora G, Valente M, Fontanella L, Rhee MH, Blumer KJ and Scheideler MA (2003) Up-regulation of regulator of G protein signaling 4 expression in a model of neuropathic pain and insensitivity to morphine. *J Pharmacol Exp Ther* **304**:1299-1306.

- Garzon J, de Antonio I and Sanchez-Blazquez P (2000) *In vivo* modulation of G proteins and opioid receptor function by antisense oligodeoxynucleotides. *Methods Enzymol* **314**:3-20.
- Garzon J, Garcia-Espana A and Sanchez-Blazquez P (1997a) Opioids binding μ and δ receptors exhibit diverse efficacy in the activation of G_{i2} and $G_{x/z}$ transducer proteins in mouse periaqueductal gray matter. *J Pharmacol Exp Ther* **281**:549-557.
- Garzon J, Lopez-Fando A and Sanchez-Blazquez P (2003) The R7 subfamily of RGS proteins assists tachyphylaxis and acute tolerance at μ -opioid receptors. *Neuropsychopharmacology* **28**:1983-1990.
- Garzon J, Martinez-Pena Y and Sanchez-Blazquez P (1994) Dissimilar efficacy of opioids to produce μ -mediated analgesia: role of $G_{x/z}$ and G_{i2} transducer proteins. *Life Sci* **55**:PL205-212.
- Garzon J, Martinez-Pena Y and Sanchez-Blazquez P (1997b) $G_{x/z}$ is regulated by μ but not δ opioid receptors in the stimulation of the low K_m GTPase activity in mouse periaqueductal grey matter. *Eur J Neurosci* **9**:1194-1200.
- Garzon J, Rodriguez-Diaz M, DeAntonio I, DeFelipe J, Rodriguez JR and Sanchez-Blazquez P (1999) Myr^+ - G_{i2} α and G_o α subunits restore the efficacy of opioids, clonidine and neurotensin giving rise to antinociception in G-protein knock-down mice. *Neuropharmacology* **38**:1861-1873.
- Garzon J, Rodriguez-Diaz M, Lopez-Fando A and Sanchez-Blazquez P (2001) RGS9 proteins facilitate acute tolerance to μ -opioid effects. *Eur J Neurosci* **13**:801-811.
- Garzon J, Rodriguez-Munoz M, Lopez-Fando A, Garcia-Espana A and Sanchez-Blazquez P (2004) RGSZ1 and GAIP regulate μ - but not δ -opioid receptors in mouse CNS: role in tachyphylaxis and acute tolerance. *Neuropsychopharmacology* **29**:1091-1104.
- Garzon J, Rodriguez-Munoz M, Lopez-Fando A and Sanchez-Blazquez P (2005) The RGSZ2 protein exists in a complex with μ -opioid receptors and regulates the desensitizing capacity of G_z proteins. *Neuropsychopharmacology* **30**:1632-1648.
- Garzon J and Sanchez-Blazquez P (2001) Administration of myr^+ - G_{i2} α subunits prevents acute tolerance (tachyphylaxis) to μ -opioid effects in mice. *Neuropharmacology* **40**:560-569.
- Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, Eisch AJ, Yanagisawa M, Nestler EJ and DiLeone RJ (2003) Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. *J Neurosci* **23**:3106-3111.

- Georgoussi Z, Leontiadis L, Mazarakou G, Merkouris M, Hyde K and Hamm H (2006) Selective interactions between G protein subunits and RGS4 with the C-terminal domains of the μ - and δ -opioid receptors regulate opioid receptor signaling. *Cell Signal* **18**:771-782.
- Gierschik P, Milligan G, Pines M, Goldsmith P, Codina J, Klee W and Spiegel A (1986) Use of specific antibodies to quantitate the guanine nucleotide-binding protein G_o in brain. *Proc Natl Acad Sci U S A* **83**:2258-2262.
- Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**:615-649.
- Gintzler AR and Chakrabarti S (2006) Post-opioid receptor adaptations to chronic morphine; altered functionality and associations of signaling molecules. *Life Sci* **79**:717-722.
- Goldenstein BL, Nelson BW, Xu K, Luger EJ, Pribula JA, Wald JM, O'Shea LA, Weinshenker D, Charbeneau RA, Huang X, Neubig RR and Doze VA (2009) Regulator of G protein signaling protein suppression of G α_o protein-mediated α_{2A} adrenergic receptor inhibition of mouse hippocampal CA3 epileptiform activity. *Mol Pharmacol* **75**:1222-1230.
- Goldstein A, Lowney LI and Pal BK (1971) Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc Natl Acad Sci U S A* **68**:1742-1747.
- Goldstein A, Tachibana S, Lowney LI, Hunkapiller M and Hood L (1979) Dynorphin-(1-13), an extraordinarily potent opioid peptide. *Proc Natl Acad Sci U S A* **76**:6666-6670.
- Grillet N, Pattyn A, Contet C, Kieffer BL, Goridis C and Brunet JF (2005) Generation and characterization of *Rgs4* mutant mice. *Mol Cell Biol* **25**:4221-4228.
- Gu H, Marth JD, Orban PC, Mossmann H and Rajewsky K (1994) Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* **265**:103-106.
- Gustafsson H, Afrah AW and Stiller CO (2001) Morphine-induced *in vivo* release of spinal cholecystinin is mediated by δ -opioid receptors--effect of peripheral axotomy. *J Neurochem* **78**:55-63.
- Han MH, Renthal W, Ring RH, Rahman Z, Psifogeorgou K, Howland D, Birnbaum S, Young K, Neve R, Nestler EJ and Zachariou V (2010) Brain region specific actions of regulator of G protein signaling 4 oppose morphine reward and dependence but promote analgesia. *Biol Psychiatry* **67**:761-769.

- Handa BK, Land AC, Lord JA, Morgan BA, Rance MJ and Smith CF (1981) Analogues of β -LPH61-64 possessing selective agonist activity at μ -opiate receptors. *Eur J Pharmacol* **70**:531-540.
- Hao S, Liu S, Zheng X, Zheng W, Ouyang H, Mata M and Fink DJ (2011) The role of TNF α in the periaqueductal gray during naloxone-precipitated morphine withdrawal in rats. *Neuropsychopharmacology* **36**:664-676.
- Harris JD (2008) Management of expected and unexpected opioid-related side effects. *Clin J Pain* **24 Suppl 10**:S8-S13.
- Hefner K, Whittle N, Juhasz J, Norcross M, Karlsson RM, Saksida LM, Bussey TJ, Singewald N and Holmes A (2008) Impaired fear extinction learning and cortico-amygdala circuit abnormalities in a common genetic mouse strain. *J Neurosci* **28**:8074-8085.
- Heinke B, Gingl E and Sandkuhler J (2011) Multiple targets of μ -opioid receptor-mediated presynaptic inhibition at primary afferent A δ - and C-fibers. *J Neurosci* **31**:1313-1322.
- Heinricher MM and Morgan MM (1999) Supraspinal mechanisms of opioid analgesia, in *Opioids in pain control : basic and clinical aspects* (Stein C ed) pp 46-69, Cambridge University Press, Cambridge, UK.
- Heinricher MM, Morgan MM and Fields HL (1992) Direct and indirect actions of morphine on medullary neurons that modulate nociception. *Neuroscience* **48**:533-543.
- Heinricher MM, Morgan MM, Tortorici V and Fields HL (1994) Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. *Neuroscience* **63**:279-288.
- Heinricher MM and Tortorici V (1994) Interference with GABA transmission in the rostral ventromedial medulla: disinhibition of off-cells as a central mechanism in nociceptive modulation. *Neuroscience* **63**:533-546.
- Hendry IA, Kelleher KL, Bartlett SE, Leck KJ, Reynolds AJ, Heydon K, Mellick A, Megirian D and Matthaei KI (2000) Hypertolerance to morphine in G $_z$ α -deficient mice. *Brain Res* **870**:10-19.
- Hescheler J, Rosenthal W, Trautwein W and Schultz G (1987) The GTP-binding protein, G $_o$, regulates neuronal calcium channels. *Nature* **325**:445-447.
- Hinton DR, Blanks JC, Fong HK, Casey PJ, Hildebrandt E and Simons MI (1990) Novel localization of a G protein, G $_z$ α , in neurons of brain and retina. *J Neurosci* **10**:2763-2770.

- Hollinger S and Hepler JR (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev* **54**:527-559.
- Huang X, Fu Y, Charbeneau RA, Saunders TL, Taylor DK, Hankenson KD, Russell MW, D'Alecy LG and Neubig RR (2006) Pleiotropic phenotype of a genomic knock-in of an RGS-insensitive G184S *Gnai2* allele. *Mol Cell Biol* **26**:6870-6879.
- Huff RM, Axton JM and Neer EJ (1985) Physical and immunological characterization of a guanine nucleotide-binding protein purified from bovine cerebral cortex. *J Biol Chem* **260**:10864-10871.
- Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA and Morris HR (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* **258**:577-580.
- Hwang JI, Choi S, Fraser ID, Chang MS and Simon MI (2005) Silencing the expression of multiple G β -subunits eliminates signaling mediated by all four families of G proteins. *Proc Natl Acad Sci U S A* **102**:9493-9498.
- Ingram SL, Macey TA, Fossum EN and Morgan MM (2008) Tolerance to repeated morphine administration is associated with increased potency of opioid agonists. *Neuropsychopharmacology* **33**:2494-2504.
- Irwin S, Houde RW, Bennett DR, Hendershot LC and SeEVERS MH (1951) The effects of morphine methadone and meperidine on some reflex responses of spinal animals to nociceptive stimulation. *J Pharmacol Exp Ther* **101**:132-143.
- Jiang M and Bajpayee NS (2009) Molecular mechanisms of G_o signaling. *Neurosignals* **17**:23-41.
- Jiang M, Gold MS, Boulay G, Spicher K, Peyton M, Brabet P, Srinivasan Y, Rudolph U, Ellison G and Birnbaumer L (1998) Multiple neurological abnormalities in mice deficient in the G protein G_o. *Proc Natl Acad Sci U S A* **95**:3269-3274.
- Jiang M, Spicher K, Boulay G, Martin-Requero A, Dye CA, Rudolph U and Birnbaumer L (2002) Mouse gene knockout and knockin strategies in application to α subunits of G_i/G_o family of G proteins. *Methods Enzymol* **344**:277-298.
- Jiang M, Spicher K, Boulay G, Wang Y and Birnbaumer L (2001) Most central nervous system D2 dopamine receptors are coupled to their effectors by G_o. *Proc Natl Acad Sci U S A* **98**:3577-3582.
- Julius D and Basbaum AI (2001) Molecular mechanisms of nociception. *Nature* **413**:203-210.
- Karim F and Roerig SC (2000) Differential effects of antisense oligodeoxynucleotides directed against G_z α and G_o α on antinociception produced by spinal opioid and α_2 adrenergic receptor agonists. *Pain* **87**:181-191.

- Katada T, Amano T and Ui M (1982) Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. *J Biol Chem* **257**:3739-3746.
- Kaur K, Kehrl JM, Charbeneau RA and Neubig RR (2011) RGS-insensitive G α subunits: probes of G α subtype-selective signaling and physiological functions of RGS proteins. *Methods Mol Biol* **756**:75-98.
- Kehrl JM, Dalton HM, Kohut KT, Huang X, Charbeneau RA and Neubig RR (2012) *Seizure sensitivity and sudden death in mice with enhanced G $_o$ signaling reveal a new Chr17 epilepsy modifier locus*. Unpublished manuscript. University of Michigan, Ann Arbor, MI.
- Kest B, Hopkins E, Palmese CA, Adler M and Mogil JS (2002a) Genetic variation in morphine analgesic tolerance: a survey of 11 inbred mouse strains. *Pharmacol Biochem Behav* **73**:821-828.
- Kest B, Hopkins E, Palmese CA, Chen ZP, Mogil JS and Pintar JE (2001) Morphine tolerance and dependence in nociceptin/orphanin FQ transgenic knock-out mice. *Neuroscience* **104**:217-222.
- Kest B, Lee CE, McLemore GL and Inturrisi CE (1996) An antisense oligodeoxynucleotide to the δ opioid receptor (DOR-1) inhibits morphine tolerance and acute dependence in mice. *Brain Res Bull* **39**:185-188.
- Kest B, Palmese CA, Hopkins E, Adler M, Juni A and Mogil JS (2002b) Naloxone-precipitated withdrawal jumping in 11 inbred mouse strains: evidence for common genetic mechanisms in acute and chronic morphine physical dependence. *Neuroscience* **115**:463-469.
- Kest B, Smith SB, Schorscher-Petcu A, Austin JS, Ritchie J, Klein G, Rossi GC, Fortin A and Mogil JS (2009) *Gnao1* (G α_o protein) is a likely genetic contributor to variation in physical dependence on opioids in mice. *Neuroscience* **162**:1255-1264.
- Kieffer BL (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell Mol Neurobiol* **15**:615-635.
- Kieffer BL (1999) Opioids: first lessons from knockout mice. *Trends Pharmacol Sci* **20**:19-26.
- Kieffer BL, Befort K, Gaveriaux-Ruff C and Hirth CG (1992) The δ -opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U S A* **89**:12048-12052.
- Kieffer BL and Evans CJ (2009) Opioid receptors: from binding sites to visible molecules *in vivo*. *Neuropharmacology* **56 Suppl 1**:205-212.

- Kimble AJ, Bosch DE, Giguère PM and Siderovski DP (2011) Regulators of G-protein signaling and their G α substrates: promises and challenges in their use as drug discovery targets. *Pharmacol Rev* **63**:728-749.
- Kindler LL, Sibille KT, Glover TL, Staud R, Riley JL, 3rd and Fillingim RB (2011) Drug response profiles to experimental pain are opioid and pain modality specific. *J Pain* **12**:340-351.
- Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lubbert H and Bujard H (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* **93**:10933-10938.
- Kolesnikov Y, Jain S, Wilson R and Pasternak GW (1998) Lack of morphine and enkephalin tolerance in 129/SvEv mice: evidence for a NMDA receptor defect. *J Pharmacol Exp Ther* **284**:455-459.
- Kornetsky C and Bain G (1968) Morphine: single-dose tolerance. *Science* **162**:1011-1012.
- Kuhn R, Streif S and Wurst W (2007) RNA interference in mice. *Handb Exp Pharmacol*:149-176.
- Lamberts JT, Jutkiewicz EM, Mortensen RM and Traynor JR (2011) μ -Opioid receptor coupling to G α_o plays an important role in opioid antinociception. *Neuropsychopharmacology* **36**:2041-2053.
- Lan KL, Sarvazyan NA, Taussig R, Mackenzie RG, DiBello PR, Dohlman HG and Neubig RR (1998) A point mutation in G α_o and G α_{i1} blocks interaction with regulator of G protein signaling proteins. *J Biol Chem* **273**:12794-12797.
- Lan KL, Zhong H, Nanamori M and Neubig RR (2000) Rapid kinetics of regulator of G-protein signaling (RGS)-mediated G α_i and G α_o deactivation. G α specificity of RGS4 and RGS7. *J Biol Chem* **275**:33497-33503.
- Lariviere WR, Chesler EJ and Mogil JS (2001) Transgenic studies of pain and analgesia: mutation or background genotype? *J Pharmacol Exp Ther* **297**:467-473.
- Laugwitz KL, Offermanns S, Spicher K and Schultz G (1993) μ and δ Opioid receptors differentially couple to G protein subtypes in membranes of human neuroblastoma SH-SY5Y cells. *Neuron* **10**:233-242.
- Law PY, Wong YH and Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40**:389-430.
- Le Bars D, Gozariu M and Cadden SW (2001) Animal models of nociception. *Pharmacol Rev* **53**:597-652.

- Leck KJ, Bartlett SE, Smith MT, Megirian D, Holgate J, Powell KL, Matthaai KI and Hendry IA (2004) Deletion of guanine nucleotide binding protein α_z subunit in mice induces a gene dose dependent tolerance to morphine. *Neuropharmacology* **46**:836-846.
- Leo S, Straetemans R, D'Hooge R and Meert T (2008) Differences in nociceptive behavioral performance between C57BL/6J, 129S6/SvEv, B6 129 F1 and NMRI mice. *Behav Brain Res* **190**:233-242.
- Leontiadis LJ, Papakonstantinou MP and Georgoussi Z (2009) Regulator of G protein signaling 4 confers selectivity to specific G proteins to modulate μ - and δ -opioid receptor signaling. *Cell Signal* **21**:1218-1228.
- Lester PA and Traynor JR (2006) Comparison of the *in vitro* efficacy of μ , δ , κ and ORL1 receptor agonists and non-selective opioid agonists in dog brain membranes. *Brain Res* **1073-1074**:290-296.
- Loeser JD and Treede RD (2008) The Kyoto protocol of IASP basic pain terminology. *Pain* **137**:473-477.
- Lord JA, Waterfield AA, Hughes J and Kosterlitz HW (1977) Endogenous opioid peptides: multiple agonists and receptors. *Nature* **267**:495-499.
- Macht DI, Herman NB and Levy CS (1915) A quantitative study of cutaneous analgesia produced by various opium alkaloids. *Proc Natl Acad Sci U S A* **1**:582-585.
- Madia PA, Navani DM and Yoburn BC (2012) [35 S]GTP γ S binding and opioid tolerance and efficacy in mouse spinal cord. *Pharmacol Biochem Behav* **101**:155-165.
- Maher CE, Eisenach JC, Pan HL, Xiao R and Childers SR (2001) Chronic intrathecal morphine administration produces homologous μ receptor/G-protein desensitization specifically in spinal cord. *Brain Res* **895**:1-8.
- Mains RE, Eipper BA and Ling N (1977) Common precursor to corticotropins and endorphins. *Proc Natl Acad Sci U S A* **74**:3014-3018.
- Maldonado R, Negus S and Koob GF (1992) Precipitation of morphine withdrawal syndrome in rats by administration of μ -, δ - and κ -selective opioid antagonists. *Neuropharmacology* **31**:1231-1241.
- Mansour A, Fox CA, Akil H and Watson SJ (1995) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci* **18**:22-29.
- Mansour A, Khachaturian H, Lewis ME, Akil H and Watson SJ (1988) Anatomy of CNS opioid receptors. *Trends Neurosci* **11**:308-314.

- Martin M, Matifas A, Maldonado R and Kieffer BL (2003) Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct μ , δ and κ tones. *Eur J Neurosci* **17**:701-708.
- Martin WR, Eades CG, Thompson JA, Huppler RE and Gilbert PE (1976) The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther* **197**:517-532.
- Massotte D, Brillet K, Kieffer B and Milligan G (2002) Agonists activate G₁₁ alpha or G₁₂ alpha fused to the human μ opioid receptor differently. *J Neurochem* **81**:1372-1382.
- Matsuoka M, Itoh H, Kozasa T and Kaziro Y (1988) Sequence analysis of cDNA and genomic DNA for a putative pertussis toxin-insensitive guanine nucleotide-binding regulatory protein α subunit. *Proc Natl Acad Sci U S A* **85**:5384-5388.
- Matthaei KI (2007) Genetically manipulated mice: a powerful tool with unsuspected caveats. *J Physiol* **582**:481-488.
- Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dollé P, Tzavara E, Hanoune J, Roques BP and Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ -opioid-receptor gene. *Nature* **383**:819-823.
- McNally GP and Akil H (2002) Role of corticotropin-releasing hormone in the amygdala and bed nucleus of the stria terminalis in the behavioral, pain modulatory, and endocrine consequences of opiate withdrawal. *Neuroscience* **112**:605-617.
- McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, Krasel C, Dewey WL, Bailey CP, Rosethorne EM, Charlton SJ, Henderson G and Kelly E (2010) μ -Opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol Pharmacol* **78**:756-766.
- Melnikova I (2010) Pain market. *Nat Rev Drug Discov* **9**:589-590.
- Mendelson J, Flower K, Pletcher MJ and Galloway GP (2008) Addiction to prescription opioids: characteristics of the emerging epidemic and treatment with buprenorphine. *Exp Clin Psychopharmacol* **16**:435-441.
- Millan MJ (2002) Descending control of pain. *Prog Neurobiol* **66**:355-474.
- Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147 Suppl** S46-55.
- Mogil JS and Pasternak GW (2001) The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol Rev* **53**:381-415.

- Moises HC, Rusin KI and Macdonald RL (1994) μ -Opioid receptor-mediated reduction of neuronal calcium current occurs *via* a G_o -type GTP-binding protein. *J Neurosci* **14**:3842-3851.
- Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, Caput D, Vassart G and Meunier JC (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett* **341**:33-38.
- Moreau JL and Fields HL (1986) Evidence for GABA involvement in midbrain control of medullary neurons that modulate nociceptive transmission. *Brain Res* **397**:37-46.
- Morgan MM and Christie MJ (2011) Analysis of opioid efficacy, tolerance, addiction and dependence from cell culture to human. *Br J Pharmacol* **164**:1322-1334.
- Morgan MM, Clayton CC and Boyer-Quick JS (2005) Differential susceptibility of the PAG and RVM to tolerance to the antinociceptive effect of morphine in the rat. *Pain* **113**:91-98.
- Morgan MM, Clayton CC and Lane DA (2003) Behavioral evidence linking opioid-sensitive GABAergic neurons in the ventrolateral periaqueductal gray to morphine tolerance. *Neuroscience* **118**:227-232.
- Mortensen RM, Conner DA, Chao S, Geisterfer-Lowrance AA and Seidman JG (1992) Production of homozygous mutant ES cells with a single targeting construct. *Mol Cell Biol* **12**:2391-2395.
- Mosberg HI, Hurst R, Hrubby VJ, Gee K, Yamamura HI, Galligan JJ and Burks TF (1983) Bis-penicillamine enkephalins possess highly improved specificity toward δ opioid receptors. *Proc Natl Acad Sci U S A* **80**:5871-5874.
- Murthy KS and Makhlof GM (1996) Opioid μ , δ , and κ receptor-induced activation of phospholipase C- β 3 and inhibition of adenylyl cyclase is mediated by G_{i2} and G_o in smooth muscle. *Mol Pharmacol* **50**:870-877.
- Narita M, Shibasaki M, Mizuo K and Suzuki T (2003) Changes in G-protein activity mediated through the stimulation of dopamine and GABA_B receptors in the mesolimbic dopaminergic system of morphine-sensitized mice. *Addict Biol* **8**:319-325.
- Neer EJ, Lok JM and Wolf LG (1984) Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylyl cyclase. *J Biol Chem* **259**:14222-14229.
- Nestler EJ, Erdos JJ, Terwilliger R, Duman RS and Tallman JF (1989) Regulation of G proteins by chronic morphine in the rat locus coeruleus. *Brain Res* **476**:230-239.

- Neubig RR and Siderovski DP (2002) Regulators of G-protein signalling as new central nervous system drug targets. *Nat Rev Drug Discov* **1**:187-197.
- Nitsche JF, Schuller AG, King MA, Zengh M, Pasternak GW and Pintar JE (2002) Genetic dissociation of opiate tolerance and physical dependence in δ -opioid receptor-1 and preproenkephalin knock-out mice. *J Neurosci* **22**:10906-10913.
- O'Callaghan JP and Holtzman SG (1975) Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. *J Pharmacol Exp Ther* **192**:497-505.
- Papachatzaki MM, Antal Z, Terzi D, Szucs P, Zachariou V and Antal M (2011) RGS9-2 modulates nociceptive behaviour and opioid-mediated synaptic transmission in the spinal dorsal horn. *Neurosci Lett* **501**:31-34.
- Peckham EM and Traynor JR (2006) Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague-Dawley rats. *J Pharmacol Exp Ther* **316**:1195-1201.
- Pert CB and Snyder SH (1973) Opiate receptor: demonstration in nervous tissue. *Science* **179**:1011-1014.
- Potenza MN, Gold SJ, Roby-Shemkowitz A, Lerner MR and Nestler EJ (1999) Effects of regulators of G protein-signaling proteins on the functional response of the μ -opioid receptor in a melanophore-based assay. *J Pharmacol Exp Ther* **291**:482-491.
- Psifogeorgou K, Papakosta P, Russo SJ, Neve RL, Kardassis D, Gold SJ and Zachariou V (2007) RGS9-2 is a negative modulator of μ -opioid receptor function. *J Neurochem* **103**:617-625.
- Psifogeorgou K, Terzi D, Papachatzaki MM, Varidaki A, Ferguson D, Gold SJ and Zachariou V (2011) A unique role of RGS9-2 in the striatum as a positive or negative regulator of opiate analgesia. *J Neurosci* **31**:5617-5624.
- Raffa RB, Goode TL, Martinez RP and Jacoby HI (1996) A $G_{i2\alpha}$ antisense oligonucleotide differentiates morphine antinociception, constipation and acute dependence in mice. *Life Sci* **58**:PL 73-76.
- Raffa RB, Martinez RP and Connelly CD (1994) G-protein antisense oligodeoxyribonucleotides and μ -opioid supraspinal antinociception. *Eur J Pharmacol* **258**:R5-7.
- Reichling DB, Kwiat GC and Basbaum AI (1988) Anatomy, physiology and pharmacology of the periaqueductal gray contribution to antinociceptive controls. *Prog Brain Res* **77**:31-46.

- Reisine T and Bell GI (1993) Molecular biology of opioid receptors. *Trends Neurosci* **16**:506-510.
- Roman DL and Traynor JR (2011) Regulators of G protein signaling (RGS) proteins as drug targets: modulating G-protein-coupled receptor (GPCR) signal transduction. *J Med Chem* **54**:7433-7440.
- Romer D, Buscher H, Hill RC, Maurer R, Petcher TJ, Welle HB, Bakel HC and Akkerman AM (1980) Bremazocine: a potent, long-acting opiate κ -agonist. *Life Sci* **27**:971-978.
- Rosenblum A, Marsch LA, Joseph H and Portenoy RK (2008) Opioids and the treatment of chronic pain: controversies, current status, and future directions. *Exp Clin Psychopharmacol* **16**:405-416.
- Ross EM and Wilkie TM (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* **69**:795-827.
- Rossi GC, Standifer KM and Pasternak GW (1995) Differential blockade of morphine and morphine-6 β -glucuronide analgesia by antisense oligodeoxynucleotides directed against MOR-1 and G-protein α subunits in rats. *Neurosci Lett* **198**:99-102.
- Sanchez-Blazquez P, Garcia-Espana A and Garzon J (1995) *In vivo* injection of antisense oligodeoxynucleotides to G α subunits and supraspinal analgesia evoked by μ and δ opioid agonists. *J Pharmacol Exp Ther* **275**:1590-1596.
- Sanchez-Blazquez P and Garzon J (1994) Antibodies directed against alpha subunits of G_{i1} , $G_{x/z}$, G_o and G_s transducer proteins reduced the morphine withdrawal syndrome in mice. *Life Sci* **55**:PL445-450.
- Sanchez-Blazquez P, Gomez-Serranillos P and Garzon J (2001) Agonists determine the pattern of G-protein activation in μ -opioid receptor-mediated supraspinal analgesia. *Brain Res Bull* **54**:229-235.
- Sanchez-Blazquez P, Juarros JL, Martinez-Pena Y, Castro MA and Garzon J (1993) $G_{x/z}$ and G_{i2} transducer proteins on μ/δ opioid-mediated supraspinal antinociception. *Life Sci* **53**:PL381-386.
- Sanchez-Blazquez P, Rodriguez-Diaz M, DeAntonio I and Garzon J (1999) Endomorphin-1 and endomorphin-2 show differences in their activation of μ opioid receptor-regulated G proteins in supraspinal antinociception in mice. *J Pharmacol Exp Ther* **291**:12-18.

- Sánchez-Blázquez P, Rodríguez-Muñoz M, Montero C and Garzón J (2005) RGS-RZ and RGS9-2 proteins control μ -opioid receptor desensitisation in CNS: the role of activated $G\alpha_z$ subunits. *Neuropharmacology* **48**:134-150.
- Satoh M and Minami M (1995) Molecular pharmacology of the opioid receptors. *Pharmacol Ther* **68**:343-364.
- Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, Kieffer BL and Basbaum AI (2009) Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* **137**:1148-1159.
- Scoto GM, Arico G, Ronsisvalle S and Parenti C (2007) Blockade of the nociceptin/orphanin FQ/NOP receptor system in the rat ventrolateral periaqueductal gray potentiates DAMGO analgesia. *Peptides* **28**:1441-1446.
- Selley DE, Liu Q and Childers SR (1998) Signal transduction correlates of μ opioid agonist intrinsic efficacy: receptor-stimulated [35 S]GTP γ S binding in mMOR-CHO cells and rat thalamus. *J Pharmacol Exp Ther* **285**:496-505.
- Selley DE, Nestler EJ, Breivogel CS and Childers SR (1997a) Opioid receptor-coupled G-proteins in rat locus coeruleus membranes: decrease in activity after chronic morphine treatment. *Brain Res* **746**:10-18.
- Selley DE, Sim LJ, Xiao R, Liu Q and Childers SR (1997b) μ -Opioid receptor-stimulated guanosine-5'-O-(γ -thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. *Mol Pharmacol* **51**:87-96.
- Sethakorn N, Yau DM and Dulin NO (2010) Non-canonical functions of RGS proteins. *Cell Signal* **22**:1274-1281.
- Shen J, Shah S, Hsu H and Yoburn BC (1998) The effects of antisense to $G_{i2}\alpha$ on opioid agonist potency and $G_{i2}\alpha$ protein and mRNA abundance in the mouse. *Brain Res Mol Brain Res* **59**:247-255.
- Sherrington CS (1906) Observations on the scratch-reflex in the spinal dog. *J Physiol* **34**:1-50.
- Silva AJ, Simpson EM, Takahashi JS, Lipp H, Nakanishi S, Wehner JM, Giese KP, Tully T, Abel T, Chapman PF, Fox K, Grand S, Itohara S, Lathe R, Mayford M, McNamara JO, Morris RJ, Picciotto MR, Roder H, Shin H, Slesinger PA, Storm DR, Stryker MP, Tonegawa S, Wang Y and Wolfer DP (1997) Mutant mice and neuroscience: recommendations concerning genetic background. Banbury Conference on genetic background in mice. *Neuron* **19**:755-759.

- Sim-Selley LJ, Scoggins KL, Cassidy MP, Smith LA, Dewey WL, Smith FL and Selley DE (2007) Region-dependent attenuation of μ opioid receptor-mediated G-protein activation in mouse CNS as a function of morphine tolerance. *Br J Pharmacol* **151**:1324-1333.
- Sim LJ, Selley DE, Dworkin SI and Childers SR (1996) Effects of chronic morphine administration on μ opioid receptor-stimulated [35 S]GTP γ S autoradiography in rat brain. *J Neurosci* **16**:2684-2692.
- Simon EJ, Hiller JM and Edelman I (1973) Stereospecific binding of the potent narcotic analgesic (3 H) Etorphine to rat-brain homogenate. *Proc Natl Acad Sci U S A* **70**:1947-1949.
- Sjögren B and Neubig RR (2010) Thinking outside of the "RGS box": new approaches to therapeutic targeting of regulators of G protein signaling. *Mol Pharmacol* **78**:550-557.
- Smith DJ, Perrotti JM, Crisp T, Cabral ME, Long JT and Scalzitti JM (1988) The μ opiate receptor is responsible for descending pain inhibition originating in the periaqueductal gray region of the rat brain. *Eur J Pharmacol* **156**:47-54.
- Smith PA, Selley DE, Sim-Selley LJ and Welch SP (2007) Low dose combination of morphine and Δ^9 -tetrahydrocannabinol circumvents antinociceptive tolerance and apparent desensitization of receptors. *Eur J Pharmacol* **571**:129-137.
- Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM and Molkentin JD (2001) Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* **89**:20-25.
- Sowell MO, Ye C, Ricupero DA, Hansen S, Quinn SJ, Vassilev PM and Mortensen RM (1997) Targeted inactivation of α_{i2} or α_{i3} disrupts activation of the cardiac muscarinic K^+ channel, I_K^{+ACh} , in intact cells. *Proc Natl Acad Sci U S A* **94**:7921-7926.
- Stanasila L, Lim WK, Neubig RR and Pattus F (2000) Coupling efficacy and selectivity of the human μ -opioid receptor expressed as receptor-G α fusion proteins in *Escherichia coli*. *J Neurochem* **75**:1190-1199.
- Standifer KM and Pasternak GW (1997) G proteins and opioid receptor-mediated signalling. *Cell Signal* **9**:237-248.
- Standifer KM, Rossi GC and Pasternak GW (1996) Differential blockade of opioid analgesia by antisense oligodeoxynucleotides directed against various G protein α subunits. *Mol Pharmacol* **50**:293-298.
- Stein C and Lang LJ (2009) Peripheral mechanisms of opioid analgesia. *Curr Opin Pharmacol* **9**:3-8.

- Sternweis PC and Robishaw JD (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J Biol Chem* **259**:13806-13813.
- Talbot JN, Roman DL, Clark MJ, Roof RA, Tesmer JJG, Neubig RR and Traynor JR (2010) Differential modulation of μ -opioid receptor signaling to adenylyl cyclase by regulators of G protein signaling proteins 4 or 8 and 7 in permeabilised C6 cells is $G\alpha$ subtype dependent. *J Neurochem* **112**:1026-1034.
- Terenius L (1973) Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta Pharmacol Toxicol (Copenh)* **32**:317-320.
- Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM and Nestler EJ (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* **548**:100-110.
- Tesmer JJ, Berman DM, Gilman AG and Sprang SR (1997) Structure of RGS4 bound to AlF₄-activated G_{i1} α : stabilization of the transition state for GTP hydrolysis. *Cell* **89**:251-261.
- Traynor J (2011) μ -Opioid receptors and regulators of G protein signaling (RGS) proteins: from a symposium on new concepts in μ -opioid pharmacology. *Drug Alcohol Depend* **121**:173-180.
- Traynor JR and Nahorski SR (1995) Modulation by μ -opioid agonists of guanosine-5'-O-(3-[³⁵S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **47**:848-854.
- Traynor JR and Neubig RR (2005) Regulators of G protein signaling & drugs of abuse. *Mol Interv* **5**:30-41.
- Trescot AM, Datta S, Lee M and Hansen H (2008) Opioid pharmacology. *Pain Physician* **11**:S133-153.
- Ueda H, Harada H, Nozaki M, Katada T, Ui M, Satoh M and Takagi H (1988) Reconstitution of rat brain μ opioid receptors with purified guanine nucleotide-binding regulatory proteins, G_i and G_o. *Proc Natl Acad Sci U S A* **85**:7013-7017.
- Valenzuela D, Han X, Mende U, Fankhauser C, Mashimo H, Huang P, Pfeiffer J, Neer EJ and Fishman MC (1997) G α_o is necessary for muscarinic regulation of Ca²⁺ channels in mouse heart. *Proc Natl Acad Sci U S A* **94**:1727-1732.
- Valeri P, Martinelli B, Pimpinella G and Severini C (1989) Effects of dapiprazole, clonidine and yohimbine on the development of dependence and withdrawal behaviour in mice. *Drug Alcohol Depend* **23**:73-77.

- Vaughan CW and Christie MJ (1997) Presynaptic inhibitory action of opioids on synaptic transmission in the rat periaqueductal grey *in vitro*. *J Physiol* **498** (Pt 2):463-472.
- Vaughan CW, Ingram SL, Connor MA and Christie MJ (1997) How opioids inhibit GABA-mediated neurotransmission. *Nature* **390**:611-614.
- Vigano D, Rubino T, Di Chiara G, Ascari I, Massi P and Parolaro D (2003) μ Opioid receptor signaling in morphine sensitization. *Neuroscience* **117**:921-929.
- Wang D, Raehal KM, Lin ET, Lowery JJ, Kieffer BL, Bilsky EJ and Sadee W (2004) Basal signaling activity of μ opioid receptor in mouse brain: role in narcotic dependence. *J Pharmacol Exp Ther* **308**:512-520.
- Wang HY, Friedman E, Olmstead MC and Burns LH (2005) Ultra-low-dose naloxone suppresses opioid tolerance, dependence and associated changes in μ opioid receptor-G protein coupling and G $\beta\gamma$ signaling. *Neuroscience* **135**:247-261.
- Wang Q, Liu-Chen L-YY and Traynor JR (2009) Differential modulation of μ - and δ -opioid receptor agonists by endogenous RGS4 protein in SH-SY5Y cells. *J Biol Chem* **284**:18357-18367.
- Wang Q and Traynor JR (2012) Modulation of μ -opioid receptor signaling by RGS19 in SH-SY5Y cells. *Mol Pharmacol*.
- Watkyn-Thomas FW (1912) The action of the opium alkaloids. *Biochem J* **6**:433-444.
- Way EL and Loh HH (1976) Responsivity to naloxone during morphine dependence. *Ann N Y Acad Sci* **281**:252-261.
- Westfield GH, Rasmussen SG, Su M, Dutta S, DeVree BT, Chung KY, Calinski D, Velez-Ruiz G, Oleskie AN, Pardon E, Chae PS, Liu T, Li S, Woods VL, Jr., Steyaert J, Kobilka BK, Sunahara RK and Skiniotis G (2011) Structural flexibility of the G α_s α -helical domain in the β_2 -adrenoceptor G $_s$ complex. *Proc Natl Acad Sci U S A* **108**:16086-16091.
- Wettschureck N, Moers A and Offermanns S (2004) Mouse models to study G-protein-mediated signaling. *Pharmacol Ther* **101**:75-89.
- Wettschureck N and Offermanns S (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* **85**:1159-1204.
- Wilkie TM, Gilbert DJ, Olsen AS, Chen XN, Amatruda TT, Korenberg JR, Trask BJ, de Jong P, Reed RR, Simon MI, Jenkins NA and Copeland NG (1992) Evolution of the mammalian G protein α subunit multigene family. *Nat Genet* **1**:85-91.
- Williams JT, Christie MJ and Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* **81**:299-343.

- Wong YH, Conklin BR and Bourne HR (1992) G_z -mediated hormonal inhibition of cyclic AMP accumulation. *Science* **255**:339-342.
- Woolfe G and MacDonald AD (1944) The evaluation of the analgesic action of pethidine hydrochloride (Demerol). *J Pharmacol Exp Ther* **80**:300-307.
- Worley PF, Baraban JM, Van Dop C, Neer EJ and Snyder SH (1986) G_o , a guanine nucleotide-binding protein: immunohistochemical localization in rat brain resembles distribution of second messenger systems. *Proc Natl Acad Sci U S A* **83**:4561-4565.
- Xie Z, Li Z, Guo L, Ye C, Li J, Yu X, Yang H, Wang Y, Chen C, Zhang D and Liu-Chen L-Y (2007) Regulator of G protein signaling proteins differentially modulate signaling of μ and δ opioid receptors. *Eur J Pharmacol* **565**:45-53.
- Yaksh TL, Al-Rodhan NR and Jensen TS (1988) Sites of action of opiates in production of analgesia. *Prog Brain Res* **77**:371-394.
- Yaksh TL and Rudy TA (1978) Narcotic analgesics: CNS sites and mechanisms of action as revealed by intracerebral injection techniques. *Pain* **4**:299-359.
- Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, Manning D, Poncz M, Lucki I, Blendy JA and Brass LF (2000) Loss of signaling through the G protein, G_z , results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci U S A* **97**:9984-9989.
- Yoburn BC, Gomes BA, Rajashekara V, Patel C and Patel M (2003) Role of $G_{i2}\alpha$ -protein in opioid tolerance and μ -opioid receptor downregulation *in vivo*. *Synapse* **47**:109-116.
- Yu VC and Sadee W (1988) Efficacy and tolerance of narcotic analgesics at the μ opioid receptor in differentiated human neuroblastoma cells. *J Pharmacol Exp Ther* **245**:350-355.
- Zachariou V, Brunzell DH, Hawes J, Stedman DR, Bartfai T, Steiner RA, Wynick D, Langel U and Picciotto MR (2003a) The neuropeptide galanin modulates behavioral and neurochemical signs of opiate withdrawal. *Proc Natl Acad Sci U S A* **100**:9028-9033.
- Zachariou V, Georgescu D, Sanchez N, Rahman Z, DiLeone R, Berton O, Neve RL, Sim-Selley LJ, Selley DE, Gold SJ and Nestler EJ (2003b) Essential role for RGS9 in opiate action. *Proc Natl Acad Sci U S A* **100**:13656-13661.
- Zhao B, Wang HB, Lu YJ, Hu JW, Bao L and Zhang X (2011) Transport of receptors, receptor signaling complexes and ion channels *via* neuropeptide-secretory vesicles. *Cell Res* **21**:741-753.

Zhong H, Wade SM, Woolf PJ, Linderman JJ, Traynor JR and Neubig RR (2003) A spatial focusing model for G protein signals. Regulator of G protein signaling (RGS) protein-mediated kinetic scaffolding. *J Biol Chem* **278**:7278-7284.