Investigating Mechanisms Regulating the *In Vivo* Actions of Delta Opioid Receptor Ligands

By

Isaac Dripps

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Doctoral Committee:

Assistant Professor Emily M. Jutkiewicz, Chair
Assistant Professor Alexandre F. DaSilva
Professor John J. Tesmer
Professor John R. Traynor

Isaac J. Dripps

drippsis@umich.edu

ORCID iD: 0000-0002-1381-3292

To Rebecca Reimann, my first academic mentor, for cultivating my love of learning and stuff

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[^{35}S]GTPγS: [^{35}S] guanosine 5'-O-[gamma-thio]triphosphate

5'-NTII: Naltrindole-5'-isothiocyanate

β-FNA: β-funaltrexamine

BDNF: Brain derived neurotrophic factor

BNTX: 7-Benzylidenenaltrexone

CFA: Complete Freund's adjuvant

DADLE: [DAla^2, DLeu^5]-enkephalin

DOPA: dihydroxyphenylalanine

DOR: Delta opioid receptor

DPDPE: DPen^{2,5}-enkephalin

ECT: Electroconvulsive therapy

ERK: Extracellular signal-regulated kinase

FST: Forced swim test

GIRK: G protein-coupled inwardly-rectifying potassium channel

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

IP: Intraperitoneal

KOR: Kappa opioid receptor

MAOI: Monoamine oxidase inhibitor

MAPK: Mitogen-activated protein kinase

MDD: Major depressive disorder

MOR: Mu opioid receptor

NTG: Nitroglycerin

NTI: Naltrindole

PAG: Periaqueductal Gray

POMC: Preopiomelanocortin

PTZ: Pentylenetetrazol

RGS: Regulator of G protein signaling

RGS4: Regulator of G protein signaling 4

Gαo RGSi: Regulator of G protein signaling-insensitive Gαo protein

SNRI: Serotonin and norepinephrine reuptake inhibitor

SSRI: Selective serotonin reuptake inhibitor

SC: Subcutaneous

TST: Tail suspension test

Abstract

Chronic pain and depression are widespread and debilitating diseases that, for many people, cannot be adequately addressed with current treatment options. Delta opioid receptor (DOR) agonists have been proposed as novel treatments for these disorders. DOR is a member of the opioid receptor family of G protein-coupled receptors (GPCRs). DOR signals through inhibitory $G\alpha_{i/o}$ proteins that are negatively regulated by regulator of G protein signaling (RGS) proteins. Activation of DOR induces antihyperalgesia and antidepressant-like effects in animal models without the constipation, respiratory depression, and abuse liability associated with mu opioid receptor agonists such as morphine. Unfortunately, some DOR agonists cause convulsions, hindering their development as therapeutics in humans. The experiments described in this thesis sought to further characterize the intracellular signaling pathways and mechanisms underlying DOR-mediated behaviors. Specifically, these studies used a number of mouse models to determine differences in the regulation of DOR-mediated convulsions relative to the antihyperalgesic and antidepressant-like effects of DOR agonists. Antihyperalgesia was measured in a nitroglycerin-induced thermal hyperalgesia assay. Antidepressant-like effects were evaluated in the forced swim and tail suspension tests. Mice were also observed for convulsive activity post-agonist treatment.

To assess the role of G protein signaling in DOR-mediated behaviors, we compared behaviors induced by the DOR agonist SNC80 in RGS4 knockout, $G\alpha_o$ RGS-insensitive (RGSi) knock-in, and $G\alpha_o$ knockout mice. SNC80-induced antihyperalgesia was enhanced in RGS4 knockout and $G\alpha_o$ RGSi mice. SNC80-induced antidepressant-like effects were also potentiated in both RGS4 knockout and $G\alpha_o$ RGSi mice. However, SNC80-induced convulsions were not changed in either strain. In $G\alpha_o$ heterozygous knockout mice, SNC80-induced antihyperalgesia was abolished while the antidepressant-like effects and convulsions were unaltered. Taken together, these data demonstrate that DOR-mediated antihyperalgesia and antidepressant-like effects, but not convulsions, are regulated by $G\alpha_o$ and RGS4.

To further characterize the pharmacological properties mediating behavioral outcomes of DOR agonists, we compared the behavioral effects of SNC80 with those of the DOR partial agonist BU48. BU48 produced convulsions with similar potency to SNC80. BU48 also produced antidepressant-like effects with reduced potency relative to SNC80 and failed to elicit antihyperalgesia. These results suggest that the efficacy requirement for DOR-mediated convulsions may be low relative to other DOR-mediated behaviors. The efficacy requirements for DOR-mediated behaviors were further evaluated by comparing the shifts in the SNC80 dose response curves for each of these behaviors following decreases in DOR receptor reserve. Decreases in receptor reserve were produced using DOR heterozygous knockout mice as well as by treating mice with the DOR irreversible antagonist naltrindole-5'-isothiocyanate (5'-NTII). SNC80-induced antihyperalgesia displayed the largest potency shift in DOR heterozygous and 5'-NTII treated mice. Antidepressant-like effects displayed the next largest shift followed by convulsions. These findings suggest that DOR-mediated behaviors display the following rank order of efficacy requirement: convulsions < antidepressant like effects < antihyperalgesia. Furthermore, the DOR competitive antagonist naltrindole differentially shifted the SNC80 dose response curves of these behaviors, suggesting that different DOR receptor populations may mediate these behaviors.

Overall, the work presented in this thesis suggests that DOR-mediated behaviors are generated by distinct signaling mechanisms and receptor populations. The possibility of pharmacologically targeting receptor populations or signaling pathways responsible for DOR-mediated analgesic and antidepressant-like effects without activating receptors that mediated convulsions should greatly aid the clinical viability of DOR agonists.

Chapter I

General Introduction

The use of opium, extracted from the poppy plant *Papaver somniferum*, predates recorded history. It was used for the treatment of pain and diarrhea, as well as for its euphoric effects. In the early 19th century, the opiates primarily responsible for these effects, morphine and codeine, were isolated. Over the next century and a half, additional opioids were discovered and synthesized including heroin, meperidine and methadone. By the 1970s it had become apparent that the effects of opioids could best be explained by the presence of multiple opioid receptor types, which in turn suggested that these receptors were the target of endogenous neurotransmitters (Goldstein et al. 1971; Kuhar et al. 1973). Three types of opioid receptors—mu (MOR), delta (DOR), and kappa (KOR)—were identified and found to be activated by several endogenous opioid peptides (Martin et al. 1976; Lord et al. 1977). β-endorphin is derived from prepro-opiomelanocortin (POMC) and activates MOR and DOR with equal potency. Leu- and met-enkephalin are cleaved from preproenkephalin and bind and activate DOR with approximately 10-fold selectivity over MOR. Dynorphin A and B are derived from preprodynorphin and potently activate KOR with minor activity at MOR (Corbett et al. 1993).

The three opioid receptor types are each associated with pronounced and distinct behavioral effects. Activation of MOR produces robust analgesia, but also causes adverse effects including constipation, respiratory depression, and itch. In addition, MOR agonists have a strong reinforcing effects that can lead to dependence and addiction. Although KOR agonists also produce analgesia, they induce feelings of dysphoria and hallucinations, limiting widespread clinical use. Activation of DOR produces weak analgesia in models of acute pain—though these effects are more robust in models of inflammatory and neuropathic pain —as well as antidepressant-like, anxiolytic, stimulant, and anti-Parkinsonian effects. Some DOR agonists also produce convulsions which has limited their clinical development (for review see Chu Sin Chung and Kieffer 2013; Al-Hasani and Bruchas, 2011). It has been proposed that multiple subtypes (i.e. DOR1, DOR2) of each opioid receptor exist and these subtypes may mediated only certain

behavioral effects of the receptor, but this hypothesis remains controversial (for review, see Dietis et al. 2011).

In the 1980s, opioid receptors were thought to belong to the family of G protein coupled receptors (GPCRs) and couple to inhibitory $G_{i/o}$ proteins due their sensitivity to pertussis toxin and their ability to activate inwardly rectifying potassium channels (Burns et al. 1983; North et al. 1987). This hypothesis was later confirmed in the early 1990s with the cloning of each receptor type (Evans et al. 1992; Chen et al. 1993; Minami et al. 1993). All GPCRs transmit extracellular stimuli into intracellular signaling through coupling to heterotrimeric G proteins, each comprised of an α and βγ subunit (Figure 1.1; Gilman, 1987). In an inactive state, the heterotrimeric G protein exists as a single complex with Gα bound to GDP. Activation of a receptor by a ligand produces a conformational change in the Gα subunit, causing it to exchange GDP for GTP and dissociate from the βγ subunit. The α and βγ subunits go on to participate in various signaling cascades depending on the G protein subtype. The subunits remain active until the GTP bound to Gα is hydrolyzed to GDP, increasing the affinity of Gα for Gβγ and allowing the heterotrimer to reform. Gα possesses intrinsic GTPase activity that hydrolyzes GTP at a slow rate. This activity is greatly accelerated by regulator of G protein signaling (RGS) proteins. Following agonist activation, GPCRs are typically phosphorylated by G protein-coupled receptor kinases (GRKs) which facilitates arrestin binding to the receptor. Once bound to the phosphorylated GPCR, arrestin can mediate receptor internalization and recruit other signaling proteins to promote G protein-independent signaling pathways (Galandrin et al. 2007; Reiter et al. 2012).

Agonists that act at the same orthosteric site on a GPCR can stabilize distinct active conformations that preferentially signal through G protein or arrestin, a phenomenon known as functional selectivity or biased agonism (Kenakin 2003). These distinct G protein- and arrestin-dependent pathways may produce different behavioral effects. For example, targeted knockdown of specific G protein subunits using antisense nucleotides inhibited DOR-mediated antinociception in mice, suggesting that this effect is produced by a G protein-dependent signaling pathway (Standifer et al. 1996; Sánchez-Blázquez and Gárzon 1998). In addition, DOR agonists have been shown to differentially recruit arrestin isoforms and tolerance to the antinociceptive effects of an agonist depends on the arrestin isoform recruited (Pradhan et al. 2016). Loss of regulator of G protein signaling 4 (RGS4) potentiated the antidepressant-like

effects of the DOR agonist SNC80 suggesting that this behavior may be generated through G protein signaling (Stratinaki et al. 2013). The behavioral consequences of biased agonism in the DOR system are only beginning to be explored, and it remains unclear whether other behavioral effects of DOR, such as convulsive effects, are mediated by G protein or arrestin.

DOR represents a promising therapeutic target due its combination of beneficial effects and the prevalence of people with comorbid pain and depression. Nevertheless, the development of DOR drugs without convulsive effects is still needed. Determining the mechanisms and intracellular signaling pathways that give rise to DOR-mediated behaviors, and DOR-mediated convulsions in particular, is critical for the development of such drugs. Therefore, the current project explored the role of distinct signaling pathways and agonist efficacy in eliciting DOR-mediated behavioral outcomes using a variety of transgenic mouse models and behavioral pharmacology techniques.

Chronic Pain and Depression

Chronic pain and depression are widespread and debilitating diseases that, for many people, cannot be adequately addressed with current treatment options. Major depressive disorder (MDD) is a psychiatric disease in which those affected experience a depressed mood (i.e. feelings of sadness, emptiness, or hopelessness) and/or a loss of interest or pleasure in everyday activities. The recently released fifth edition of the Diagnostic and Statistical Manual of Mental Disorders defines a person as having a major depressive episode when they exhibit one of the former symptoms and at least four of the following within a two week period: 1) significant changes in weight or 2) sleeping pattern, 3) agitation, 4) fatigue, 5) feelings of worthlessness or excessive guilt, 6) diminished concentration, and/or 7) recurring thoughts of death or suicide (APA, 2013). In addition, MDD often presents with comorbidities such as chronic pain and anxiety disorders.

Major depression is a pervasive disease, affecting around 350 million (1 in 20) people worldwide (WHO, 2012). It is the top cause of disability in terms of total years lost to to the illness and is the leading cause of disease burden in women regardless of income (WHO, 2008). Furthermore, this disease appears to have lasting, cross-generational effects as evidence points to a mother with depression being a risk factor for poor growth and development in children (Rahman et al. 2008). However, the gravest concern with a depressed patient is an increased risk

of suicide. MDD is responsible for approximately half of all suicides in the United States with 15% of people with depression eventually committing suicide (Loosen and Shelton 2008).

The etiology of depression is unclear. Until recently, the prevailing hypothesis posited that depression was caused by deficiencies in the neurotransmission of monoamines, specifically serotonin and norepinephrine. Therefore, current treatments for depression have focused on enhancing monoaminergic signaling. Although there are currently several methods for treating major depression, no single pharmacological treatment has been widely effective or without serious shortcomings. The selective serotonin reuptake inhibitors (SSRIs) are the first line therapy and most commonly used drug class for the treatment of major depression. SSRIs act by blocking the reuptake of serotonin into presynaptic nerve terminals thereby increasing neurotransmitter signaling. Although this action of SSRIs occurs immediately, it can take up to six weeks for an SSRI to reach full effect in depressed patients (Trivedi et al. 2006a). This finding suggests that monoamine deficiency alone is not sufficient to produce depression or that enhancement of monoaminergic signaling is not solely necessary for producing therapeutic effects. In further support of this hypothesis, 70% of patients do not achieve remission with an SSRI alone (Trivedi et al. 2006a). Augmenting SSRI treatment with a second antidepressant can be helpful although only a third of patients who do not respond to SSRI monotherapy achieve remission with combination therapy (Trivedi et al. 2006b). Furthermore, SSRI treatment can lead to serious complications including tinnitus, insomnia, akathisia, and sexual dysfunction.

The tricyclic antidepressants (TCAs) are an alternative treatment for major depression and were the primary therapy prior to the development of SSRIs. TCAs act through a variety of mechanisms, however the majority block serotonin and/or norepinephrine reuptake. Like SSRIs, TCAs can also take several weeks to reach their full effect and effectively treat a small percentage of patients. In the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) clinical trial, about 20% of patients not adequately treated with SSRI mono- or combination therapy achieve remission with a TCA (Fava et al. 2006). TCAs can also have effects on the cardiovascular system including changes in heart rate or rhythm and orthostatic hypotension. Furthermore, the therapeutic index of TCAs is small and overdoses can be lethal making administration of these drugs to suicidal patients concerning. Other traditional medications used for the treatment of depression include serotonin receptor agonists, serotonin and norepinephrine reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors (MAOIs).

An alternative to pharmacotherapy, and arguably the most effective treatment for major depression currently available, is electroconvulsive therapy (ECT; Polyakova et al. 2015). ECT works by using an electric current to induce a generalized seizure in the central nervous system of the patient. The mechanism by which this seizure alleviates depressive symptoms is currently unknown, although there is evidence that it involves modulation of the opioid system (Emrich et al. 1979; Inturrisi et al. 1982). The primary side effects associated with ECT are confusion and memory loss. Although rare, this memory loss is potentially permanent. Other problems surrounding ECT include insufficient patient understanding and public disapproval of its use (Eisendrath and Lichtmacher 2014).

Although the majority of antidepressant therapies function via augmentation of aminergic neurotransmission, the monoamine deficiency hypothesis of depression is likely overly simplistic as several alternative targets including GABA, glutamate, adenosine, stress hormones, and opioids have been proposed to be involved in mediating depressive symptoms. Changes in opioid signaling have already been observed with currently used treatments for depression, such as ECT, and the atypical antidepressant tianeptine, which was recently shown to be an agonist at both MOR and DOR opioid receptors, albeit at large concentrations (Gassaway et al. 2014). Case studies have also reported that the opioid ligand buprenorphine has antidepressant actions in patients with refractory depression, potentially through inhibition of kappa opioid receptors (for review, see Stanciu et al. 2017). Despite these findings, the use of opioids for the treatment of depression is rarely discussed.

Depression and chronic pain are often comorbid. Among people suffering from chronic pain, it is estimated that 30-54% of them also suffer from MDD (Gieseke et al. 2005). Conversely, in a study of 150 MDD patients, 76% of them exhibited multiple pain symptoms (Corruble and Guelfi, 2000). In addition, this study found that pain complaints correlated with the severity of depression symptoms. Pain can be difficult to characterize and can be due to a number of factors but is commonly defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (IASP, 2014). Chronic pain, defined as pain persisting for at least three months, is a condition that affects roughly 100 million Americans (Henschke et al. 2015). The economic burden of chronic pain is enormous, costing the United States over $500 billion annually in healthcare costs and lost productivity, a total greater than the cost of heart disease, diabetes, and cancer combined (Gaskin and Richard 2012).

MOR agonists, such as hydrocodone and fentanyl, are widely used for the treatment of chronic pain. MOR agonists act in a number of brain regions to stimulate descending inhibitory pathways and blunt nociceptive transmission from the periphery to the CNS. Although these drugs have demonstrated efficacy for some types of pain, including postoperative and breakthrough cancer pain, MOR agonists are significantly less effective at treating other types of pain, such as neuropathic and inflammatory pain. Switching between different MOR agonists and/or routes of administration, a strategy known as opioid rotation, may maintain analgesia while limiting adverse effects though there is a lack of strong evidence in support of this strategy (Nalamachu 2012). Many physicians still utilize opioid rotation, however finding equi-analgesic doses can be difficult and there are no evidence-based guidelines regarding opioid choice (Smith and Peppin 2014).

The GABA analogues gabapentin and pregabalin are commonly used for the treatment of neuropathic pain. It is unclear how these drugs function, but it has been proposed to be due to inhibition of voltage-gated sodium channels through interaction with the α2δ subunit. While gabapentin and pregabalin are typically well tolerated, they are not particularly efficacious and produce a 50% reduction of pain levels in only 20-40% of patients (Gilron and Flatters 2006; et al. 2014). TCAs, amytriptyline in particular, are also used to treat neuropathic man. Like the GABA analogues, TCAs are effective in only 20-30% of patients (Kremer et al. 2016). In addition, TCAs require prolonged treatment to achieve efficacy and are associated with serious adverse effects as previously discussed. Given the inadequacy of available treatments, there is a demonstrable need for alternative therapies for major depression and chronic pain.

Antidepressant-like Effects and DOR Activation

Evidence from Preclinical Models

In 1975, peptides with opiate-like properties were observed in the aqueous extracts of pig brain and human cerebrospinal fluid (Hughes 1975a; Terenius and Wahlström 1975). These compounds were quickly identified as the pentapeptides leu- (Tyr-Gly-Gly-Phe-Leu) and met-enkephalin (Tyr-Gly-Gly-Phe-Met; Hughes et al. 1975b). The following year, Plotnikoff et al. (1976) showed that met-enkephalin potentiated increases in motor activity produced by racemic dihydroxyphenylalanine (DOPA). Because the tricyclic antidepressants were also effective in this assay, it was used as an early screening technique for antidepressant drugs (Everett, 1966).

The antidepressant-like effects of opioids were further supported when enkephalins and endorphins were shown to decrease immobility in the forced swim test and the learned helplessness paradigm, again demonstrating effects similar to clinically used antidepressants (Kastin et al. 1978; Tejedor-Real et al. 1995). Later, numerous experiments showed that preventing the breakdown of endogenous opioid peptides using enkephalinase inhibitors produced antidepressant-like effects. Tejedor-Real et al. (1993) demonstrated that RB38A, a mixed enkephalinase inhibitor, and RB38B, a selective endopeptidase EC 3.4.24.11 inhibitor, reduced escape failures in the learned helplessness paradigm, and that these effects were blocked by the nonselective opioid receptor antagonist naloxone, suggesting an opioid receptor-mediated effect. In the mouse forced swim test, the enkephalinase inhibitor BL-2401 produced naloxone-reversible antidepressant-like effects, again indicating an opioid receptor-mediated effect (Kita, et al. 1997).

However, these experiments did not necessarily demonstrate a role for the DOR in mediating these behaviors. Inhibition of the effects of RB101, a mixed enkephalinase inhibitor, and the DOR selective peptide agonist BUBU (Tyr-D.Ser-(*O-tert*-butyl)-Gly-Phe-Leu-Thr(O-Tet-butyl-OH)), in the learned helplessness paradigm by the DOR selective antagonist naltrindole (NTI), suggested that the antidepressant-like effects of these drugs were DOR-mediated in mice (Baamonde et al. 1992) and rats (Tejedor-Real et al. 1998). RB101 was later shown to consistently produce DOR-mediated antidepressant-like effects in the rat forced swim test (Jutkiewicz et al. 2006). Recently, opiorphin, an endogenously expressed inhibitor of human neutral endopeptidase and aminopeptidase-N, was found to induce antidepressant-like effects in the rat forced swim test (Javelot et al. 2010). These effects were blocked by NTI as well as the MOR selective antagonist β-funaltrexamine (β-FNA), indicating a role for both DORs and MORs (Javelot et al. 2010; Yang et al. 2011). Although these studies demonstrate that stimulation of the DOR produces antidepressant-like effects in animal models, they do not indicate the role of the endogenous DOR system or endogenous opioids in regulating mood states. To this end, König and colleagues (1996) found that mice lacking preproenkephalin displayed anxiety-related behaviors and the males were also hyperaggressive. Later, Filliol et al. (2000) demonstrated a role for endogenous delta opioid tone in the regulation of mood states by showing that DOR knockout mice (*OPRD1*-deficient) exhibited anxiogenic and prodepressive behaviors.

The development of nonpeptidic DOR selective agonists greatly aided the investigation of DOR-mediated antidepressant-like effects by allowing for the study of centrally mediated behaviors using peripherally administered compounds. The nonpeptidic DOR selective agonists (+)BW373U86 ((±)-4-((α-R*)-α-(2S*,5R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-N,N-diethylbenzamide), SNC80 ((+)-4-[(alpha R)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3- methoxybenzyl]-N,N-diethylbenzamide), and TAN-67 ((-)-2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydorquinolino[2,3,3g]isoquiniline dihydrobromide) all demonstrated antidepressant-like effects in the rat forced swim test (Table 1.1; Broom et al. 2002a; Nagase et al. 2002). The effects of (+)BW373U86 and SNC80 were shown to be NTI-reversible indicating a DOR-mediated effect (Broom et al. 2002a). Additionally, SNC80 has been found to elicit other antidepressant-like effects, including improving the emotionality score in olfactory bulbectomized rats (Saitoh et al. 2008) and reversing pain depressed responding of intracranial self-stimulation in rats (Negus et al. 2012). Furthermore, SNC80 is not self-administered by monkeys (Negus et al. 1998), does not facilitate intracranial self-stimulation (Do Carmo et al. 2009), and does not promote dopamine efflux in the nucleus accumbens (Longoni et al. 1998), suggesting a low abuse potential. Unlike typical antidepressants, which require multiple administrations to generate an effect in many animal models of depression, these DOR agonists were effective after a single, acute dose, suggesting a faster onset of action. Although tolerance develops to some of the effects of DOR agonists after a single dose, DOR agonists continue to produce antidepressant-like effects after repeated administration (Jutkiewicz et al. 2005a; Saitoh et al. 2008; Nozaki et al. 2014).

Due to the effectiveness of ECT in treating depression in humans, it was hypothesized that DOR agonist-induced convulsions were required for their antidepressant-like effects, similar to that produced by other convulsive agents that had been used clinically prior to the development of ECT, such as metrazol or insulin-induced seizure. DOR agonist-induced convulsions consist of brief, non-lethal, generalized seizure activity, are NTI-sensitive, and absent in DOR knockout mice (Comer et al. 1993; Broom et al. 2002b). Broom et al. (2002b) showed that pretreatment with the short acting benzodiazepine midazolam blocked (+)BW373U86-induced convulsions without affecting (+)BW373U86-induced antidepressant-like effects. By slowing the rate at which SNC80 was administered, Jutkiewicz et al. (2005b) eliminated the convulsive effects of SNC80 while maintaining its antidepressant-like effects.

Additionally, they were able to elicit convulsions without observable antidepressant-like effects in the forced swim test in rats at a dose of 1 mg/kg SNC80 via rapid (20 sec) intravenous infusions. Moreover, tolerance to the convulsive effects of DOR agonists develops after a single administration, whereas the antidepressant-like effects remain after chronic administration. Recently, it was shown that loss of DOR expression in GABAergic forebrain neurons in mice eliminated SNC80-induced convulsions and EEG disturbances (Chu Sin Chung et al. 2015). Taken together, these data suggest that it is possible to observe antidepressant-like effects of DOR agonists without generating convulsions.

In further support of this hypothesis, nonpeptidic DOR agonists that produce antidepressant-like effects without generating convulsions, including ADL5859 and KNT-127, have been developed. ADL5859 (N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl) benzamide; Table 1.1) significantly reduced immobility and increased swimming when administered at 3 mg/kg orally in a rat forced swim test (Le Bourdonnec et al. 2008). These antidepressant-like effects were not accompanied by any convulsions, hyperlocomotion, or stereotypy in rats or mice at doses up to 1 g/kg. In addition, no EEG disturbances were observed in rats at doses up to 30 mg/kg i.v. (Le Bourdonnec et al. 2008) or in mice at doses up to 300 mg/kg p.o. (Chu Sin Chung et al. 2015). ADL5859 passed phase I clinical trials and was evaluated in human clinical trials for the treatment of rheumatoid arthritis and neuropathic pain but was not found effective (Spahn and Stein 2017). To this point, studies evaluating ADL5859 as a treatment for depression in humans have not been conducted.

KNT-127 (1,2,3,4,4a,5,12,12a-octahydro-2-methyl-4aβ,1β-([1,2]benzenomethano)-2,6-diazanaphthacene-12aβ,17-diol; Table 1.1) has been extensively investigated in animal models of depression and anxiety. In the mouse forced swim test, KNT-127 significantly decreased immobility and increased swimming behavior without affecting overall locomotor activity or eliciting convulsions (Saitoh et al. 2011). These antidepressant-like effects of KNT-127 were reversed by NTI as well as the putative DOR2 antagonist naltriben. Daily injections of 5 mg/kg KNT-127 did not affect the ability of acute administration of 3 mg/kg KNT-127 to reduce immobility in the mouse forced swim test suggesting that chronic administration of KNT-127 does not induce tolerance to its antidepressant-like effects (Nozaki et al. 2014). Furthermore, daily administration of KNT-127 significantly decreased hyperemotionality scores in olfactory-bulbectomized rats throughout the 14 day test period (Gotoh et al. 2016).

Possible Mechanisms of Action of DOR-Mediated Antidepressant-like Effects

Although monoamines, namely dopamine, norepinephrine and serotonin, play a well-established role in regulating emotion and cognition (Robbins and Arnsten 2009), the etiology of depression goes beyond deficiencies in the levels of these neurotransmitters in the brain. There are two primary problems with the monoamine deficiency hypothesis of depression. First, although clinically used antidepressants typically take weeks to achieve a therapeutic effect, they block reuptake and/or metabolism of monoamines within hours or days of first use. Second, loss of serotonin or norepinephrine does not readily cause depression in healthy controls suggesting that monoamine deficiency is not sufficient to produce depression. Therefore, alternatives to the monoamine hypothesis of depression have been put forward.

One such hypothesis proposes that depression is caused by dysfunction of glutamatergic neurotransmission. Excess glutamate leads to neurotoxicity and this loss of neurons is thought to promote a depressive phenotype, even though many studies have shown that inhibition of neurogenesis does not lead to a depressive phenotype (for review see Petrik et al. 2012). Many clinical studies have shown elevated levels of glutamate in the plasma, CSF, and brain tissue of depressed patients that are reduced after antidepressant treatment (for review see Sanacora et al. 2012). In addition, low doses of the noncompetitive NMDA receptor antagonist ketamine have been shown to elicit rapid antidepressant-like effects in human patients (Monteggia and Zarate 2015) and animal models (Browne and Lucki 2012). These effects last for up to two weeks after a single dose of ketamine suggesting a synaptic plasticity-mediated mechanism.

The interactions between the delta opioid and glutamatergic systems are not well characterized and differ across brain regions. SNC80 has been found to increase glutamate release in rat striatum (Bosse et al. 2014). KNT-127 increased glutamate release within the striatum, nucleus accumbens, and medial prefrontal cortex of male Sprague-Dawley rats (Tanahashi et al. 2012). The peptidic delta agonist DPDPE enhanced the glutamate content of intrastriatal dialysate (Billet et al. 2004), but also inhibited glutamate release in the rat anterior cingulate cortex (Tanaka and North 1994) and in the amygdala of morphine treated rats (Bie et al. 2009). The peptidomimetic DOR agonist UFP-512 decreased glutamate release in the rat substantia nigra (Mabrouk et al. 2009). Further research is needed to characterize the role of

glutamate and glutamate circuitry in depression and in eliciting DOR-mediated antidepressant-like effects.

Another putative mechanism for the actions of antidepressant drugs is through upregulation of brain derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family of growth factors and promotes the growth, survival, and differentiation of neurons. Many studies have shown that stress decreases BDNF expression and promotes cell death in brain regions that regulate mood (Duman 2003; Lee and Kim 2010). Serum levels of BDNF in depressed patients are significantly lower compared to healthy controls (Bocchio-Chiavetto et al. 2010). In postmortem studies, BDNF expression was decreased in the hippocampus and prefrontral cortex of depressed patients and suicide victims (Dwivedi et al. 2003; Karege et al. 2005). Furthermore, antidepressant treatment has been shown to increase BDNF expression in preclinical and clinical studies (Duman 2003; Lee and Kim 2010).

There are few reports examining the effects of DOR agonists on BDNF. DPDPE increased BDNF mRNA expression in the rat frontal cortex (Torregrossa et al. 2006) while (+)BW373U86 increased BDNF mRNA expression in the hippocampus, amygdala, and frontal cortex (Torregrossa et al. 2004). For both drugs, these changes were NTI-sensitive and occurred at doses that also produced antidepressant-like effects in the forced swim test. Interestingly, upregulation of BDNF in response to DOR agonists was observed before increases could be observed with traditional antidepressants, suggesting a faster onset of action. Elevated levels of BDNF mRNA in the frontal cortex persisted after 8 days of daily (+)BW373U86 injections but returned to basal levels after 21 days of treatment. However, it is unclear whether or not BDNF protein levels were also changed (Torregrossa et al. 2005). UFP-512 attenuated hypoxia-induced decreases in BDNF expression in mouse cortex exposed to hypoxic (10% O_2) conditions for 3 or 10 days (Tian et al. 2013). The nonpeptidic DOR agonist AZD2327 significantly increased BDNF expression in the rat hippocampus but not in the frontal cortex and plasma BDNF levels remained unchanged (Richards et al. 2016). AZD2327 also failed to alter plasma BDNF levels in human patients, albeit in a small, underpowered cohort (Richards et al. 2016). Taken together, these data suggest that BDNF expression may correlate with the antidepressant actions of DOR agonists, although future studies should examine whether BDNF plays a causal role in mediating these effects.

Pain Relieving Effects and DOR Activation

Evidence from Preclinical Models

Following the isolation of leu- and met-enkephalin, these pentapeptides were tested for analgesic properties. Both peptides produce antinociception in the hot plate and tail flick tests in rats when given intraventricularly (Frederickson 1977). However, these effects were produced with low potency and only for a short duration due to rapid degradation. Single peptide substitutions, such as replacing the first glycine of met-enkephalin with D-alanine, greatly increased the potency and duration of antinociceptive action. Naloxone antagonized enkephalin-induced antinociception with potency comparable to morphine, suggesting that these effects are mediated by MOR (Yaksh et al. 1978).

Evidence for DOR-mediated antinociception was first found with intrathecal administration of the peptide DADLE (Tyr-D-Ala-Gly-Phe-D-Leu). Naloxone antagonized DADLE-induced antinociception in the rat hot plate test with significantly lower potency compared to morphine-induced antinociception (Tung and Yaksh, 1982). In addition, this report also found that DADLE produced antinociception with similar potency in naïve and morphine tolerant rats. Taken together, these data suggest that DADLE-induced antinociception is mediated by a receptor other than MOR. Jensen and Yaksh (1986) found that injecting DADLE directly into the periaqueductal gray (PAG) or the medullary reticular formation was sufficient to produce antinociception in the tail flick and acetic acid-induced stretching tests. DPDPE (Tyr-D-Pen-Gly-Phe-D-Pen), a cyclized peptide with greater selectivity for DOR than MOR, produced antinociception in models of acute pain including the tail flick and paw pressure tests (Rodriguez et al. 1986). This report also showed that the DOR selective antagonist ICI 174,864 blocked DPDPE-mediated, but not morphine-mediated, antinociception, indicating a DOR-mediated effect. In a formalin-induced inflammation model in rats, DPDPE reduced nocifensive paw licking suggesting DOR agonists could be effective at treating inflammatory pain (Calgnetti et al. 1988). DPDPE also alleviated cold water allodynia in rats with a crushed sciatic nerve, a model of neuropathic pain (Mika et al. 2001). This effect of DPDPE was blocked by pretreatment with the putative DOR1 antagonist 7-benzylidenenaltrexone (BNTX), indicating a DOR-mediated effect.

As with antidepressant-like effects, evaluation of DOR-mediated analgesia benefited from the development of small molecule DOR selective agonists and antagonists. Peripheral

administration of SNC80 or BW373U86 did not produce DOR-mediated antinociception in models of acute thermal nociception such as the hot plate, tail flick, and warm water tail withdrawal assays in rodents (Wild et al. 1993; Bilsky et al. 1995; Gallantine and Meert 2005) or in monkeys (Negus et al. 1998). However, these drugs did produce dose-dependent DOR-mediated antinociception in other pain models such as the acetic acid-induced stretch assay (Wild et al. 1993; Broom et al. 2002c; Gallantine and Meert 2005).

SNC80 has demonstrated efficacy in several models of continuous inflammatory or neuropathic pain. SNC80 blocked capsaicin-induced thermal hyperalgesia in rhesus monkeys in a NTI-sensitive manner (Brandt et al. 2001). In addition, thermal hyperalgesia in rats produced by complete Freund's adjuvant (CFA) was alleviated by SNC80 (Fraser et al. 2000). SNC80 also reduced nocifensive behavior induced by formalin and tactile allodynia following sciatic nerve injury in NTI reversible manners (Obara et al. 2009). In mice, SNC80 reversed mechanical allodynia and thermal hyperalgesia produced by CFA in wild-type but not DOR knockout animals (Gavériaux-Ruff et al. 2008; Nozaki et al. 2012). Furthermore, Gavériaux-Ruff et al. (2008) found that DOR knockout mice showed enhanced allodynic and hyperalgesic responses to CFA, suggesting a role for endogenous DOR tone in suppressing the response to inflammatory pain.

These studies demonstrate that a single dose of a DOR agonist is effective at treating pain symptoms. However, the effects of a drug after repeated dosing is also critical to its clinical utility. Unfortunately, acute tolerance develops to the antinociceptive effects of some DOR agonists. Mice receiving repeated intercerebroventricular injections of DPDPE for 2 or 4 days displayed significant tolerance to the antinociceptive effects of DPDPE in the tail flick assay (Zhao and Bhargava 1997). A 24 hr pretreatment of SNC80 significantly decreased the antinociceptive effects of a second dose of SNC80 in the acetic acid-induced stretch assay in mice (Hong et al. 1998) or the lactic acid-induced stretch assay in rats (Negus et al. 2012). A single injection of SNC80 produced acute tolerance to the antihyperalgesic effects of a second dose of SNC80 given 4 or 12 hrs after the initial dose (Pradhan et al. 2010; Pradhan et al. 2016). Interestingly, arrestin2 knockout mice did not show tolerance to SNC80, suggesting arrestins may play a role in DOR desensitization (Pradhan et al. 2016). Waiting 24 hrs to give the second dose allowed SNC80 to produce a full response in wild-type mice, however the antihyperalgesic effects were completely lost after 5 daily injections of SNC80 (Pradhan et al. 2010).

It is possible that the antinociceptive effects of DOR are dependent on its convulsive effects, however this does not appear to be the case. BU48 (N-Cyclopropylmethyl-[7α,8α,2′,3′]-cyclohexano-1′[*S*]-hydroxy-6,14-endo-ethenotetrahydronororipavine; Table 1.1), a buprenorphine analog that acts as a partial agonist at DOR and KOR, produces DOR-mediated convulsions in mice without producing DOR-mediated antinociception (Broom et al. 2000). This finding suggests that DOR-mediated convulsions are not sufficient to produce antinociception. Furthermore, other DOR agonists have been shown to provide antinociception without producing convulsions.

ARM390 (N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; Table 1.1) has been shown to produce antihyperalgesia with reduced tolerance relative to SNC80. In mice treated with CFA, ARM390 reversed thermal antihyperalgesia acutely and in mice pretreated with ARM390 12 or 24 hrs prior to a second dose (Pradhan et al. 2010). However, after 5 days of daily treatment ARM390 failed to inhibit thermal or mechanical hyperalgesia. ARM390 had similar effects on nitroglycerin-induced hyperalgesia after both acute and repeated dosing (Pradhan et al. 2014). There are no reports of ARM390 producing convulsions and it did not generate changes in EEG recordings at doses up to 60 mg/kg p.o. (Chu Sin Chung et al. 2015).

JNJ-20788560 (9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide; Table 1.1) is a DOR agonist of particular interest because it appears to produce antinociception with little development of tolerance. Acutely, 30 mg/kg p.o. JNJ-20788560 inhibited CFA-induced thermal hyperalgesia in rats and acetylcholine bromide-induced stretching in mice (Codd et al. 2009). Tolerance to these effects did not develop after 5 days of daily treatment with JNJ-20788560. Pradhan et al. (2014) showed that JNJ-20788560 could reverse nitroglycerin-induced mechanical hyperalgesia in mice with no significant tolerance when given every other day for 10 days (Pradhan et al. 2014). There are no reports of JNJ-20788560 producing convulsions.

KNT-127 also displays a variety of antinociceptive effects. KNT-127 reduced acetic acid-induced stretching as well as paw licking in the formalin test (Nagase et al. 2010; Saitoh et al. 2011). These behaviors were blocked by pretreatment with NTI and naltriben demonstrating that the antinociceptive effects of KNT-127 are DOR-mediated. KNT-127 also reversed thermal and mechanical hyperalgesia in mice treated with CFA (Nozaki et al. 2014). This study also found

that daily treatment of KNT-127 for 5 days produced full tolerance to the antihyperalgesic effects of KNT-127 and cross-tolerance to the antihyperalgesic effects of SNC80.

Possible Mechanisms of Action of DOR-Mediated Antinociceptive Effects

The circuits governing DOR-mediated antinociception are well characterized. There are two primary neural circuits—the ascending and descending pathways—that regulate pain processing (Figure 1.2). Activation of the ascending pathway occurs when nociceptors, a class of afferent sensory neurons, relay noxious stimuli from the periphery to the spinal cord. There are two primary types of nociceptors that relay information to the spinal cord. Aδ fibers are myelinated, fast conducting neurons that release glutamate. Their signaling is thought to result in sharp, localized pain. C fibers are unmyelinated, slow conducting neurons whose signaling manifests as burning or throbbing pain that is poorly localized. They release neuropeptides such as substance P and calcitonin gene-related peptide. In the dorsal horn of the spinal cord, nociceptors synapse onto interneurons that signal to second order neurons that relay information up the spinal cord to the brain stem—specifically the PAG and raphe nuclei—and thalamus. Third order neurons receive signals in the thalamus and relay them to the somatosensory cortex of the brain where the noxious stimuli is interpreted as pain. The descending pathway involves efferent signaling from the PAG and raphe nuclei to the spinal cord that inhibits the ascending pathway.

Multiple reports have located DORs in a variety of places along the ascending and descending pathways, namely in presynaptic terminals of C fiber neurons (Cheng et al. 1995; Mennicken et al. 2003), the cell bodies and dendrites of dorsal horn interneurons (Minami et al. 1995; Cahill et al. 2001), and the PAG (Jenab et al. 1995; Kalyuzhny et al. 1996). Under basal conditions, a substantial portion of DORs are stored in intracellular vesicles that are trafficked to the plasma membrane in response to pain states such as chronic inflammation (Cahill et al. 2003; Gendron et al. 2006). DOR activation triggers a signaling cascade that inhibits voltage-dependent calcium channels (Acosta and López, 1999; Law et al. 2000; Pradhan et al. 2013). Because Ca^{2+} facilitates trafficking and fusion of synaptic vesicles to the plasma membrane, inhibition of Ca^{2+} influx significantly reduces neurotransmitter release. This reduction of neurotransmitter release in turn prevents the relay of pain signals to subsequent neurons in the circuit and is theorized to be responsible for analgesia. DOR activation has also been linked to potassium efflux through

modulation of G protein-coupled inwardly rectifying potassium channels (GIRKs) which will hyperpolarize the neuron and further inhibit neurotransmitter release (Lüscher and Slesinger 2010). Although there is strong evidence for DORs in the spinal cord mediating antinociception (Cahill et al. 2003; Gendron et al. 2006; Kouchek et al. 2013), DOR activation within the PAG appears to produce only mild antinociception (Ossipov et al. 1995; Morgan et al. 2009).

In summary, depression and pain are serious and intractable conditions that are not adequately treated with current therapies. DOR agonists have been shown to produce antidepressant-like effects and antinociception in a variety of animal models. The convulsive activity of several DOR agonists has heretofore limited their clinical utility, however the development of DOR agonists that do not produce convulsions should allow for more thorough testing in humans. Although some DOR agonists alter glutamatergic neurotransmission and others upregulate BDNF expression, consistent with modern hypotheses on the etiology of depression, the mechanism by which DOR-mediated antidepressant-like effects are generated is not known. In terms of DOR-mediated antinociception, the neuronal circuits governing these effects have been reasonably well characterized, however the intracellular signaling partners involved have not been elucidated. At the intracellular level, DOR-mediated behaviors may be generated via distinct intracellular signaling pathways. Determining the signal transduction mechanisms that give rise to DOR-mediated behaviors is critical for the development of DOR drugs with improved safety and clinical utility and future work should be devoted to elucidating these pathways.

Experimental Objectives

The experiments described in this thesis sought to further characterize the intracellular signaling pathways and mechanisms underlying DOR-mediated behaviors. Specifically, these studies focused on determining differences in the regulation of DOR-mediated convulsions relative to the potential therapeutic effects of DOR agonists.

Aim 1: Characterize the role of RGS4 in the regulation of DOR-mediated behaviors

The second Chapter of this thesis investigates how RGS4 regulates DOR-mediated behaviors by comparing the potency of SNC80 to produce antinociception, antihyperalgesia, antidepressant-like effects, and convulsions in wild-type and RGS4 knockout mice. Stratinaki et

al. (2013) previously showed that a single 5 mg/kg dose of SNC80 decreased immobility in the mouse forced swim test to a greater degree in RGS4 knockout mice compared to wild-type mice. This group of studies expands on those findings by evaluating other DOR-mediated behaviors using a wide range of doses. It was hypothesized that RGS4 negatively regulates all DOR-mediated behaviors as evidenced by an increase in the potency of SNC80. Changes in SNC80 potency were validated *ex vivo* by examining SNC80-induced phosphorylation of MAP kinase in striatum from RGS4 knockout mice. Potential changes in receptor density were measured using [^{3}H]DPDPE saturation binding in mouse forebrain tissue. The relative contributions of DOR in the CNS and periphery to SNC80-induced behaviors were also evaluated using the peripherally-limited antagonist N-methylnaltrexone. The results from this first study showed that DOR-mediated antihyperalgesia and antidepressant-like effects in the forced swim test are regulated by RGS4, suggesting they are generated through a G protein signaling mechanism.

Aim 2: Investigate the role of G protein- and arrestin-mediated signaling in DOR-mediated behaviors

The third Chapter of this thesis investigates which G protein mediates these behaviors, as well as whether convulsions are produced via a G protein-dependent or –independent mechanism. It was hypothesized that $G\alpha_o$ mediates the antihyperalgesic and antidepressant-like effects of DOR while convulsions are mediated by arrestins. To evaluate the role of $G\alpha_o$ in DOR-mediated behaviors, the potency of SNC80 to produce antihyperalgesia, antidepressant-like effects, and convulsions was assessed in $G\alpha_o$ RGS-insensitive heterozygous knock-in mice and $G\alpha_o$ heterozygous knockout mice. The effects of SNC80 were compared to the tricyclic antidepressant desipramine and the anti-migraine drug sumatriptan. DOR-mediated behaviors were also evaluated in arrestin2 and arrestin3 knockout mice. Potential changes in receptor density were measured using [^{3}H]DPDPE saturation binding in mouse forebrain tissue. The study demonstrated that antidepressant-like effects and antihyperalgesia are likely mediated by Gαo protein signaling pathways but failed to find a signaling pathway that positively regulates DOR-mediated convulsions.

Aim 3: Compare the efficacy requirements of DOR-mediated behaviors

The final data Chapter of this thesis evaluated the role of ligand efficacy as related to DOR-mediated convulsions. It was hypothesized that convulsions have the lowest efficacy requirement among the DOR-mediated behaviors tested, followed by antidepressant-like effects, with antihyperalgesia having the highest efficacy requirement. Efficacy requirements were assessed, in part, by comparing the behavioral effects of the DOR partial agonist BU48 and the DOR full agonist SNC80. Efficacy requirements were further evaluated by comparing the shifts in the SNC80 dose response curves for each of these behaviors in mice with decreased DOR number, such as DOR heterozygous knockout mice and following treatment with the DOR irreversible antagonist naltrindole-5'-isothiocyanate (5'-NTII). The potency of NTI to antagonize each of these SNC80-induced behaviors was also investigated. Overall, this study found that the observed behavioral effects of SNC80 display the following rank order of efficacy requirement: convulsions < antidepressant-like effects < antihyperalgesia. NTI antagonized these behaviors with distinct potencies, suggesting that discrete populations of DORs may mediate these different behavioral effects.

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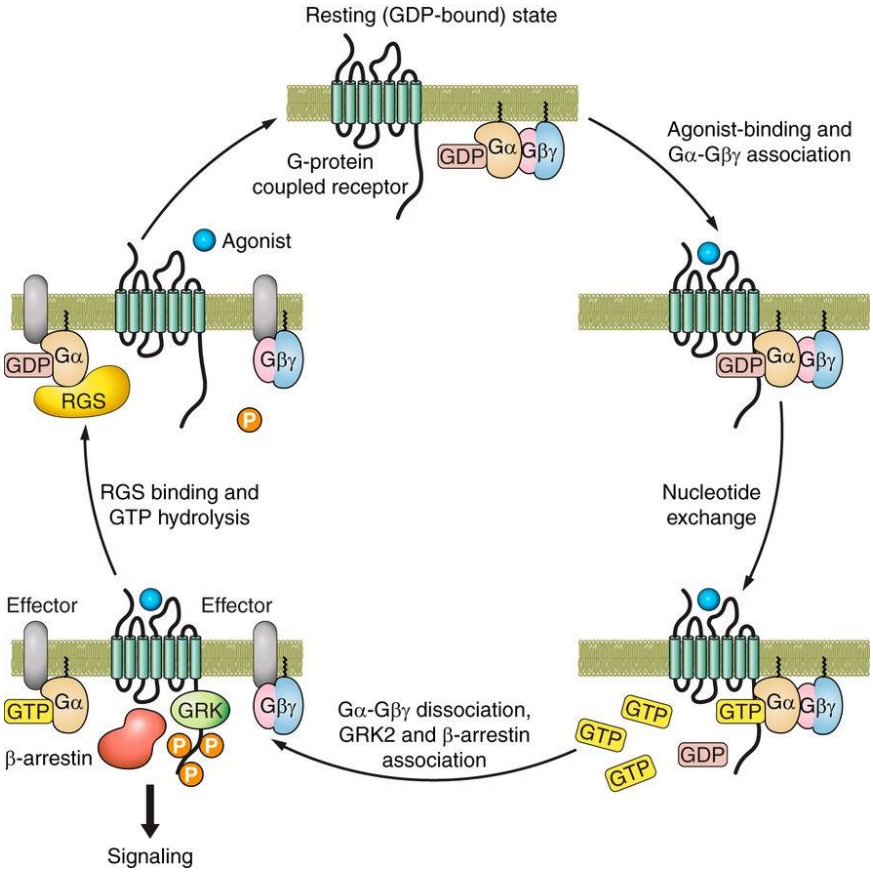


Figure 1.1. GPCR-mediated signaling mechanisms. In the inactive state, heterotrimeric G proteins exist as a single complex with Gα bound to GDP. Activation of a GPCR by a ligand produces a conformational change in the Gα subunit, causing it to exchange GDP for GTP and dissociate from the βγ subunit. The α and βγ subunits go on to activate various downstream effectors. The subunits remain active until the GTP bound to Gα is hydrolyzed to GDP, a process that is accelerated by RGS proteins. In addition to signaling through G proteins, GPCRs can also activate G protein-independent signaling via recruitment of arrestin proteins. Figure adapted from Sánchez-Fernández et al. 2014.

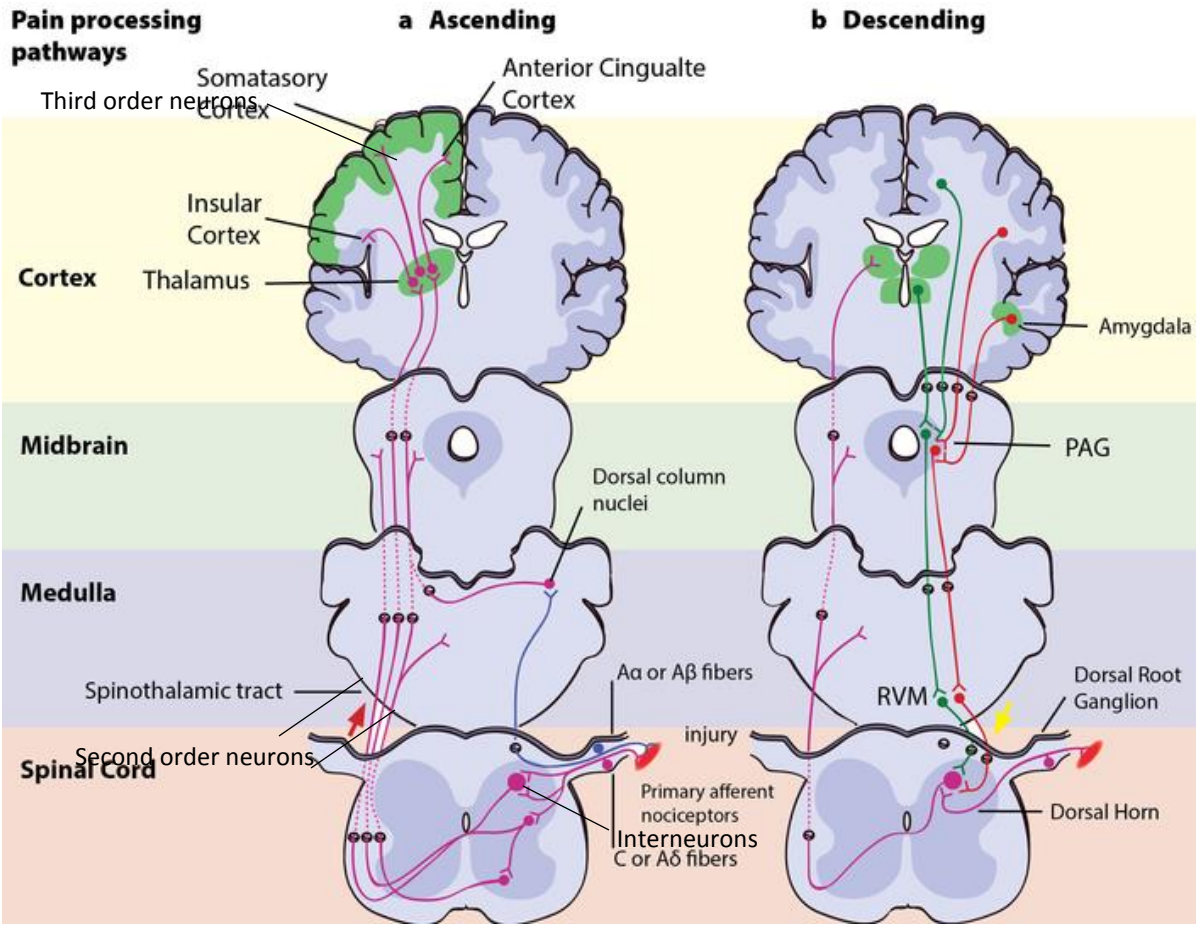
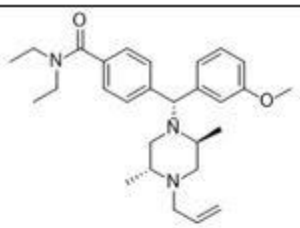
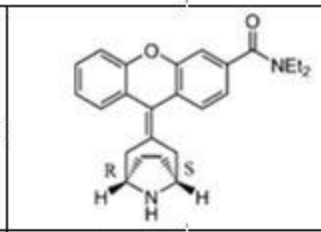
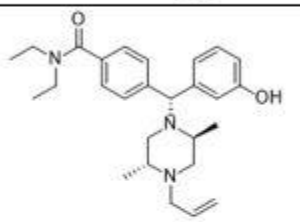
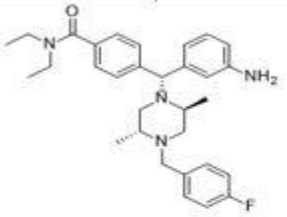
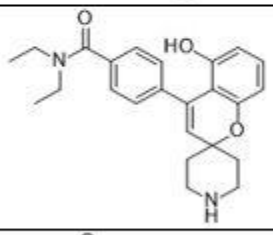
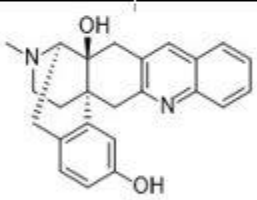
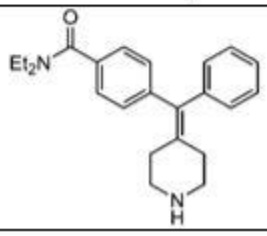
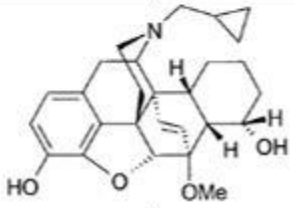


Figure 1.2. Ascending and descending pain pathways. Primary afferent nociceptors relay noxious stimuli from the periphery to the spinal cord. In the dorsal horn of the spinal cord, nociceptors synapse onto interneurons that signal to second order neurons that relay information up the spinal cord to the brain stem and thalamus. Third order neurons receive signals in the thalamus and relay them to the somatosensory cortex of the brain where the noxious stimuli is interpreted as pain. The descending pathway involves efferent signaling from the PAG and raphe nuclei to the spinal cord that inhibits the ascending pathway (yellow arrow). Figure adapted from Zhou and Verne 2014).

Table 1.1. Chemical Structures of Representative Delta Opioid Receptor Agonists

SNC80		JNJ-20788560	
BW373U86		AZD2327	
ADL5859		KNT-127	
ARM390		BU48	

Chapter II

The Role of Regulator of G Protein Signaling 4 in Delta-Opioid Receptor-Mediated Behaviors

Introduction

Regulator of G protein signaling 4 (RGS4) is a member of the R4 subfamily of RGS proteins and interacts with $G\alpha_{i/o}$ proteins (Hollinger and Hepler 2002). Like other RGS proteins, RGS4 inhibits G protein signaling by binding Gα and accelerating Gα-mediated GTP hydrolysis. This acceleration of GTP hydrolysis shortens the lifetime of the active Gα-GTP complex and limits signal transduction to downstream effectors. RGS4 is highly expressed in multiple brain regions including the cerebral cortex, amygdala, hippocampus and striatum (Nomoto et al. 1997) and negatively regulates signaling at multiple G protein-coupled receptor (GPCR) types including 5-HT1A (Gu et al. 2007), M3 muscarinic (Blazer et al. 2015), and delta (DOR) and mu (MOR) opioid receptors (Wang et al. 2009; Leontiadis et al. 2009).

Opioid receptors are class A GPCRs that couple to $G\alpha_{i/o}$ proteins. In rodents, activation of DOR induces antinociception, antihyperalgesia, and antidepressant-like effects without the constipation, respiratory depression, and abuse liability associated with mu opioid receptor agonists (for review see Chu Sin Chung and Kieffer 2013). Some DOR agonists also produce convulsions, hindering their development as therapeutics (Comer et al. 1993; Hong et al. 1998). Little is known about the signaling molecules and pathways involved in DOR-mediated behaviors and potential therapeutic effects. DOR is abundantly expressed in brain regions with high RGS4 expression (Lutz and Kieffer 2013). There are multiple, albeit somewhat discrepant, reports on the role of RGS4 in modulating opioid receptor function. In transfected HEK293 cells, RGS4 is recruited to the plasma membrane upon agonist-induced activation of DOR or MOPr where it associates with either receptor (Leontiadis et al. 2009). RGS4 also attenuated DOR- and MOPr-mediated phosphorylation of ERK in those cells. However, in a SH-SY5Y neuroblastoma

cell line endogenously expressing RGS4, DOR, and MOPr, siRNA-induced inhibition of RGS4 potentiated ERK phosphorylation and inhibition of cAMP accumulation mediated by DOR, but not MOPr (Wang et al. 2009). *In vivo*, a single dose of the DOR agonist SNC80 (5 mg/kg) in the mouse forced swim test (FST) decreased immobility to a greater degree in RGS4 knockout mice relative to wild-type mice (Stratinaki et al. 2013), suggesting that RGS4 may also regulate DOR signaling *in vivo*; however, its function is not clear based on this single dose experiment.
The role of RGS proteins in modulating other DOR-mediated behaviors has not been fully elucidated. Determining the intracellular signaling pathways that give rise to DOR-mediated behaviors, and DOR-mediated convulsions in particular, is critical for the development of DOR drugs with improved safety and clinical utility. Therefore, to better understand the downstream signaling mechanisms, specifically the role of RGS4, contributing to DOR-mediated behaviors, we evaluated the effects of the DOR selective agonist SNC80 to induce antinociception, antihyperalgesia, antidepressant-like effects, and convulsions in wildtype and RGS4 knockout mice.

Materials and Methods

Subjects

The Rgs4tm1Dgen/J mouse strain was obtained from The Jackson Laboratory (Bar Harbor, Maine, http://jaxmice.jax.org/strain/ 005833.html; Cifelli et al. 2008). Mice were backcrossed at least six generations into a C57BL/6 background and maintained in-house as heterozygote harem (1 male, 2 female) breeding groups. Wild-type littermates (+/+) were used as controls in all experiments involving C57RGS4 heterozygote (+/R4) and homozygote (R4/R4) knockout mice. For studies in which transgenic mice were not required, C57BL/6N mice (17-30g) were obtained from Envigo (formerly Harlan, Indianapolis, IN). Mice were housed in groups of two to four animals per cage. Animals were used between 8 and 15 weeks of age at time of experiment and weighed 15-32 g. Mice had free access to standard lab chow and water and were maintained in a temperature- and humidity-controlled environment on a 12-h dark/light cycle with lights on at 7:00 AM. All animal use procedures complied with the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health, and were approved by the

University of Michigan Institutional Committee on the Use and Care of Animals. Mice were tested only once, and all analyses are between-subject.

Drugs

All drugs were injected at a volume of 10 ml/kg unless otherwise noted. SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was dissolved in 1 M HCl and diluted in sterile water to a concentration of 3% HCl. Naltrindole 5' Naltrindole was dissolved in sterile water. N-methylnaltrexone (Sigma-Aldrich, St. Louis, MO) was dissolved in saline. The RGS4-selective RGS inhibitor CCG-203769 (2-ethyl-4-butyl-1,2,4-thiadiazolidine-3,5-dione) was synthesized as previously described and dissolved in saline (Turner et al. 2012). Nitroglycerin (NTG) was provided by Dr. Adam Lauver (Department of Pharmacology, University of Michigan) and was diluted in saline. Glacial acetic acid (Mallinckrodt Specialty Chemicals, Paris, KY) was diluted in sterile water to a concentration of 0.6% and given as a standard 0.4 ml similar to published methods (Broom et al. 2000, 2002b). All drugs were administered subcutaneously (sc) except for NTG and acetic acid which were given by intraperitoneal (ip) injection.

Acetic Acid Stretch Assay

The acetic acid stretch assay protocol was performed as previously described (Hong et al. 1998; Broom et al. 2000; Broom et al. 2002). Male and female mice were injected with SNC80 (1, 3.2, 10, 32 mg/kg), morphine (0.1, 1 mg/kg), or vehicle and immediately placed in separate cages (18 X 28 X 13 cm). 30 minutes following drug injection, 0.4 ml of 0.6% acetic acid was administered ip. Animals were observed for abdominal stretches for 30 min beginning 5 min after acetic acid administration. Abdominal stretches were characterized by contraction of the abdominal musculature and extension of the hind limbs. CCG-203769 (0.01, 0.1, 1 mg/kg) was administered 3 min after injection of acetic acid (2 min before observation of stretching). Naltrindole (3.2 mg/kg) was injected 30 min prior to, and naltrexone (3.2 mg/kg) and N-methylnaltrexone (10 mg/kg) were injected 10 min prior to SNC80 administration.

Tail Suspension Test

The procedure for the tail suspension test (TST) was adapted from Steru et al (1985). Male C57RGS4 mice were given SNC80 (0.32, 1, 3.2, 10 mg/kg) or vehicle. After 30 min, mice were suspended by their tail from a height of ~35cm using self-sticking tape and their behavior was recorded for 6 minutes using a Sony HDR-CX220 digital camcorder. In one experiment for comparison with the forced swim test (described below), mice were given SNC80 (1, 3.2, 10 mg/kg) 60 min prior to the tail suspension test. Videos were analyzed by individuals blind to the experimental conditions, and the time (out of the total 6 min) which the animals spent immobile was quantified. Immobility was defined as the animal remaining motionless or making only minor, non-escape related movements.

Forced Swim Test

The forced swim test was adapted from Porsolt et al (1977). Sixty min after SNC80 (0.1, 0.32, 1, 3.2, 10, 32 mg/kg) or vehicle injection, each mouse was placed in a 4L beaker filled with 15 cm of 25±1°C water and its behavior was recorded for 6 min using a Sony HDR-CX220 digital camcorder. Videos were analyzed by individuals blind to the experimental conditions and the amount of time the animals spent immobile was quantified. Immobility was defined as the mouse not actively traveling through the water and making only movements necessary to stay afloat. The time the mouse spends immobile after the first 30 sec of the assay was recorded.

Nitroglycerin-Induced Hyperalgesia

The NTG-induced hyperalgesia assay was adapted from Bates et al (2010) using modifications described in Pradhan et al (2014). Male C57BL6 or male and female C57RGS4 mice were used to evaluate NTG-induced hyperalgesia. Hyperalgesia was assessed by immersing the tail (~5cm from the tip) in a 46°C water bath and determining the latency for the animal to withdraw its tail with a cut-off time of 60 sec. After determining baseline withdrawal latencies, 10 mg/kg NTG (ip) was administered to each animal. Tail withdrawal latency was assessed again 1 hr after NTG administration. At 90 min post-NTG, animals received an injection of SNC80 (0.32, 1, 3.2, 10 mg/kg) or vehicle, and mice were observed continuously in individual cages for 20 min to observe for convulsions (see section below). Tail withdrawal latencies were assessed again 30 min after SNC80 administration. Naltrindole (3.2 mg/kg) was injected 30 min prior to

SNC80 administration. Naltrexone (3.2 mg/kg) and N-methylnaltrexone (10 mg/kg) were injected 10 min prior to SNC80 administration (see Figure 2.4A).

SNC80-Induced Convulsions

Mice were observed continuously in individual cages for convulsions. NTG treatment had no effect on the frequency or nature of SNC80-induced convulsions (data not shown). Convulsions were comprised of a tonic phase characterized by sudden tensing of the musculature and extension of the forepaws followed by clonic contractions that extended the length of the body. Convulsions were followed by a period of catalepsy that lasted 2-5 min after which the animals were indistinguishable from untreated controls. Post-convulsion catalepsy was assessed by placing a horizontal rod under the forepaws of the mouse and a positive catalepsy score was assigned if the mouse did not remove its forepaws after 30 sec.

DOR Saturation Binding and Signaling Assay

RGS4 protein was identified in whole striatum of R4/R4 mice and wild-type littermates by Western blot as compared to purified RGS4 protein with a specific RGS4 antibody as previously described (Wang et al. 2009). α-Tubulin was used as a loading control. For saturation binding assays, mice were decapitated and whole brain or striatum was removed and membranes were freshly prepared as previously described (Broom et al. 2002b). Protein concentrations were determined with a BCA assay kit (Thermo Scientific, Rockford, IL). Specific binding of the DOR agonist [^{3}H]DPDPE was determined as described using 10μM of the opioid antagonist naloxone to define non-specific binding as described (Broom et al. 2002b). Reactions were incubated for 60 min at 26ºC and stopped by rapid filtration through GF/C filter mats using a MLR-24 harvester (Brandel, Gaithersburg, MD). Bound [^{3}H]DPDPE was determined by scintillation counting and B_{max} and K_d values calculated using nonlinear regression analysis with GraphPad Prism version 6.02 (GraphPad, San Diego, CA).

Whole striatal tissues from R4/R4 mice or wild-type littermates were incubated with or without 10 μM SNC80 for 5 min at 37 °C. Samples were prepared and subjected to gel electrophoresis as previously described (Wang et al. 2009). The level of activation of the MAPK pathway was determined by Western blotting with anti-phospho-p44/42MAPK (Thr202/Tyr204) antibody or anti-p44/42 MAPK antibody (Cell Signaling Technology, Danvers, MA). Images

were acquired and quantified using an Odyssey FC imaging system (Li-COR Biosciences, Lincoln, NE). MAPK activity was calculated as the ratio of normalized arbitrary units (a.u.) of phosphorylated ERK1/2 over total ERK1/2 and presented as percent of vehicle-treated control.

Data Analysis

Three-way ANOVA was performed using SPSS Statistics 22 (IBM, Armonk, NY). All other data analysis was performed using GraphPad Prism version 6.02 (GraphPad, San Diego, CA). Post hoc analysis was conducted using the Tukey's post hoc test to correct for multiple comparisons. For all tests, level of significance (α) was set to 0.05. ED_{50} values were calculated using GraphPad Prism version 6.02 by extrapolating the 50% maximum effect from the straight line analysis of the averaged treatment group data used to generate each dose effect function.

Results

Effects of RGS4 on DOR-Mediated Antinociception

To determine whether RGS4 plays a role in DOR-mediated antinociception, the effects of the DOR agonist SNC80 were evaluated in RGS4 wild-type (+/+), heterozygous (+/R4), and homozygous (R4/R4) mutant mice in the acetic acid stretch assay (Figure 2.1A). Two-way ANOVA revealed a significant interaction (SNC80 dose [1-32 mg/kg only] X genotype, $F(8,86) = 3.2$, $p = 0.0034$), as well as significant main effects of SNC80 dose ($F(4,86) = 15.4$, $p < 0.0001$) and genotype ($F(2,86) = 25.07$, $p < 0.0001$). Overall, the potency of SNC80 to reduce stretching was significantly increased in the RGS4 mutant mice as evidenced by an approximate 6-fold leftward shift in the +/R4 dose effect curve and 27-fold leftward shift in the dose effect curve of the R4/R4 mice (ED_{50} values: +/+: 41 mg/kg; +/R4: 6.9 mg/kg; R4/R4: 1.5 mg/kg).

Because elimination or reduced levels of RGS4 enhanced SNC80-induced antinociception, we evaluated the effects of pharmacological inhibition of RGS4 with CCG-203769 (Blazer et al. 2015) on SNC80-induced antinociception in C57BL6 wild-type mice (Figure 2.1B). Two-way ANOVA revealed a significant interaction (SNC80 dose X CCG-203769 dose, $F(3,42) = 3.9$, $p = 0.02$) and significant main effects of SNC80 dose ($F(1,42) = 24.3$, $p < 0.0001$) and CCG-203769 dose ($F(3,42) = 6.9$, $p = 0.0007$). Administration of either an ineffective SNC80 dose (3.2 mg/kg) or various doses of CCG-203769 alone did not alter

stretching behavior; however, CCG-203769 dose-dependently enhanced antinociception produced by 3.2 mg/kg SNC80, such that the combination of 3.2 mg/kg SNC80 with either 0.1 mg/kg or 1 mg/kg CCG-203769 significantly reduced stretching.

To assess the role of DOR in SNC80-induced antinociception in the acetic acid stretch assay, RGS4 knockout mice were pretreated with the DOR-selective antagonist naltrindole (Figure 2.1C). Three-way ANOVA revealed a significant main effect of SNC80 ($F(1,63) = 38.7$, $p < 0.0001$) and a trend towards a significant SNC80 X genotype interaction ($F(2,63) = 2.5$, $p = 0.087$). SNC80-induced reduction of stretching was completely blocked upon pretreatment with 3.2 mg/kg naltrindole (SNC80 dose X naltrindole dose, $F(1,63) = 52.2$, $p < 0.0001$), indicating a DOR-mediated effect. This effect of naltrindole did not differ between RGS4 wild-type and mutant mice.

To investigate whether the effects of SNC80 in the acetic acid stretch assay were peripherally- or centrally-mediated, C57BL6 wild-type mice were pretreated with the nonspecific opioid antagonist naltrexone or its peripherally-limited analog N-methylnaltrexone in the acetic acid stretch assay (Figure 2.1D). Administration of 32 mg/kg SNC80 significantly reduced stretching in vehicle-pretreated animals, but pretreatment with either 3.2 mg/kg naltrexone or 10 mg/kg N-methylnaltrexone significantly attenuated the SNC80-induced reduction in stretching (one-way ANOVA: $F(3,20) = 14.6$, $p < 0.0001$).

To determine whether the role of RGS4 in opioid-induced antinociception was limited to DOR-mediated antinociception, the mu opioid receptor agonist actions of morphine were evaluated in RGS4 transgenic mice in the acetic acid stretch assay (Figure 2.1E). The main effect of morphine dose was significant ($F(2,45) = 28.5$, $p < 0.0001$) but there was no main effect of genotype and no morphine dose X genotype interaction. Increasing doses of morphine produced similar decreases in stretching in RGS4 +/+, +/R4, and R4/R4 mice. In addition, pharmacological inhibition of RGS4 by CCG-203769 did not alter the effects of morphine in the acetic acid stretch assay (Figure 2.1F). Treatment of C57BL6 wild-type mice with 0.1 mg/kg morphine significantly reduced stretching relative to vehicle treated controls ($F(1,26) = 13.2$, $p = 0.001$) and this decrease was not enhanced by administration of 1 mg/kg CCG-203769.

Effects of RGS4 on DOR-Mediated Antidepressant-like Effects

The effects of SNC80 on immobility time in the forced swim test were evaluated in RGS4 +/+, +/R4, and R4/R4 mice (Figure 2.2A). Two-way ANOVA revealed a significant interaction (SNC80 dose [0.1-10 mg/kg only] X genotype, $F(10,102) = 6,1$, $p < 0.0001$) and a significant main effect of SNC80 dose ($F(5,102) = 17.1$, $p < 0.0001$), but no effect of genotype. In all three genotypes, SNC80 produced a U-shaped dose effect curve with similar magnitude of maximum effect, albeit at different doses (+/+: 10 mg/kg, +/R4 and R4/R4: 1 mg/kg). Both the descending and ascending limbs of this U-shaped curve were shifted to the left in both the +/R4 and R4/R4 mice, indicating an increase in the potency, but not efficacy, of SNC80.

To further probe the role of RGS4 in DOR-mediated antidepressant-like effects, the effects of SNC80 were evaluated in the tail suspension test (TST) in RGS4 +/+, +/R4, and R4/R4 mice (Figure 2.2B). Doses up to 3.2 mg/kg SNC80 produced dose-dependent decreases in immobility in RGS4 +/+, +/R4, and R4/R4 mice; however, the largest dose tested failed to further decrease immobility. The effect of SNC80 dose was significant ($F(4,98) = 18.2$, $p < 0.0001$) but there was only a non-significant trend for genotype ($F(2,98) = 2.8$, $p = 0.07$) and a non-significant SNC80 dose X genotype interaction. Although the magnitude of effect of SNC80 on immobility reduction was slightly greater in the R4/R4 mice, this effect was not statistically significant. There was no difference in the magnitude of effect or shape of the dose effect curve in RGS4 heterozygous mutant mice treated with SNC80 30 or 60 minutes prior to the tail suspension test.

Effects of RGS4 on DOR-Mediated Convulsions

SNC80 produced dose-dependent increases in convulsion frequency in RGS4 +/+, +/R4, and R4/R4 mice (Figure 2.3A). These convulsions were blocked by pretreatment with 3.2 mg/kg naltrindole in all genotypes (Figure 2.3B). There were no significant differences in the frequency, time of onset, and duration (data not shown) of convulsions in the +/R4 or R4/R4 animals relative to wild-type littermates for a given dose of SNC80. There were also no differences between genotypes in the frequency of convulsions produced by the chemical convulsant pentylenetetrazol (PTZ; data not shown).

To evaluate whether SNC80-induced convulsions are centrally-mediated, C57BL6 wild-type mice were pretreated with the nonspecific opioid antagonist naltrexone or its peripherally-

restricted analog N-methylnaltrexone (Figure 2.3C). 10 mg/kg and 32 mg/kg SNC80 produced convulsions that were blocked by pretreatment with naltrexone but not N-methylnaltrexone.

Effects of RGS4 on DOR-Mediated Antihyperalgesia

In order to determine if RGS4 can modulate DOR-mediated antihyperalgesia, the potency of SNC80 to reverse NTG-induced thermal hyperalgesia in RGS4 +/+, +/R4, and R4/R4 mice was assessed (Figure 2.4B). There were no differences between genotypes in the baseline tail withdrawal latencies prior to NTG treatment (+/+: 39.1 ± 2.4 s, +/R4: 43.3 ± 2.2 s, R4/R4: 42.4 ± 2.3 s). Administration of 10 mg/kg NTG (ip) significantly decreased tail withdrawal latency to a similar degree in all genotypes (+/+: 7.4 ± 1.2 s, +/R4: 9.0 ± 1.1 s, R4/R4: 8.3 ± 0.6 s). Reduction of RGS4 enhanced the ability of SNC80 to increase tail withdrawal latency. Two-way ANOVA revealed a significant interaction (SNC80 dose X genotype, $F(8,92) = 2.6$, $p = 0.01$), as well as significant main effects of SNC80 dose ($F(4,92) = 25.3$, $p < 0.0001$) and genotype ($F(2,92) = 20.9$, $p < 0.0001$). This enhanced effect of SNC80 resulted in a pronounced leftward shift (~10-fold) in the dose response curve, indicating a significant increase in the potency of SNC80.

To evaluate the role of DOR in SNC80-induced antihyperalgesia, C57BL6 wild-type mice were pretreated with the DOR selective antagonist naltrindole (Figure 2.4C). Two-way repeated measure ANOVA revealed a significant interaction (NTI dose X time point, $F(2,20) = 8.7$, $p = 0.01$), and a significant main effect of NTI dose ($F(1,10) = 5.0$, $p = 0.05$), indicating a DOR-mediated effect. To evaluate the relative contributions of central and peripheral opioid receptors to SNC80-induced antihyperalgesia, C57BL6 wild-type mice were pretreated with the nonselective opioid antagonist naltrexone or the peripherally-limited analog N-methylnaltrexone (Figure 2.4D). Two-way repeated measure ANOVA revealed a significant interaction (pretreatment X time point, $F(4,32) = 3.0$, $p = 0.03$) and a significant main effect of pretreatment ($F(2,16) = 4.4$, $p = 0.03$). Post-hoc analysis further revealed that SNC80-induced increases in tail withdrawal latency were blocked by naltrexone ($p < 0.01$) but not by N-methylnaltrexone.

Elimination of RGS4 Enhances DOR Signaling But Does Not Affect DOR Number

To evaluate the effect of RGS4 on DOR signaling in brain, whole striatum of R4/R4 mice or their wild-type littermate controls were treated with the DOR agonist SNC80 (1.0 μM) and phosphorylation of ERK1/2 was examined. This concentration of SNC80 did not increase

ERK1/2 phosphorylation in striatum as compared with vehicle treatment in RGS4 +/+ mice but caused a marked increase in ERK1/2 phosphorylation as compared with vehicle in striatal tissue from R4/R4 knockout mice (125 ± 67%) (Figure 2.5B).

Western blot analysis confirmed a lack of RGS4 protein expression in the RGS4 knockout mice (Figure 2.5A). However, it is possible that the enhanced behavioral effects of SNC80 in RGS4 mutant mice are due to elevated density or agonist affinity of DOR relative to their wild-type littermates. Saturation binding with the radiolabeled DOR agonist [^{3}H]DPDPE was performed with brain tissue from RGS4 +/+, +/R4, and R4/R4 mice to assess potential changes in DOR density and agonist affinity (Figure 2.5C). There were no significant differences in total receptor number between the three genotypes (Table 2.1). There were also no changes in the K_d of [^{3}H]DPDPE for DOR in the RGS4 mutant mice (Table 2.1).

Discussion

In this report, we demonstrate that RGS4 differentially regulates DOR-mediated behaviors acting as a negative regulator of some, but not all, behavioral outcomes. In the acetic acid stretch assay (antinociception), SNC80 alleviated acid-induced stretches at a relatively large dose (32 mg/kg) consistent with previous reports (Broom et al. 2002b; Gallantine and Meert 2005; Negus et al. 2012). Large doses of SNC80 (10 and 32 mg/kg) were also required to reverse NTG-induced thermal hyperalgesia, consistent with the findings of Pradhan et al. (2014). SNC80-induced antinociception, but not hyperalgesia, was blocked by pretreatment with the peripherally-restricted opioid antagonist N-methylnaltrexone, suggesting peripheral DOR likely mediate the antinociceptive actions of SNC80 in the acetic acid stretch assay while central DOR mediated SNC80-induced antihyperalgesia. Genetic loss of RGS4 or acute pharmacological inhibition of RGS4 with CCG-203769 increased the potency of SNC80 to produce antinociception and antihyperalgesia. The fact that significant changes are seen in both the heterozygote (+/R4) and homozygote (R4/R4) knockout mice suggests that DOR-mediated signaling is exquisitely sensitive to the actions of RGS4. Overall, these observations suggest that central and peripheral DOR within various pain pathways and neurocircuits are likely co-localized with RGS4 proteins to modulate DOR signaling and DOR-induced pain relief *in vivo*.

Interestingly, pharmacological inhibition or genetic alterations of RGS4 did not alter the potency of the MOPr agonist morphine to produce antinociception in the acetic acid stretch assay, similar to other reports *in vivo* (Han et al. 2010) and in SHSY5Y cells endogenously expressing opioid receptors and RGS4 proteins (Wang et al. 2009). However, RGS4 proteins have been shown to alter MOPr signaling *in vitro* and *in vivo*, depending on the cell type, brain region, or MOPr agonist used. For example, RGS4 was shown to regulate the rewarding effects of morphine and the antinociceptive effects of the MOPr agonists methadone and fentanyl but not morphine (Han et al. 2010). In addition, in heterologous expression systems, RGS4 can regulate MOPr signaling (Leonidas et al. 2009; Talbot et al. 2010). Together, these data suggest that RGS4 proteins may have the capacity to regulate MOPr-mediated signaling and behaviors as long as the appropriate proteins are co-expressed in the necessary cells and/or circuits.

It has previously been shown that loss of RGS4 altered SNC80 action in the mouse forced swim test (Stratinaki et al. 2013); however, this report only examined a single dose of SNC80 (5 mg/kg) in one behavioral assay making it difficult to fully assess the role of RGS4 in SNC80-induced antidepressant-like effects. This report sought to expand on those findings and shows that RGS4 plays a complex role in regulating DOR-mediated antidepressant-like effects, depending on the type of assay employed. In wild-type mice, SNC80 produced decreases in immobility in the forced swim and tail suspension tests, consistent with previous work showing that DOR agonists produce antidepressant-like effects in these assays (Broom et al. 2002a; Naidu et al. 2007; Saitoh et al. 2011). In both assays, SNC80 produced U-shaped dose effect curves, such that larger doses of SNC80 (10 mg/kg in TST, 32 mg/kg in FST) failed to produce significant antidepressant-like effects. The lack of effect observed with large SNC80 doses is possibly due to competing behaviors, such as possible recovery from recent seizure events or excessive locomotor stimulation (Chu Sin Chung et al. 2015). In the forced swim test, elimination of RGS4 shifted the entire U-shaped function to the left, indicating that RGS4 acts as a negative regulator of DOR-mediated antidepressant-like effects (as well as any possible competing behaviors) in this assay. However, loss of RGS4 activity had no significant effect on SNC80-induced antidepressant-like effects in the tail suspension test. It is unlikely that different SNC80 pretreatment times were responsible for the different effects of RGS4 in the TST and FST because even longer pretreatments (60 min) in the TST did not alter the effects of SNC80 in RGS4 heterozygous knockout mice. Also, relatively similar doses of SNC80 produced

antidepressant-like effects in both assays, so it is unlikely that differences in receptor occupancy or efficacy requirement could account for this disparity between the TST and FST. Differences in drug responses between these two behavioral assays have been reported previously (for review see, Cryan et al. 2005), and it has been argued that the biological substrates underlying these behaviors may be distinct. Therefore, it is possible that the behavioral effects of SNC80 in the two assays may be governed by separate brain regions, behavioral mechanisms, and/or signaling pathways that are differentially dependent on RGS4 signaling.

In contrast to the role of RGS4 in antinociception, antihyperalegsia, and antidepressant-like effects in the FST (but not the TST), reductions in RGS4 did not alter the potency of SNC80 to induce convulsions. The ability of RGS4 to alter a behavioral endpoint does not appear to be correlated with the potency of SNC80, since similar doses of SNC80 were required to produce convulsions, antinociception, and antihyperalgesia in wild-type mice. Alternatively, if we measured more subtle electroencephalographic activity rather than overt convulsive behavior (Jutkiewicz et al. 2006), we may have been able to observe an effect of RGS4 on this endpoint and future studies will evaluate this. In the present study, convulsions were evaluated in mice that received injections of NTG to induce hyperalgesia in an attempt to reduce the number of animals used. While NTG did not alter the frequency or nature of SNC80-induced convulsions in wild-type mice, it is possible that NTG masked or altered the dependency of convulsions on RGS4. Nevertheless, these results suggest that RGS4 selectively regulates signaling pathways mediating different behavioral outcomes of DOR activation and is likely not involved in the signaling mechanisms mediating convulsions.

RGS proteins function as negative regulators of G protein signaling by binding Gα-GTP and accelerating Gα-mediated GTP hydrolysis which returns Gα to an inactive state. Loss of RGS function should prolong the lifetime of active Gα and increase downstream signaling. Consistent with this theory, the present study demonstrated that loss of RGS4 potentiated DOR-mediated: 1) phosphorylation of ERK1/2 in mouse striatal tissue, 2) peripheral antinociception, 3) central antihyperalgesia, and 4) antidepressant-like effects measured in the FST, likely due to prolongation of DOR-mediated G protein signaling and amplification of downstream effectors. These findings identify some specific DOR-mediated behaviors and downstream signaling molecules in certain brain regions that are regulated by RGS4. However, DOR-mediated convulsions and behaviors in the TST were not significantly altered by reductions in RGS4,

suggesting that different signaling pathways may underlie these behaviors. DOR and RGS4 may not be expressed in the same neurons within circuits mediating these behavioral outcomes. For example, while DOR and RGS4 are both highly expressed in the hippocampus, the proposed origin site of convulsions (Simmons and Chavkin 1996; Chung et al. 2015), they may not be co-expressed in the same cells and, therefore, may not functionally interact. It is also possible that RGS proteins other than RGS4 modulate the DOR-induced convulsive effects and behaviors in the TST. Alternatively, these specific DOR-mediated behaviors may be generated by a G protein-independent, arrestin-mediated signaling mechanism (Violin 2014). Previous studies have demonstrated that DOR activation leads to signaling through G protein-dependent and -independent pathways (Bradbury et al. 2009; Charfi et al. 2014; Charfi et al. 2015). However, there are few reports connecting these distinct signaling mechanisms to specific behavioral outputs (Chiang et al. 2016; Pradhan et al. 2016). In conclusion, this study demonstrates that RGS4 differentially regulates SNC80-induced behaviors, suggesting that different molecular or cellular signaling pathways or neurocircuitry mediate these behavioral outcomes. Future work will investigate the role of other RGS proteins in DOR-mediated convulsions and the underlying signaling mechanism and pathways mediating the convulsive and other behavioral effects of DOR agonists.

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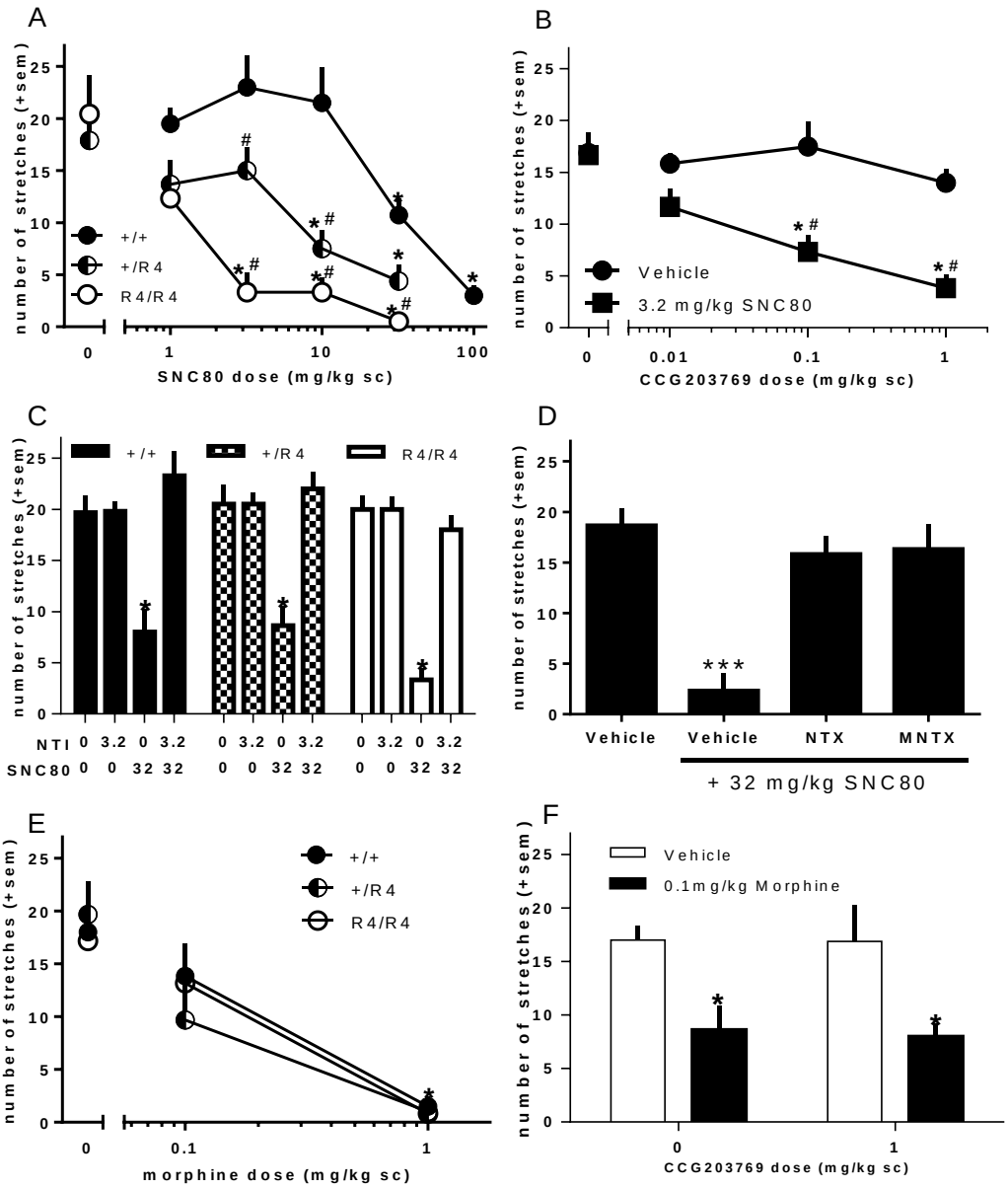

Figure 2.1 Effects of RGS4 on opioid-mediated antinociception in the mouse acetic acid stretch assay. Number of acetic acid-induced stretches after treatment with (A) different doses of SNC80 in RGS4 wild-type and mutant (+/R4, R4/R4) mice, (B) different doses of the RGS4 inhibitor CCG-203769 in combination with an inactive SNC80 dose (3.2 mg/kg) or SNC80 vehicle in C57BL6 wild-type mice, (C) SNC80 following pretreatment with 3.2 mg/kg of the DOR antagonist naltrindole in RGS4 wild-type and mutant mice (D) the MOPr agonist morphine in RGS4 wild-type and mutant mice, (E) different doses of the RGS4 inhibitor CCG-203769 in combination with a low dose of morphine or vehicle, (F) SNC80 following pretreatment with 3.2 mg/kg of the opioid antagonist naltrexone or 10 mg/kg of the peripherally-restricted opioid antagonist N-methylnaltrexone. n = 6-9 mice per group for all experiments. Data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle treatment in the same genotype, *** $p < 0.001$ compared to vehicle treatment, # $p < 0.05$ compared to wild-type mice or control condition with same drug dose.

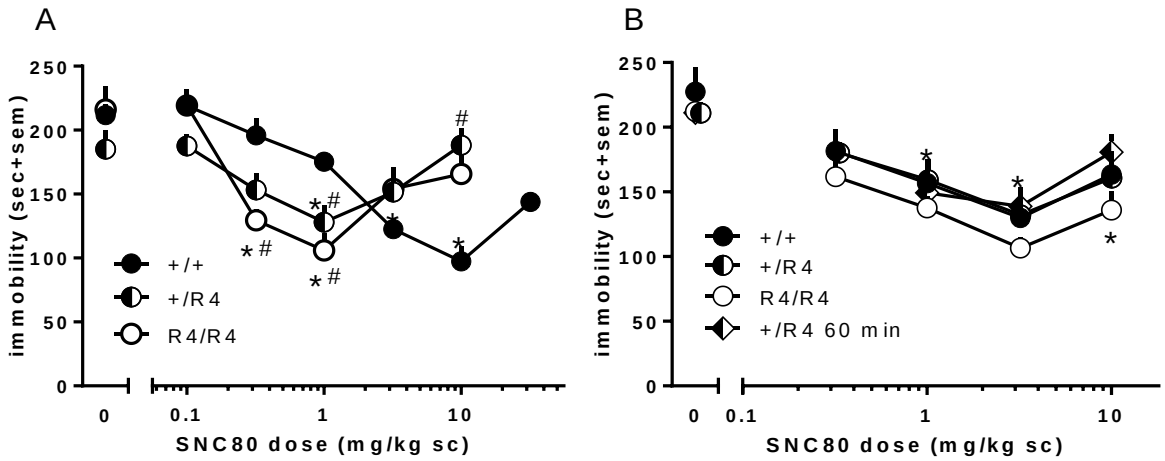

Figure 2.2 Effects of RGS4 on DOR-mediated antidepressant-like effects. Immobility scores of RGS4 wild-type and mutant (+/R4, R4/R4) mice following treatment with different doses of SNC80 in the (A) forced swim test, and (B) tail suspension test. n = 6-10 mice per group and data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle treatment in the same genotype, # $p < 0.05$ compared to wild-type mice with same drug dose.

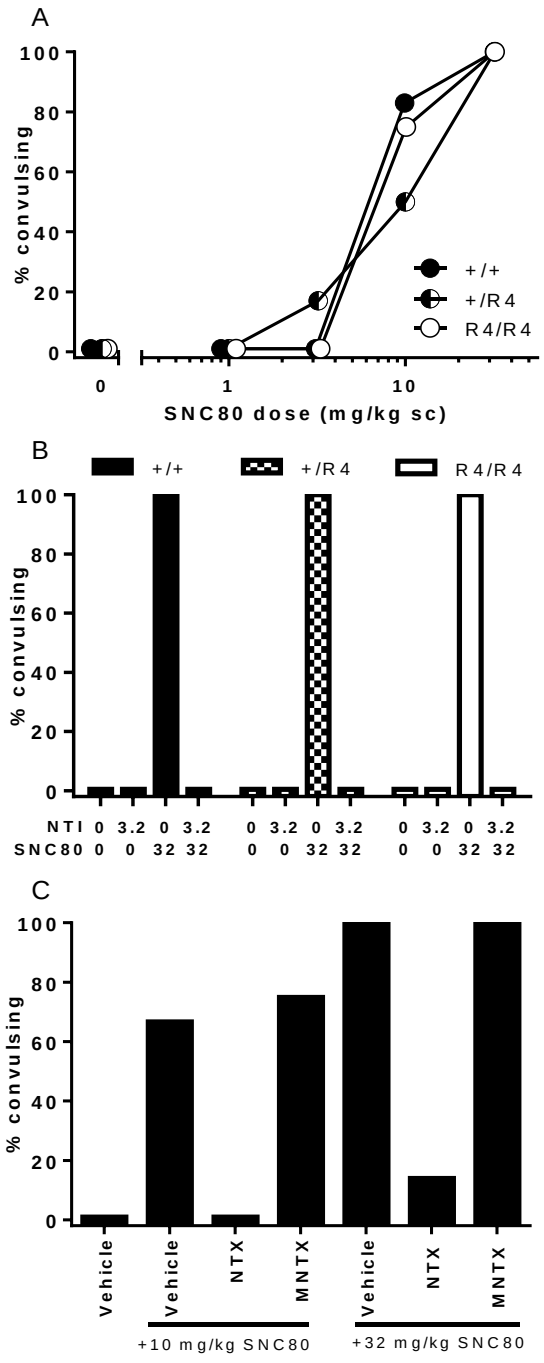

Figure 2.3 Frequency of SNC80-induced convulsions (A) in RGS4 wild-type and mutant (+/R4, R4/R4) mice, (B) following pretreatment with 3.2 mg/kg of the DOR antagonist naltrindole in RGS4 wild-type and mutant mice, (C) following pretreatment with 3.2 mg/kg of the opioid antagonist naltrexone or 10 mg/kg of the peripherally-restricted opioid antagonist N-methylnaltrexone in C57BL6 wild-type mice. n = 6-12 mice per group for all experiments and data are shown as average per treatment condition with standard error of the mean (sem).

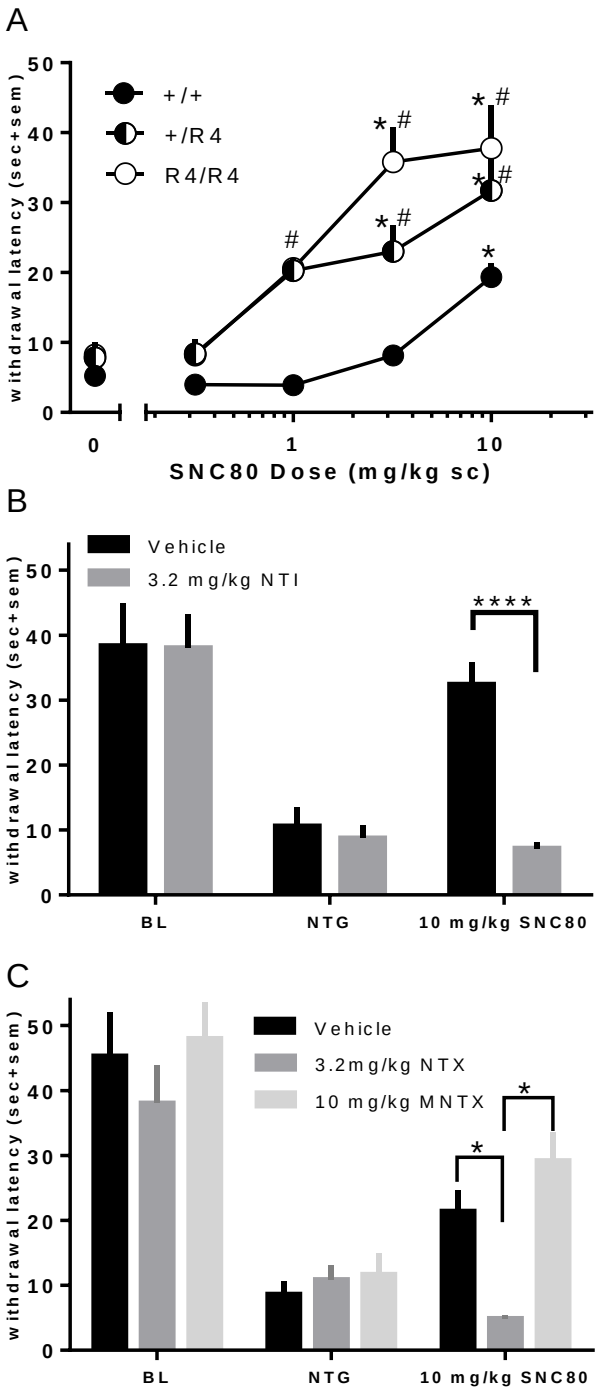

Figure 2.4. (A) Diagrams of NTG-induced hyperalgesia test schedule, depending on antagonist treatment. (B) Effect of different doses of SNC80 on tail withdrawal latency in NTG treated RGS4 wild-type and mutant (+/R4, R4/R4) mice. (C) Effect of the DOR antagonist NTI or vehicle on SNC80-induced antihyperalgesia in C57BL6 mice. (D) Effect of pretreatment with vehicle, 3.2 mg/kg NTX, or 10 mg/kg MNTX on SNC80-induced antihyperalgesia in C57BL6 mice. n = 6-10 mice per group for all experiments and data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle or NTX treatment in the same genotype, # $p < 0.05$ compared to wild-type mice with same drug dose.

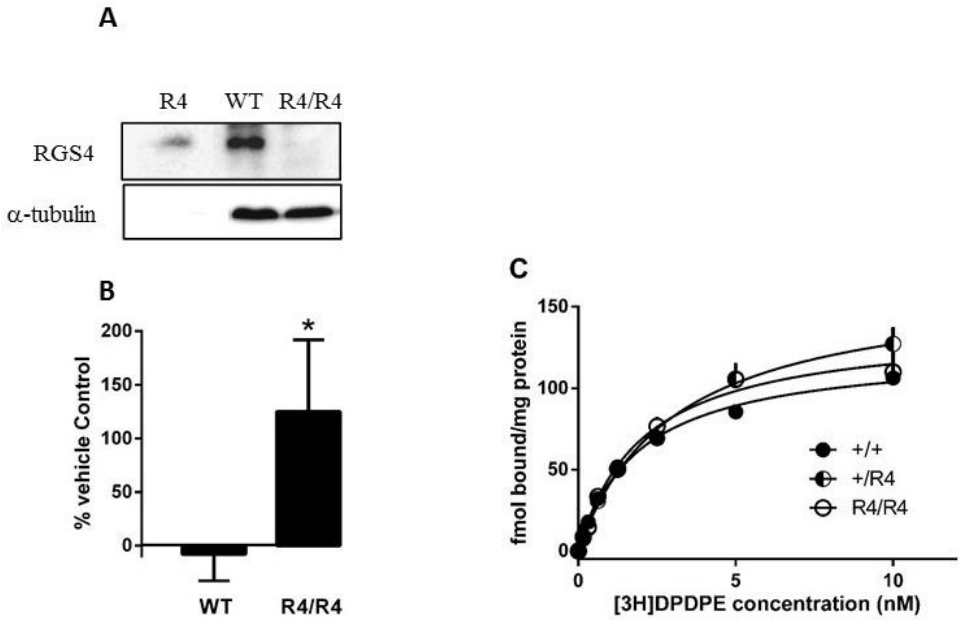

Figure 2.5. (A) RGS4 protein expression by Western blot. Lysates were prepared from striatal brain tissue from wildtype and R4/R4 mice as compared with RGS4 purified protein. (B) SNC80-mediated stimulation of ERK1/2 phosphorylation over vehicle control in striatal tissue from wild-type and R4/R4 mice ($t(5.4)=1.9$, $p=0.05$). (C) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of RGS4 wild-type or mutant (+/R4, R4/R4) mice. Points represent data averaged (shown with standard error of the mean) from 3-4 mice, each assayed in triplicate.

Table 2.1 DOR density and agonist affinity in RGS4 knockout mice

Genotype	B_{max} (fmol/mg ± sem)	[^{3}H] DPDPE K_d (nM)
+/+	124 ± 8	2 ± 0.4
+/R4	165 ± 12	2 ± 0.5
R4/R4	138 ± 9	3 ± 0.3

Chapter III

In Vivo Consequences of Functional Selectivity at the Delta Opioid Receptor

Introduction

G protein-coupled receptors (GPCRs) are a diverse family of membrane bound receptors that regulate a wide array of biological functions. Canonically, GPCRs regulate these processes through activation of G proteins which subsequently interact with a variety of downstream effectors. Following agonist activation, a GPCR is phosphorylated by G protein-coupled receptor kinases (GRKs) and internalized by arrestins. In recent years, it has become apparent that GPCRs can signaling through G protein-independent mechanisms (Galandrin et al. 2007) by directly recruiting arrestins that can also promote signaling from GPCRs (Reiter et al. 2012). Furthermore, ligands that act at the same orthosteric site on a receptor can stabilize distinct active conformations that preferentially signal through distinct G protein or arrestin subtypes. This phenomenon, known as functional selectivity or biased agonism, has been observed with multiple GPCRs including the β2 adrenergic receptor (Drake et al. 2008), the CB1 cannabinoid receptor (Hudson et al. 2010), as well as mu, kappa, and delta opioid receptors (Pradhan et al. 2012).

The delta opioid receptor (DOR) is a class A GPCR and interacts with $G\alpha_{i/o}$ proteins. Activation of DOR in rodents has been shown to produce antinociception, antihyperalgesia, and antidepressant-like effects without the constipation, respiratory depression, and abuse liability observed with mu opioid receptor agonists (for review see Chu Sin Chung and Kieffer 2013). In addition, some DOR agonists also cause convulsions, which has limited their clinical utility (Comer et al. 1993; Hong et al. 1998).

The signaling pathways that bring about DOR-mediated behaviors are only beginning to be understood. Targeted knockdown of specific G protein subunits using antisense nucleotides

inhibited DOR-mediated spinal and supraspinal antinociception in mice, implicating multiple $G\alpha_{i/o}$ subtypes in the regulation of these effects (Standifer et al. 1996; Sánchez-Blázquez and Gárzon 1998). Loss of regulator of G protein signaling 4 (RGS4) potentiated the antinociceptive, antihyperalgesic, and antidepressant-like effects of the DOR agonist SNC80 suggesting that these behaviors are generated through G protein signaling (Dripps et al. 2017). However, this study also found that the frequency of SNC80-induced convulsions was not altered in RGS4 knockout mice suggesting that DOR-mediated convulsions may signal through a G protein-independent mechanism. Loss of arrestin 2 (β-arrestin 1) increased the potency of SNC80 to induce mechanical antihyperalgesia, whereas loss of arrestin 3 (β-arrestin 2) produced acute tolerance to the antihyperalgesic effects of the DOR agonists ARM390 and JNJ20788560 (Pradhan et al. 2016).

Use of a drug that is biased towards producing the analgesic and antidepressant-like effects of DOR could be an effective strategy for improving the safety and clinical utility of DOR agonists. A detailed understanding of the intracellular signaling pathways that give rise to DOR-mediated behaviors, and DOR-mediated convulsions in particular, is critical for the development of such drugs. Therefore, to gain a better understanding of the downstream signaling mechanisms that give rise to DOR-mediated behaviors, we evaluated the potency of SNC80 to produce antihyperalgesia, antidepressant-like effects, and convulsions in $G\alpha_o$ heterozygous knockout mice, $G\alpha_o$ RGS-insensitive heterozygous knock-in mice, as well as arrestin 2 and arrestin 3 knockout mice.

Materials and Methods

Subjects

All animal use procedures complied with the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health, and were approved by the *University of Michigan Institutional Committee on the Use and Care of Animals*. All animal studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.,* 2010; McGrath *et al.,* 2010). Mice were housed in groups of four to five animals per cage. All mice were used between 8 and 15 weeks of age at time of experiment and weighed 16-32 g. Mice had free access to standard lab chow and water and were maintained in a temperature- and

humidity-controlled environment on a 12-h dark/light cycle with lights on at 7:00 AM. Mice were tested only once, and all analyses are between-subject with the exception of the hot plate test (within-subject analysis).

The arrestin 3 knockout mouse strain ($Arrb2^{tm1Rjl/J}$) was obtained from The Jackson Laboratory (Bar Harbor, Maine, https://www.jax.org/strain/011130). Arrestin 2 knockout mice ($Arrb1^{tm1jse}$, https://www.jax.org/strain/011131) were provided by Dr. Amynah A. Pradhan (University of Illinois at Chicago). $G\alpha_o$ RGS-insensitive heterozygous knock-in mice (Goldstein et al. 2009) were obtained from Dr. Richard Neubig and $G\alpha_o$ knockout mice were obtained from Dr. Richard Mortensen (Duan et al. 2007). Mice were backcrossed at least six generations into a C57BL/6 background and maintained in-house as heterozygote harem (1 male, 2 female) breeding groups except for arrestin 2 knockout mice which were maintained as homozygote harem breeding groups. Wild-type littermates (+/+) were used as controls for all strains except arrestin 2 knockout mice in which case arrestin 3 wild-type littermates were used. For studies in which transgenic mice were not required, C57BL/6N mice (17-30g) were obtained from Envigo (formerly Harlan, Indianapolis, IN).

Drugs

All drugs were injected at a volume of 10 $ml \cdot kg^{-1}$unless otherwise noted. SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was dissolved in 1 M HCl and diluted in sterile water to a concentration of 3% HCl. Nitroglycerin (NTG) was provided by Dr. Adam Lauver (Department of Pharmacology and Toxicology, Michigan State University) at a concentration of 5mg/ml and was diluted in saline. Desipramine hydrochloride (Sigma-Aldritch, St. Louis, MO), sumatriptan succinate (Sigma-Aldritch, St. Louis, MO) and morphine sulfate (RTI International, Research Triangle Park, NC) were dissolved in saline. All drugs were given subcutaneously (sc) except for NTG which was administered via intraperitoneal (ip) injection.

Forced Swim Test

The forced swim test (FST) was adapted from Porsolt et al (1977) and performed as previously described (Dripps et al. 2017). Briefly, sixty min after SNC80 (0.1, 0.32, 1, 3.2, 10, or 32 $mg \cdot kg^{-1}$) or vehicle injection, each mouse was placed in a 4L beaker filled with 15 cm of

25±1°C water and its behavior was recorded for 6 min using a Sony HDR-CX220 digital camcorder. Videos were analyzed by individuals blind to the experimental conditions and the amount of time the animals spent immobile was quantified. Immobility was defined as the mouse not actively traveling through the water and making only movements necessary to stay afloat. The time the mouse spends immobile after the first 30 sec of the assay was recorded.

Nitroglycerin-Induced Hyperalgesia

The NTG-induced hyperalgesia assay was adapted from Bates et al (2010) using modifications described in Pradhan et al (2014) and performed as previously described (Dripps et al. 2017). In brief, male and female C57RGS4 mice were used to evaluate NTG-induced hyperalgesia. Hyperalgesia was assessed by immersing the tail (~5cm from the tip) in a 46°C water bath and determining the latency for the animal to withdraw its tail with a cut-off time of 60 sec. After determining baseline withdrawal latencies, 10 $mg \cdot kg^{-1}$ NTG (ip) was administered to each animal. Tail withdrawal latency was assessed again 1 hr after NTG administration. At 90 min post-NTG, animals received an injection of SNC80 (0.32, 1, 3.2, 10, or 32 $mg \cdot kg^{-1}$) or vehicle, and mice were observed continuously in individual cages for 30 min to observe for convulsions (see section below). Tail withdrawal latencies were assessed again 30 min after SNC80 administration.

SNC80-Induced Convulsions

Mice were observed continuously for 30 min in individual cages for convulsions. NTG treatment had no effect on the frequency or nature of SNC80-induced convulsions (data not shown). Convulsions were comprised of a tonic phase characterized by sudden tensing of the musculature and extension of the forepaws followed by clonic contractions that extended the length of the body. Convulsions were followed by a period of catalepsy that lasted 2-5 min after which the animals were hyperlocomotive but otherwise indistinguishable from untreated controls. The severity of each convulsion was quantified using the following modified Racine scale: 1- teeth chattering or face twitching; 2- head bobbing or twitching; 3- tonic extension or clonic convulsion lasting less than 3 sec; 4- tonic extension or clonic convulsion lasting longer than 3 sec; 5- tonic extension or clonic convulsion lasting more than 3 sec with loss of balance. Post-convulsion catalepsy-like behavior was assessed by placing a horizontal rod under the

forepaws of the mouse and a positive catalepsy score was assigned if the mouse did not remove its forepaws after 30 sec.

Hot Plate Test

The hot plate test was adapted from Lamberts et al. (2011). Briefly, male wild-type C57BL/6N mice were placed on a 52°C hot plate and the latency to lick forepaw(s) or jump was measured with a cutoff time of 60s in order to prevent tissue damage. To determine baseline latency, mice were placed on the hot plate 30 min after each of two injections of saline. Following an injection of 32 $mg \cdot kg^{-1}$ morphine, latency was assessed every 30 min.

DOR Saturation Binding

Mice were decapitated, whole brain was removed, and membranes were freshly prepared as previously described (Broom et al. 2002a). Protein concentrations were determined with a BCA assay kit (Thermo Scientific, Rockford, IL). Specific binding of the DOR agonist [^{3}H]DPDPE was determined as described using 10μM of the opioid antagonist naloxone to define non-specific binding (Broom et al. 2002a). Reactions were incubated for 60 min at 26ºC and stopped by rapid filtration through GF/C filter mats using a MLR-24 harvester (Brandel, Gaithersburg, MD). Bound [^{3}H]DPDPE was determined by scintillation counting and B_{max} and K_d values calculated using nonlinear regression analysis with GraphPad Prism version 6.02 (GraphPad, San Diego, CA).

Data Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al. 2015). All data analysis was performed using GraphPad Prism version 6.02 (GraphPad, San Diego, CA). Post hoc analysis was conducted using the Tukey's post hoc test to correct for multiple comparisons. For all tests, level of significance (α) was set to 0.05. All values in the text are reported as mean ± SEM. ED_{50} values were calculated using GraphPad Prism version 6.02 by extrapolating the 50% maximum effect from the straight line analysis of the averaged treatment group data used to generate each dose effect function.

Results

DOR-Mediated Behaviors in $G\alpha_o$ RGS-Insensitive Mice

It has previously been demonstrated that loss of RGS4 potentiates DOR-mediated antihyperalgesia and antidepressant-like effects, but not DOR-mediated convulsions (Dripps et al, 2017). To further investigate the signaling mechanisms involved in these behaviors, we characterized the behavioral effects of SNC80 in $G\alpha_o$ RGS-insensitive heterozygous mice. The $G\alpha_o$ RGS-insensitive heterozygous mice have one copy of *GNAO1* with a G184S point mutation that prevents binding of all RGS proteins to $G\alpha_o$ and should enhance signaling from those G proteins (Goldstein et al. 2009; Lamberts et al. 2013). First, the potency of SNC80 to reverse NTG-evoked thermal hyperalgesia was evaluated in $G\alpha_o$ RGS-insensitive heterozygous mice (+/GS) and their wild-type littermates (+/+; Figure 3.1A). The +/GS mice did not differ significantly from wild-type littermates in their baseline tail withdrawal latencies prior to NTG treatment (+/+: 42.4 ± 2.5 s, +/GS: 41.4 ± 1.4 s). Administration of 10 $mg \cdot kg^{-1}$ NTG significantly decreased tail withdrawal latency to a similar degree in both genotypes (+/+: 6.1 ± 1.3 s, +/GS: 5.4 ± 0.3 s). Two-way ANOVA revealed a significant interaction (SNC80 dose X genotype, $F(5,60) = 7.61$, $p < 0.0001$), as well as significant main effects of SNC80 dose ($F(5,60) = 56.15$, $p < 0.0001$) and genotype ($F(1,60) = 53.07$, $p < 0.0001$). There was an approximately 4-fold leftward shift in the SNC80 dose effect curve and a slight increase in the maximum effect observed in the +/GS mice compared with their wild-type littermates. Overall, the potency and efficacy of SNC80 to increase tail withdrawal latency was enhanced in $G\alpha_o$ RGS-insensitive heterozygous mice.

The potency of SNC80-induced antidepressant-like effects in $G\alpha_o$ RGS-insensitive heterozygous mice was evaluated in the FST (Figure 3.1B). In the absence of drug treatment, +/GS mice had lower immobility scores than wild-type littermates. SNC80 produced significantly lower immobility scores in +/GS mice compared to wild-type littermates. Two-way ANOVA revealed significant main effects of SNC80 dose ([vehicle and 0.32-10 mg/kg only] $F(4,51) = 17.7$, $p < 0.0001$) and genotype ($F(1,51) = 45.34$, $p < 0.0001$), as well as a significant interaction (SNC80 dose X genotype, $F(4,51) = 5.74$, $p = 0.0007$). Due to the basal differences in immobility scores, scores were normalized to a percentage relative to vehicle treated mice of the appropriate genotype (Figure 3.1C). Two-way ANOVA revealed significant main effects of SNC80 dose ([vehicle and 0.32-10 mg/kg only] $F(4,51) = 17.1$, $p < 0.0001$) and genotype

($F(1,51) = 4.80$, $p = 0.0331$), as well as a significant interaction (SNC80 dose X genotype, $F(4,51) = 6.23$, $p = 0.0004$). To investigate whether $G\alpha_o$ RGS-insensitive heterozygous mice were hyperresponsive to a wider array of antidepressive drugs, the effects of the tricyclic antidepressant desipramine were evaluated in the FST (Figure 3.1D). Although desipramine produced decreases in immobility (main effect of desipramine dose: $F(2,31) = 12.43$, $p = 0.0001$), there was no effect of genotype and no significant interaction.

Although loss of RGS4 did not alter SNC80-induced convulsions, other RGS proteins may play a role in regulating this behavior. Therefore, we evaluated SNC80-induced convulsions in the strain of mice with $G\alpha_o$ subunits insensitive to all RGS proteins. The severity of SNC80-induced convulsions in $G\alpha_o$ RGS-insensitive wild-type and heterozygous mice were evaluated using a modified Racine scale (Figure 3.1E). SNC80 produced similar dose-dependent increases in convulsion severity in both genotypes. If a convulsion occurred, it was typically within 15 min of SNC80 administration and lasted 6-15 sec.

It is possible that the enhanced behavioral effects of SNC80 in $G\alpha_o$ RGS-insensitive heterozygous mice are due to elevation of density or agonist affinity of DOR relative to their wild-type littermates. To evaluate potential changes in receptor density or agonist affinity, saturation binding with the radiolabeled DOR agonist [^{3}H]DPDPE was performed using brain tissue from $G\alpha_o$ RGS-insensitive +/+ and +/GS mice. There were no significant differences in total receptor number of the +/GS mice compared to wild-type littermates (Table 3.1; Figure 3.1F). In addition, there were no changes in the affinity of [^{3}H]DPDPE for DOR in the $G\alpha_o$ RGS-insensitive heterozygous mice.

DOR-Mediated behaviors in $G\alpha_o$ Heterozygous Knockout Mice

SNC80-induced antihyperalgesia and antidepressant-like effects were enhanced in $G\alpha_o$ RGS-insensitive heterozygous mice, suggesting that these behaviors are mediated by $G\alpha_o$ proteins. To further evaluate the role of $G\alpha_o$ in DOR-mediated behaviors, we characterized DOR-mediated antihyperalgesia, antidepressant-like effects, and convulsions in $G\alpha_o$ heterozygous knockout mice. $G\alpha_o$ null mice were produced infrequently and rarely survived to weaning (Lamberts et al. 2011). Therefore we chose to only evaluate $G\alpha_o$ wild-type and heterozygous knockout mice.

Prior to NTG administration, there were no significant differences in tail withdrawal latency in wild-type and $G\alpha_o$ heterozygous knockout mice (+/+: 41.2 ± 1.8 s, +/-: 40.3 ± 2.0 s). Administration of 10 $mg \cdot kg^{-1}$ NTG produced similar decreases in tail withdrawal latency in both genotypes (+/+: 4.9 ± 0.5 s, +/-: 4.1 ± 0.3 s). In $G\alpha_o$ wild-type mice, SNC80 produced dose-dependent increases in tail withdrawal latency following NTG administration (Figure 3.2A). This effect was abrogated in $G\alpha_o$ heterozygous knockout mice. Two-way ANOVA revealed significant main effects of SNC80 dose [vehicle and 10-56 $mg \cdot kg^{-1}$ only] ($F(3,40) = 23.55$, $p < 0.0001$) and genotype ($F(1,40) = 167.6$, $p < 0.0001$), as well as a significant interaction (SNC80 dose X genotype, $F(3,40) = 22.40$, $p < 0.0001$). To investigate whether the antihyperalgesic effects of non-DOR drugs were altered in $G\alpha_o$ heterozygous knockout mice, the effects of the 5-$HT_{1B/1D}$ agonist sumatriptan on NTG-induced thermal hyperalgesia were examined (Figure 3.2B). Sumatriptan produced similar robust increases in tail withdrawal latency in wild-type and $G\alpha_o$ heterozygous knockout mice (two-way ANOVA main effect of sumatriptan dose: $F(2,30) = 91.28$, $p < 0.0001$ but no main effect of genotype and no interaction).

In the FST, SNC80 produced significant decreases in immobility in both the $G\alpha_o$ wild-type and heterozygous knockout mice (Figure 3.2C; Two-way ANOVA main effect of SNC80 dose: $F(4,50) = 22.05$, $p < 0.0001$)). However, there were no significant differences between genotypes in the immobility scores produced in response to a given dose of SNC80. SNC80 also produced similar dose-dependent increases in convulsion severity in $G\alpha_o$ wild-type and heterozygous knockout mice (Figure 3.2D).

The diminished effect of SNC80 on NTG-induced hyperalgesia in $G\alpha_o$ heterozygous knockout mice could be due to decreased density of or agonist affinity at DOR relative to wild-type littermates. To evaluate potential changes in receptor density or agonist affinity, saturation binding with the radiolabeled DOR agonist [^{3}H]DPDPE was performed using brain tissue from $G\alpha_o$ wild-type and heterozygous knockout mice. There were no significant differences in total receptor number or affinity of [^{3}H]DPDPE for DOR in the $G\alpha_o$ heterozygous knockout mice relative to wild-type littermates (Table 3.1; Figure 3.2E).

DOR-Mediated Behaviors in Arrestin 2 and Arrestin 3 Knockout Mice

To investigate G protein-independent mechanisms, we evaluated SNC80-induced antihyperalgesia, antidepressant-like effects, and convulsions in arrestin 2 and arrestin 3

knockout mice. There were no significant differences in SNC80-induced antihyperalgesia, antidepressant-like effects, or convulsions in the arrestin 3 knockout mice compared to wild-type and heterozygote knockout littermates (Figures 3.3A-C). However, the increase in hot-plate latency produced by a single bolus dose of 32 $mg \cdot kg^{-1}$ morphine in the 52 ºC hot plate test was potentiated in arrestin 3 knockout mice (Figure 3.3D) consistent with previously published data (Bohn et al. 1999; Two-way repeated measures ANOVA: main effects of time ($F(6,90) = 64.11$, $p < 0.0001$), genotype ($F(2,15) = 13.95$, $p = 0.0004$), and a significant interaction ($F(12,90) = 6.89$, $p < 0.0001$).

In arrestin 2 knockout mice, SNC80-induced increases in tail withdrawal latency following NTG administration were similar to wild-type controls (Figure 3.4A). Arrestin 2 knockout mice had no significant differences in SNC80-induced decreases in immobility in the forced swim test relative to wild-type mice (Figure 3.4B). In contrast, SNC80-induced convulsions were profoundly altered in arrestin 2 knockout mice. The potency of SNC80 to induce convulsions was significantly increased in arrestin 2 knockout mice as evidenced by a leftward shift in the dose response curve (Figure 3.4C). Two-way ANOVA revealed significant effects of genotype ($F(2,62) = 17.83$, $p < 0.0001$), SNC80 dose ($F(4,62) = 87.05$, $p < 0.0001$), and a significant interaction ($F(8,62) = 7.04$, $p < 0.0001$). In addition, several arrestin 2 knockout mice had multiple convulsions in response to a single dose of SNC80 (Figure 3.4D). These subsequent convulsions were similar in nature to the initial SNC80-induced convulsions, consisting of both tonic and clonic phases followed by a brief (2-5 min) period of catalepsy.

Discussion

In this report, we sought to further elucidate the downstream signaling mechanism that give rise to DOR-mediated behaviors. We found that $G\alpha_o$ and arrestins differentially regulate the antihyperalgesia, antidepressant-like effects, and convulsions produced by the DOR agonist SNC80. In the NTG-induced thermal hyperalgesia assay, SNC80 produced antihyperalgesia in wild-type mice at doses of at least 10 $mg \cdot kg^{-1}$, consistent with previous studies (Pradhan et al. 2014; Dripps et al. 2016). SNC80 also decreased in immobility in the forced swim test, consistent with the well-established antidepressant-like effects of DOR agonists (Broom et al. 2002b; Naidu et al. 2007; Saitoh et al. 2011). RGS proteins negatively regulate G protein signaling by binding Gα-GTP and accelerating Gα-mediated GTP hydrolysis which returns Gα

to an inactive state. This function prolongs the lifetime of active Gα and increase downstream signaling. The potency of SNC80 to produce antihyperalgesia and antidepressant-like effects was significantly increased in the $G\alpha_o$ RGS-insensitive heterozygous mice. These data indicate that these DOR-mediated behaviors signal through $G\alpha_o$ and are negatively regulated by RGS proteins, consistent with our previous finding that RGS4 negatively regulates these behaviors (Dripps et al. 2017). Furthermore, these enhanced effects of SNC80 were observed in mice with only one mutant copy of $G\alpha_o$, demonstrating that DOR-mediated signaling *in vivo* is highly sensitive to the effects of RGS proteins. Interestingly, the magnitude of these behavioral changes are consistent with those seen in RGS4 knockout mice, suggesting that other RGS proteins likely do not play a significant role in regulating the antihyperalgesic and antidepressant-like effects of DOR. We hypothesize that the enhanced DOR-mediated antihyperalgesia and antidepressant-like effects observed in the +/GS mice are likely due to prolongation of DOR-mediated G protein signaling and amplification of downstream effectors.

To confirm the role of $G\alpha_o$ in DOR-mediated behaviors, we examined the behavioral effects of SNC80 in $G\alpha_o$ heterozygous knockout mice. SNC80-induced antihyperalgesia was abolished in $G\alpha_o$ heterozygous knockout mice, suggesting that $G\alpha_o$ is required for the antihyperalgesic effects of DOR. Furthermore, this profound effect was produced by only a 50% reduction in $G\alpha_o$, indicating that DOR-mediated antihyperalgesia likely requires robust amplification of downstream signaling. It is possible that larger doses of SNC80 could produce antihyperalgesia in $G\alpha_o$ heterozygous knockout mice, however such doses are likely to be nonselective. Taken together, our findings indicate that $G\alpha_o$ plays a critical role in mediating signaling required for DOR-mediated antihyperalgesia.

In contrast, decreased expression of $G\alpha_o$ did not affect DOR-mediated antidepressant-like effects in the forced swim test. DOR could be capable of signaling through other G proteins in order to produce antidepressant-like effects and compensate for the reduction in $G\alpha_o$ expression. Alternatively, it is possible that the efficacy requirement for DOR-mediated antidepressant-like effects is relatively low compared to that for DOR-mediated antihyperalgesia in which case one functional copy of *GNAO1* and approximately 50% of $G\alpha_o$ protein subunits (Lamberts et al. 2011) could be sufficient to produce a full response in the forced swim test. Broom et al. (2002a) proposed that the efficacy requirement for DOR-mediated antinociception was higher than that

required for convulsions. The relative efficacy requirement for antidepressant-like effects has not been evaluated and should be investigated in future studies.

In the present study, DOR-mediated convulsions were not altered in $G\alpha_o$ RGSi and $G\alpha_o$ knockout mice. In addition, we previously observed that SNC80-induced convulsions were unaltered in RGS4 knockout mice (Dripps et al. 2017). Therefore, we explored the hypothesis that SNC80-induced convulsions are produced by a G protein-independent, arrestin-mediated mechanism. Although class A GPCRs are thought to preferentially interact with arrestin 3 (Oakley et al. 2000), no significant changes in DOR-mediated behaviors, including convulsions, were observed in arrestin 3 knockout mice. It should be noted that these data are the result of acute administration of SNC80 and it is possible that arrestin 3 could play a role in regulating the effects of repeated doses of SNC80. This observation is consistent with previous reports that found that loss of arrestin 3 in mice did not alter the analgesic profile of DOR agonists and had no effect on the enhanced coupling of DOR to voltage-dependent calcium channels observed in the Complete Freund's Adjuvant model of chronic inflammatory pain (Pradhan et al. 2013; Pradhan et al. 2016). Because we saw no change in DOR-mediated behaviors in arrestin 3 knockout mice, we evaluated morphine-induced antinociception in the hot plate assay as a positive control. As first shown in Bohn et al. (1999), we observed potentiation of morphine-induced antinociception in arrestin 3 knockout mice. Overall, our findings indicate that arrestin 3 is not required for DOR-mediated antihyperalgesia, antidepressant-like effects, or convulsions.

In arrestin 2 knockout mice, we observed no changes in the effects of SNC80 in response to NTG-induced thermal hyperalgesia. However, the effects of SNC80 on CFA-induced mechanical hyperalgesia were potentiated in arrestin 2 knockout mice (Pradhan et al. 2016). It is possible that the DOR-mediated responses to these distinct pain modalities are differentially regulated by arrestin 2. In contrast to the antihyperalgesic effects, the convulsive effects of SNC80 were strongly enhanced in arrestin 2 knockout mice. The potency of SNC80 to induce convulsions was enhanced in arrestin 2 knockout mice, suggesting that arrestin 2 acts as a negative regulator of DOR-mediated convulsions. Secondly, arrestin 2 knockout mice convulsed multiple times in response to a single dose of SNC80. Tolerance to DOR-mediated convulsions is typically acute and long lasting (Comer et. al 1993; Hong et al. 1998). In addition, the changes in the electroencephalographic waveform produced by SNC80 return to normal baseline activity following the end of catalepsy (Jutkiewicz et al. 2006). To our knowledge, this is the first report

of multiple convulsive events in response to a DOR agonist in rodents. One possible explanation for this observation is that loss of arrestin 2 produces these behavioral changes by upregulating DOR trafficking to the cell membrane resulting in enhanced DOR signaling (Mittal et al. 2013). However, DOR-mediated antihyperalgesia and antidepressant-like effects were not significantly altered in arrestin 2 knockout mice. Therefore, it is possible that the behavioral effects of SNC80 are differentially regulated by arrestin 2 due to differences in regional expression, behavioral mechanisms, and/or signaling pathways. Alternatively, arrestin 2 could be necessary for the rapid desensitization and tolerance to the convulsive effects of SNC80. Thus, loss of arrestin 2 could allow signaling pathways that would normally be terminated to persist and produce multiple convulsive events. Future work will examine whether arrestin 2 also regulates tolerance to other behavioral effects of DOR agonists.

Overall, our data demonstrate a role for $G\alpha_o$, but not arrestins, in regulating the acute antihyperalgesic and antidepressant-like effects of DOR. However, DOR-mediated convulsions appear to be negatively regulated by arrestin 2 and were not altered by manipulations to $G\alpha_o$ function. Taken together, these findings suggest that different signaling pathways underlie the convulsive effects of DOR relative to the antihyperalgesic and antidepressant-like effects. Perhaps due in part to this phenomenon, multiple DOR agonists have been shown to not produce convulsions at doses far exceeding those needed to produce antinociception and antidepressant-like effects (Le Bourdonnec et al. 2008; Saitoh et al. 2011; Chung et al. 2015). However, the properties of DOR agonists that determine their convulsive nature remain unclear. Future work will continue to investigate the signaling mechanisms responsible for the behavioral effects of DOR agonists.

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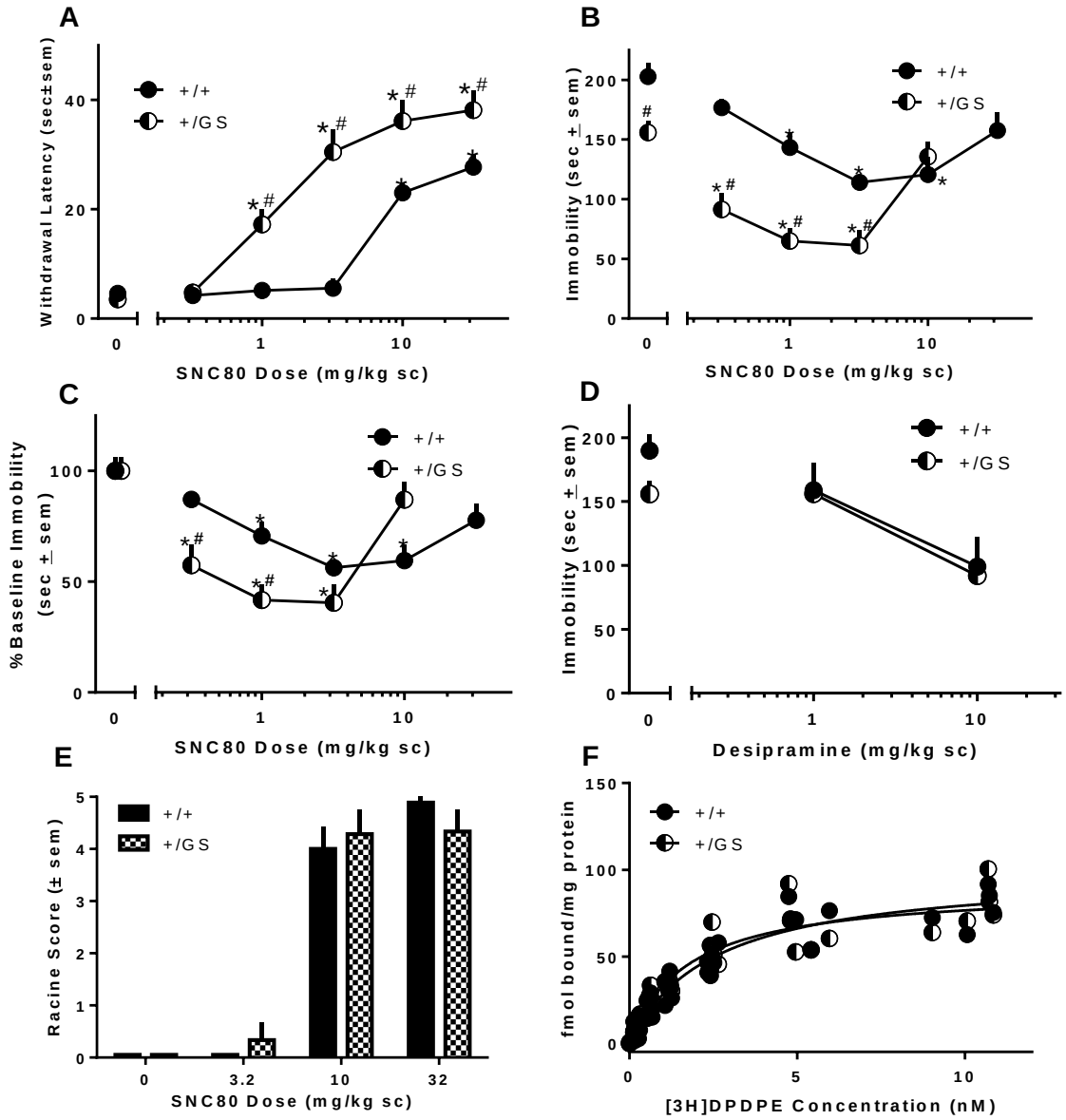

Figure 3.1. (A) Effect of different doses of SNC80 on tail withdrawal latency in NTG treated $G\alpha_o$ RGS-insensitive wild-type (+/+) and heterozygous (+/GS) mice (B,C) Immobility scores of $G\alpha_o$ RGS-insensitive +/+ and +/GS mice in response to SNC80 in the forced swim test expressed as (B) raw immobility scores or (C) immobility scores normalized to a percentage of the scores of vehicle treated mice of the appropriate genotype. (D) Effects of desipramine on immobility scores of $G\alpha_o$ RGS-insensitive +/+ and +/GS mice in the forced swim test (E) Severity of SNC80-induced convulsions in $G\alpha_o$ RGS-insensitive +/+ and +/GS mice (F) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of $G\alpha_o$ RGS-insensitive +/+ or +/GS mice. Each point represents tissue from 1 mouse assayed in triplicate. n = 5-7 mice per group for all experiments. Data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle treatment in the same genotype, # $p < 0.05$ compared to wild-type mice with same drug dose.

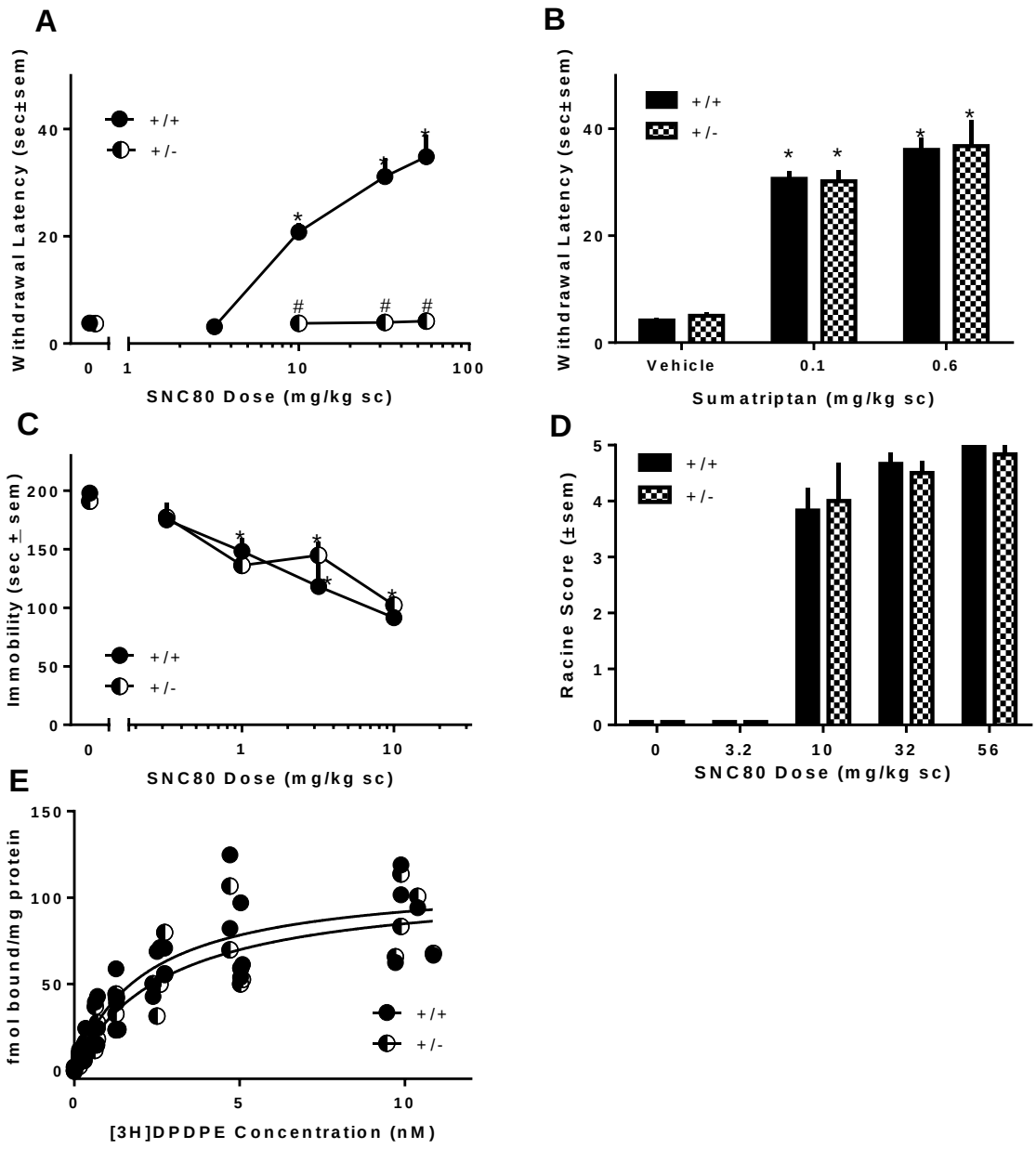

Figure 3.2. Tail withdrawal latencies in NTG treated $G\alpha_o$ wild-type (+/+) and heterozygous knockout (+/-) mice in response to (A) SNC80 or (B) sumatriptan. (C) Effects of SNC80 on immobility scores of $G\alpha_o$ +/+ and +/- mice in the forced swim test. (D) Severity of SNC80-induced convulsions in $G\alpha_o$ +/+ and +/- mice. (E) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of $G\alpha_o$ +/+ and +/- mice. Each point represents tissue from 1 mouse assayed in triplicate. n = 5-6 mice per group for all experiments. Data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle treatment in the same genotype, # $p < 0.05$ compared to wild-type mice with same drug dose.

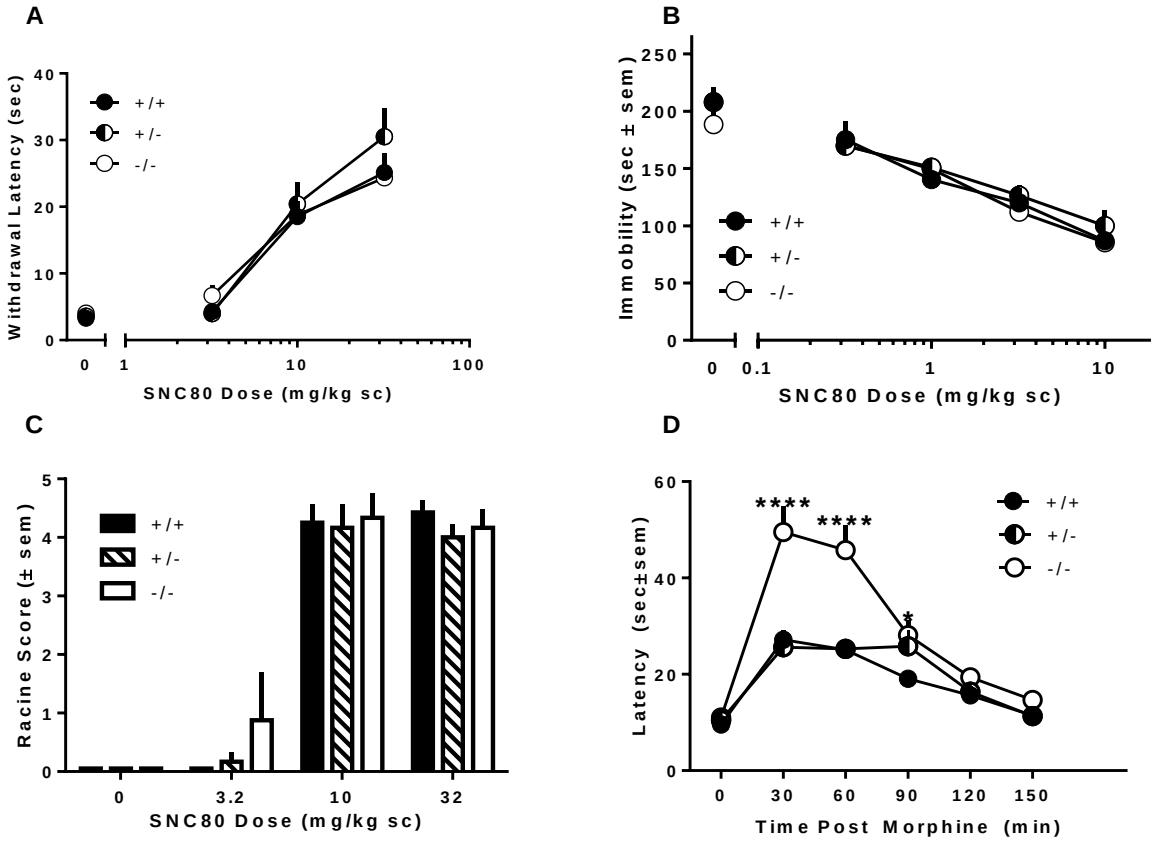

Figure 3.3. (A) Effects of SNC80 on tail withdrawal latencies in NTG treated arrestin 3 wild-type (+/+), heterozygous (+/-), and homozygous (-/-) knockout mice. (B) Immobility scores of arrestin 3 +/+, +/-, and -/- mice in the forced swim test following treatment with SNC80. (C) Severity of SNC80-induced convulsions in arrestin 3 +/+, +/-, and -/- mice (D) Time course of the effects of morphine on hot-plate latency in arrestin 3 +/+, +/-, and -/- mice. n = 6-7 mice per group for all experiments. Data are shown as average per treatment condition with standard error of the mean (sem). **** $p < 0.0001$, * $p < 0.05$ compared to wild-type at the same time point.

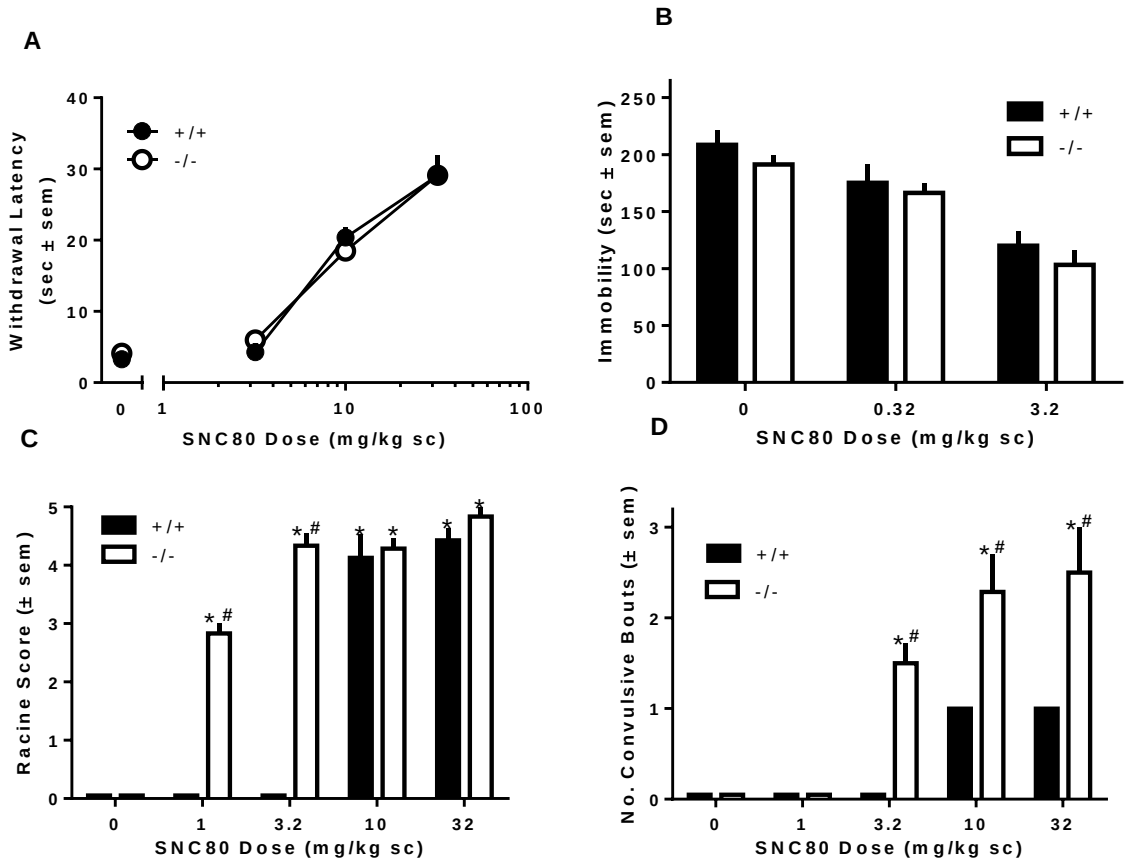

Figure 3.4. (A) Effects of SNC80 on tail withdrawal latencies in NTG treated arrestin 2 wild-type (+/+) and knockout (-/-) mice. (B) Immobility scores of arrestin 2 +/+ and -/- mice in the forced swim test following treatment with SNC80. (C) Severity of SNC80-induced convulsions in arrestin 3 +/+ and -/- mice. (D) Number of SNC80-induced convulsions observed in arrestin 3 +/+ and -/- mice. n = 6 mice per group for all experiments. Data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle treatment in the same genotype, # $p < 0.05$ compared to wild-type mice with same drug dose.

Table 3.1. DOR density and agonist affinity in $G\alpha_o$ RGSi and $G\alpha_o$ knockout mice

Genotype	Bmax (fmol mg^{-1} ± sem)	[^{3}H] DPDPE K_d (nM ± sem)
$G\alpha_o$ RGSi +/+	99 ± 6	2.5 ± 0.5
$G\alpha_o$ RGSi +/GS	90 ± 5	1.7 ± 0.3
$G\alpha_o$ +/+	111 ± 11	2.1 ± 0.6
$G\alpha_o$ +/-	108 ± 11	2.8 ± 0.7

Chapter IV

Pharmacological Properties of Delta Opioid Receptor-Mediated Behaviors: Distinct Efficacy Requirements and Receptor Populations

Introduction

The delta opioid receptor (DOR) is a class A GPCR that couples to inhibitory $G\alpha_{i/o}$ proteins. Activation of DOR has been shown to elicit a number of behavioral effects. DOR agonists produce antinociception and antihyperalgesia in mice (Hong *et al.,* 1998; Pradhan *et al.,* 2014), rats (Gallantine and Meert 2005; Fraser *et al.,* 2000), and monkeys (Negus et al. 1998; Brandt et al. 2001). They have also been shown to produce antidepressant-like effects in a number of rodent models (for review, see Lutz and Kieffer, 2013). Some, but not all, DOR agonists also produce convulsions (Comer *et al.,* 1993; Hong *et al.,* 1998).

To better understand the pharmacological properties mediating behavioral effects of DOR agonists, we sought to compare directly two structurally distinct DOR ligands that differ in intrinsic efficacy: the piperazinyl benzamide SNC80 and the morphinan derivative BU48. SNC80 is the prototypical nonpeptidic DOR agonist. It is highly efficacious at stimulating G protein activation *in vitro* in C6 glioma cells (Clark et al. 1997) and *ex vivo* (Jutkiewicz et al. 2004). *In vivo,* SNC80 has been shown to produce antihyperalgesia (Pradhan et al. 2014), antidepressant-like effects (Saitoh et al. 2004), and convulsions (Hong et al. 1998) in mice. BU48 is less efficacious than SNC80 *in vitro,* producing approximately 40% stimulation of GTPγS binding relative to SNC80 in C6 glioma cells expressing DOR (Broom et al. 2000). This report also found that BU48 produces DOR-mediated convulsions but did not produce DOR-mediated antinociception (Broom et al. 2000), suggesting that the efficacy requirement for DOR-mediated convulsions is low relative to that for antinociception. Consistent with this hypothesis, small doses (3 and 10 mg/kg sc) of the DOR irreversible antagonist naltrindole-5'-isothiocyanate (5'-NTII) antagonized the antinociceptive effects of the DOR agonist BW373U86 but did not decrease the frequency of BW373U86-induced convulsions in NIH Swiss mice (Broom et al.

2002a). In another study, SNC80 was significantly more potent at producing decreases in immobility in the rat forced swim test compared to the partial agonist SNC162 (Jutkiewicz et al. 2004), but these two compounds produced convulsions with similar potencies, also suggesting that the efficacy requirement for DOR-mediated convulsions may be low relative to that for antidepressant-like effects. However, the relationship of agonist efficacy to the behavioral effects of DOR agonists has not been thoroughly evaluated.

Therefore, the present study evaluated the role of agonist efficacy, DOR receptor reserve, and DOR receptor populations involved in DOR-mediated behaviors. To do this, we compared the behavioral effects of the DOR full agonist SNC80 and the DOR partial agonist BU48. Effects of receptor reserve were assessed by comparing the shifts in the dose response curves for SNC80-induced antihyperalgesia, antidepressant-like effects, and convulsions in DOR heterozygous knockout mice and mice treated with the irreversible DOR antagonist 5'-NTII treated wild-type mice as compared with controls. We also evaluated the ability of the competitive DOR antagonist naltrindole (NTI) to attenuate SNC80-induced antihyperalgesia, antidepressant-like effects, and convulsions to determine antagonist potency and potential mechanisms of antagonism.

Materials and Methods

Subjects

All animal use procedures complied with the *Guide for the Care and Use of Laboratory Animals* by the National Institutes of Health, and were approved by the *University of Michigan Institutional Committee on the Use and Care of Animals*. Mice were housed in groups of four to five animals per cage. All mice were used between 8 and 15 weeks of age at time of experiment and weighed 16-32 g. Mice had free access to standard lab chow and water and were maintained in a temperature- and humidity-controlled environment on a 12-h dark/light cycle with lights on at 7:00 AM. Mice were tested only once, and all analyses are between-subject.

The *Oprd1*^{tm1Kff}/J mouse strain was obtained from The Jackson Laboratory (Bar Harbor, Maine, https://www.jax.org/strain/007557; Filliol *et al.,* 2000). Mice maintained in-house as heterozygote pair or harem (1 male, 2 female) breeding groups. Male and female mice were used in all studies. Wild-type littermates (+/+) were used as controls in all experiments involving

C57DOR heterozygote (+/-) and homozygote (-/-) knockout mice. For studies in which transgenic mice were not required, C57BL/6N mice were obtained from Envigo (formerly Harlan, Indianapolis, IN).

Forced Swim Test

The forced swim test was adapted from Porsolt et al (1977) and performed as previously described (Dripps er al. 2017). In brief, sixty min after SNC80 (0.32, 1, 3.2, 10 mg/kg sc), BU48 (1, 3.2, 10 mg/kg sc) or vehicle injection, each mouse was placed in a 4L beaker filled with 15 cm of 25±1°C water and swim sessions were recorded for 6 min using a Sony HDR-CX220 digital camcorder. Videos were analyzed by individuals blind to the experimental conditions and the amount of time the animals spent immobile was quantified. Immobility was defined as the mouse not actively traveling through the water and making only movements necessary to stay afloat. The time the mouse spends immobile after the first 30 sec of the assay was recorded.

Nitroglycerin-Induced Hyperalgesia

The NTG-induced hyperalgesia assay was adapted from Bates et al (2010) using modifications described in Pradhan et al (2014) and performed as previously described (Dripps *et al.,* 2017). Hyperalgesia was assessed by immersing the tail (~5cm from the tip) in a 46°C water bath and determining the latency for the mouse to withdraw its tail with a cut-off time of 60 sec. After determining baseline withdrawal latencies, 10 mg/kg NTG (ip) was administered to each animal. Tail withdrawal latency was assessed again 1 hr after NTG administration. At 90 min post-NTG, animals received an injection of SNC80 (3.2, 10, 32, 100, 180 mg/kg sc), BU48 (3.2, 10, 32 mg/kg sc) or vehicle, and mice were observed continuously in individual cages for 20 min to observe for convulsions (see section below). Tail withdrawal latencies were assessed again 30 min after SNC80 administration.

SNC80-Induced Convulsions

Mice were observed continuously in individual cages for convulsions, catalepsy, myclonic jerks, stop-and-stare behaviors, wet dog shakes, digging and other normal or abnormal behaviors. Convulsions were comprised of a tonic phase characterized by sudden tensing of the musculature and extension of the forepaws followed by clonic contractions that extended the

length of the body. Convulsions were followed by a period of catalepsy that lasted 2-5 min after which the animals were indistinguishable from untreated controls. The severity of each convulsion was quantified using the following modified racine scale: 1- teeth chattering or face twitching; 2- head bobbing or twitching; 3- tonic extension or clonic convulsion lasting less than 3 sec; 4- tonic extension or clonic convulsion lasting longer than 3 sec; 5- tonic extension or clonic convulsion lasting more than 3 sec with loss of balance. Post-convulsion catalepsy was assessed by placing a horizontal rod under the forepaws of the mouse and a positive catalepsy score was assigned if the mouse did not remove its forepaws after 30 sec.

DOR Saturation Binding

Mice were decapitated, whole brain was removed, and membranes were freshly prepared as previously described (Broom *et al.*, 2002a). Protein concentrations were determined with a BCA assay kit (Thermo Scientific, Rockford, IL). Specific binding of the DOR agonist [^{3}H]DPDPE was determined as described using 10µM of the opioid antagonist naloxone to define non-specific binding as described (Broom *et al.*, 2002a). Reactions were incubated for 60 min at 26ºC and stopped by rapid filtration through GF/C filter mats using a MLR-24 harvester (Brandel, Gaithersburg, MD). Bound [^{3}H]DPDPE was determined by scintillation counting.

[^{35}S]GTPγS Binding Assay

Mouse brain membranes (as prepared above, 10 μg/well) were incubated for 90 min at 26 °C in buffer comprising 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 nM [^{35}S]GTPγS, 100 μM GDP (guanosine 5-diphosphate) , and 0.4 U/mL adenosine deaminase in a final volume of 200 μL. SNC80 or BU48 were also included at appropriate concentrations. 10 µM SNC80 was used as the maximal standard and assay buffer was used to assess basal [^{35}S]GTPγS binding. The reaction was terminated by filtration through glass microfiber GF/C filters (Whatman) using a Brandell harvester. The filters were rinsed, dried, and radioactivity was determined by scintillation counting.

Data Analysis

All data analysis was performed using GraphPad Prism version 6.02 (GraphPad, San Diego, CA). Unless otherwise indicated, data were compared by two-way ANOVA and Post hoc

analysis was conducted using the Tukey's post hoc test to correct for multiple comparisons. B_{max} and K_d values were compared by unpaired t test. For all tests, level of significance (α) was set to 0.05. ED_{50} values were calculated by extrapolating the 50% maximum effect from the straight line analysis of the averaged treatment group data used to generate each dose effect function. *Ex Vivo* B_{max}, K_d, and EC_{50} values calculated using nonlinear regression analysis.

Materials

SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was dissolved in 1 M HCl and diluted in sterile water to a concentration of 3% HCl. BU48 (N-Cyclopropylmethyl-[7alpha,8alpha,2', 3']-cyclohexano-1'[S]-hydroxy-6,14-endo-ethenotetrahydronororipavine) was dissolved in a solution comprised of 10% ethanol, 10% Alkamuls EL-620 (Acros Organics, Morris Plains, NJ), and 80% sterile water. NTG was provided by Dr. Adam Lauver (Department of Pharmacology and Toxicology, Michigan State University) at a concentration of 5mg/ml and was diluted in saline. Naltrindole-5'-isothiocyanate (5'-NTII; Sigma-Aldritch, St. Louis, MO) was dissolved in 10% DMSO. Naltrindole (NTI; Tocris Bioscience, Pittsburgh, PA) was dissolved in sterile water. 5'-NTII (32 mg/kg) or vehicle was injected 24 hrs prior to SNC80 administration. NTI (1, 3.2 mg/kg) or vehicle was injected 30 min prior to SNC80 administration. All drugs were injected at a volume of 10 mL/kg. All drugs were given subcutaneously (sc) except for NTG which was administered via intraperitoneal (ip) injection.

Results

Characterization of BU48-Induced Behaviors

The DOR partial agonist BU48 has previously been shown to produce DOR-mediated convulsions but not DOR-mediated antinociception (Broom *et al.*, 2000). The potential antihyperalgesic and antidepressant-like effects of BU48 have not been evaluated. Therefore, we sought to further characterize the pharmacological and behavioral effects of BU48 as compared with the full DOR agonist SNC80. In mouse forebrain tissue, SNC80 produced robust and dose-dependent stimulation of [^{35}S]GTPγS binding (EC_{50}: 210 nM; Figure 4.1A). BU48 did not produce significant stimulation of [^{35}S]GTPγS binding at concentrations up to 10 μM. *In vivo*, BU48 failed to increase tail withdrawal latency in NTG treated wild-type mice up to a dose of 32

mg/kg whereas SNC80 significantly increased tail withdrawal latency at 10 and 32 mg/kg (Figure 4.1B). Two-way ANOVA revealed significant effects of drug ($F(1,40) = 118.0$, $p < 0.0001$), dose ($F(3,40) = 50.90$, $p < 0.0001$), and a significant interaction ($F(3,40) = 45.41$, $p < 0.0001$). Additionally, pretreatment with BU48 30 min prior to SNC80 administration prevented SNC80-induced increases in tail withdrawal latency in C57BL/6N wild-type mice (Figure 4.1C). Two-way ANOVA revealed a significant interaction (BU48 dose X genotype, $F(1,20) = 67.83$, $p < 0.0001$) and significant main effects of BU48 dose ($F(1,20) = 78.38$, $p < 0.0001$) and SNC80 dose ($F(1,20) = 57.97$, $p < 0.0001$). However, in mice lacking regulator of G protein signaling 4 (RGS4), 32 mg/kg BU48 was able to increase tail withdrawal latencies relative to wild-type littermates (Figure 4.1D). Two-way ANOVA revealed significant main effects of BU48 dose ($F(1,21) = 17.67$, $p = 0.0004$) and genotype ($F(1,21) = 12.21$, $p = 0.0022$), as well as a significant interaction (BU48 dose X genotype, $F(1,21) = 23.46$, $p < 0.0001$).

In the forced swim test, BU48 produced dose-dependent decreases in immobility (Figure 4.1E). Two-way ANOVA revealed only a significant main effect of drug ($F(1,42) = 38.48$, $p < 0.0001$), suggesting a difference in potency and/or efficacy between BU48 and SNC80. To evaluate the role of DOR in BU48-induced antidepressant-like effects, C57BL6 wild-type mice were pretreated with the DOR selective antagonist NTI (Figure 4.1F). Two-way ANOVA revealed a significant main effect of BU48 dose ($F(1,21) = 7.77$, $p = 0.011$). BU48-induced decreases in immobility were blocked by pretreatment with 3.2 mg/kg NTI (BU48 dose X NTI dose, $F(1,21) = 13.04$, $p = 0.0016$), indicating a DOR-mediated effect.

BU48 produced dose-dependent increases in convulsion severity (Figure 4.2A). As with those produced by SNC80, BU48-induced convulsions were comprised of tonic and clonic phases that were followed by a period of catalepsy. For a given dose, there were no significant differences in the time of onset (Figure 4.2B) or duration (Figure 4.2C) of convulsions produced by BU48 and SNC80. BU48-induced convulsions were blocked by pretreatment with 3.2 mg/kg NTI, indicating a DOR-mediated effect (Unpaired two-tailed t test: $t(10) = 12.85$, $p < 0.0001$; Figure 4.2D).

DOR-Mediated Behaviors in DOR Knockout Mice

Changes in DOR density and agonist affinity in DOR mutant mice were assessed by saturation binding in brain tissue with the radiolabeled DOR agonist [^{3}H]DPDPE (Figure 4.3A).

Total DOR in heterozygous knockout mice was approximately 40% of that measured for wild-type mice (Table 4.1). The affinity of [^{3}H]DPDPE for DOR did not differ significantly between wild-type and heterozygous knockout mice (Table 4.1). DOR could not be detected in DOR homozygous knockout mice.

To evaluate the efficacy requirements of DOR-mediated behaviors, we compared the potency of SNC80 to induce antihyperalgesia, antidepressant-like effects, and convulsions in DOR wild-type and heterozygous knockout mice. In a NTG-induced thermal hyperalgesia assay, there were no differences between genotypes in the baseline tail withdrawal latencies prior to NTG treatment (+/+: 35.7 ± 1.7 s, +/-: 35.6 ± 1.3 s, -/-: 38.9 ± 2.9 s). Administration of 10 mg/kg NTG (ip) significantly decreased tail withdrawal latency to a similar degree in all genotypes (+/+: 3.6 ± 0.3 s, +/-: 3.6 ± 0.2 s, -/-: 4.1 ± 0.4 s). Overall, the potency of SNC80 to increase tail withdrawal latency was significantly decreased in the DOR heterozygous knockout mice as evidenced by a 7.1-fold leftward shift in the dose effect curve relative to wild-type mice (Figure 4.3B; ED_{50} values: +/+: 14 mg/kg; +/-: 100 mg/kg). Two-way ANOVA comparing the DOR wild-type and heterozygous knockout groups revealed a significant interaction (SNC80 dose [0, 3.2-100 mg/kg only] X genotype, $F(4,51) = 7.99$, $p < 0.0001$), as well as significant main effects of SNC80 dose ($F(4,51) = 23.97$, $p < 0.0001$) and genotype ($F(1,51) = 36.92$, $p < 0.0001$). SNC80 failed to increase tail withdrawal latency in DOR homozygous knockout mice at a dose of 100 mg/kg.

The potency of SNC80 to reduce immobility time in the forced swim test was evaluated in DOR mutant mice (Figure 4.3C). Two-way ANOVA comparing the DOR wild-type and heterozygous knockout groups revealed significant main effects of SNC80 dose ($F(4,59) = 34.15$, $p < 0.0001$) and genotype ($F(1,59) = 22.20$, $p < 0.0001$), and a significant interaction effect (SNC80 dose X genotype, $F(4,59) = 4.74$, $p = 0.0022$). The SNC80 dose response curve for DOR heterozygous knockout mice shifted approximately 4.2-fold to the right relative to wild-type mice, indicating a decrease in the potency of SNC80 (ED_{50} values: +/+: 1.3 mg/kg; +/-: 5.5 mg/kg). SNC80 failed to reduce immobility in DOR homozygous knockout mice at a dose of 10 mg/kg.

The potency of SNC80 to produce convulsive effects was also evaluated in DOR mutant mice (Figure 4.3D). Two-way ANOVA comparing the DOR wild-type and heterozygous knockout mice revealed significant main effects of SNC80 dose ($F(4,51) = 89.68$, $p < 0.0001$)

and genotype ($F(1,51) = 12.23$, $p = 0.001$), as well as a significant interaction effect (SNC80 dose X genotype, $F(4,51) = 7.83$, $p < 0.0001$). The SNC80 dose response curve for DOR heterozygous knockout mice was shifted approximately 1.7-fold to the right relative to wild-type mice, indicating a decrease in the potency of SNC80 (ED_{50} values: +/+: 13 mg/kg; +/-: 22 mg/kg). SNC80 failed to produce convulsions in DOR homozygous knockout mice at a dose of 100 mg/kg.

DOR-Mediated Behaviors in 5'-NTII Treated Mice

To further explore the efficacy requirements contributing to the behavioral effects of DOR agonists, we aimed to decrease DOR numbers by approximately 25%—less receptor loss than that observed in the DOR heterozygous knockout mice—using the irreversible DOR antagonist 5'-NTII. 24 hr pretreatment with 32 mg/kg 5'-NTII reduced the B_{max} of [^{3}H]DPDPE by approximately 30% (Figure 4.4A; Table 4.1). There were no significant differences in the affinity of [^{3}H]DPDPE for DOR between treatment groups (Table 4.1).

In the NTG-induced thermal hyperalgesia assay, there were no differences in the baseline tail withdrawal latencies 24 hrs after 5'-NTII or vehicle pretreatment (vehicle: 37 ± 1.4 s, 5'-NTII: 41 ± 2.2s). Administration of 10 mg/kg NTG (ip) significantly decreased tail withdrawal latency to a similar degree in both treatment groups (vehicle: 4.4 ± 0.5 s, 5'-NTII: 5.2 ± 0.4 s). Pretreatment with 32 mg/kg 5'-NTII reduced the potency of SNC80 to increase tail withdrawal latency as evidenced by an approximate 3.3-fold rightward shift in the dose response curve (Figure 4.4B; ED_{50} values: vehicle: 13 mg/kg; 5'-NTII: 43 mg/kg). Two-way ANOVA revealed a significant interaction (SNC80 dose [0, 10, 32 mg/kg only] X 5'-NTII dose, $F(2,29) = 3.80$, $p = 0.034$), as well as significant main effects of SNC80 dose ($F(2,29 = 27.01$, $p < 0.0001$) and 5'-NTII dose ($F(1,29) = 17.04$, $p = 0.0003$).

In the forced swim test, pretreatment with 32 mg/kg 5'-NTII alone did not alter immobility scores relative to vehicle pretreatment. Pretreatment with 5'-NTII produced an approximate 2-fold rightward shift in the SNC80 dose response curve relative to wild-type mice, indicating a decrease in the potency of SNC80 (Figure 4.4C; ED_{50} values: vehicle: 1 mg/kg; 5'-NTII: 2 mg/kg). Two-way ANOVA revealed a significant interaction (SNC80 dose X 5'-NTII dose, $F(2,30) = 9.22$, $p = 0.0008$), as well as significant main effects of SNC80 dose ($F(2,30 = 55.36$, $p < 0.0001$) and 5'-NTII dose ($F(1,30) = 4.62$, $p = 0.0398$). The severity of SNC80-

induced convulsions were not significantly altered by pretreatment with 5'-NTII (Figure 4.4D) nor did NTII alter the frequency, duration, or time to onset of convulsions (data not shown).

DOR-Mediated Behaviors in NTI Treated Mice

The affinity of a competitive antagonist for a given population of receptors should not change depending on the output being measured (Kenakin 1982). To further characterize the receptor populations mediating SNC80-induced behaviors, we evaluated the ability of small (1 mg/kg) or large (3.2 mg/kg) doses of the competitive DOR antagonist NTI to block SNC80-induced behaviors. In a NTG-induced thermal hyperalgesia assay, 10 and 32 mg/kg SNC80 produced significant increases in tail withdrawal latency in C57BL6 wild-type mice (Figure 4.5A). Pretreatment with either 1 or 3.2 mg/kg NTI abolished the effects of SNC80 at 10 and 32 mg/kg. Two-way ANOVA revealed a significant interaction (SNC80 dose X NTI dose, $F(4,45) = 34.44$, $p < 0.0001$), as well as significant main effects of SNC80 dose ($F(2,45 = 27.34$, $p < 0.0001$) and NTI dose ($F(2,45) = 130.0$, $p < 0.0001$).

In the forced swim test, administration of 1 or 3.2 mg/kg SNC80 significantly decreased immobility (Figure 4.5B). SNC80-induced decreases in immobility were blocked by pretreatment with 3.2 mg/kg NTI. Pretreatment with 1 mg/kg NTI blocked the decreases in immobility produced by the small SNC80 dose (1 mg/kg); however, administration of 3.2 mg/kg SNC80 was able to surmount the effects of this low dose of NTI. Two-way ANOVA revealed significant main effects of SNC80 dose ($F(2,46 = 11.22$, $p = 0.0001$) and NTI dose ($F(2,46) = 23.03$, $p < 0.0001$), and a significant interaction (SNC80 dose X NTI dose, $F(4,46) = 8.38$, $p < 0.0001$).

Administration of 10 or 32 mg/kg SNC80 alone produced pronounced convulsions (Figure 4.5C). Pretreatment with 1 mg/kg NTI failed to block SNC80-induced convulsions. Pretreatment with 3.2 mg/kg NTI eliminated convulsions produced by 10 mg/kg SNC80 and significantly decreased the severity of convulsive behavior resulting from administration of 32 mg/kg SNC80. Two-way ANOVA revealed significant main effects of SNC80 dose ($F(2,45 = 77.91$, $p < 0.0001$) and NTI dose ($F(2,45) = 43.68$, $p < 0.0001$), as well as a significant interaction (SNC80 dose X NTI dose, $F(4,45) = 11.52$, $p < 0.0001$).

Discussion

In this report, we sought to explore the pharmacological characteristics differentiating some behavioral effects of DOR agonists, such as antihyperalgesia, antidepressant-like and convulsive effects. We found that these three behaviors demonstrate a rank order of efficacy requirements with convulsions having the lowest requirement, followed by antidepressant-like effects and then antihyperalgesia. We also provide pharmacological evidence to suggest that these DOR-mediated behaviors are governed by distinct receptor populations.

The DOR partial agonist BU48 has previously been shown to elicit DOR-mediated convulsions, but not DOR-mediated antinociception (Broom *et al.,* 2000). To further characterize the behavioral effects of a DOR partial agonist, we evaluated the potency of BU48 to produce DOR-mediated antihyperalgesia, antidepressant-like effects and convulsions. BU48 produced dose-dependent increases in convulsion severity with similar potency and efficacy to SNC80, though mice treated with 3.2 mg/kg BU48 exhibited some preconvulsive behavior, such as head twitches and brief myoclonic jerks. BU48 also produced antidepressant-like effects in the forced swim test, albeit with reduced potency relative to SNC80. BU48 also appeared to be less efficacious than SNC80 in the forced swim test, consistent with a partial agonist profile, but larger doses would need to be tested to fully evaluate this claim. The reduced potency and/or efficacy of BU48 in the forced swim test could be due to its activity as a kappa opioid agonist which are known to produce prodepressant-like effects (Mague *et al.,* 2003). It would be interesting to test whether co-administration of a kappa opioid antagonist would enhance the antidepressant-like effects of BU48. *Ex vivo,* BU48 did not significantly stimulate ^{35}[S]GTPγS binding in forebrain tissue, indicating that BU48 is a low efficacy agonist, at least at the level of G protein activation. BU48 could act as a full agonist for a different, G protein-independent signaling pathway.

BU48 not only failed to reverse NTG-induced thermal hyperalgesia in wild-type mice, it antagonized SNC80-induced antihyperalgesia. These data further support the claim that BU48 is a DOR partial agonist. One alternative explanation is that BU48 is a biased agonist that cannot activate the intracellular signaling mechanisms needed to produce antihyperalgesia after engaging DOR. However, a large (32 mg/kg) dose of BU48 did produce mild antihyperalgesia in RGS4 knockout mice. RGS4 acts as a negative regulator of $G\alpha_{i/o}$ signaling and has previously been shown to enhance the potency of SNC80-induced antihyperalgesia, suggesting that under

these conditions BU48 can produce antihyperalgesia through a SNC80-like mechanism. Eliminating RGS activity has also been shown to increase the efficacy of MOR partial agonists (Clark et al. 2008). Taken together, these data strongly support BU48 as a DOR partial agonist. As a partial agonist, BU48 should more readily produce low efficacy requiring behaviors as compared to high efficacy requiring behaviors. Therefore, the ability of BU48 to produce DOR-mediated convulsions with potency comparable to a full agonist is consistent with convulsions having a low efficacy requirement. Likewise, BU48 producing antidepressant-like effects with reduced potency and failing to produce antihyperalgesia suggests that these behaviors have higher efficacy requirements relative to convulsive effects.

To further evaluate the role of receptor reserve in DOR-mediated behaviors, we evaluated the potency of SNC80 to produce antihyperalgesia, antidepressant-like effects, and convulsions in DOR heterozygous knockout mice. In wild-type littermates, SNC80 dose-dependently reversed NTG-induced hyperalgesia, consistent with previous reports (Pradhan *et al.,* 2014; Dripps *et al.,* 2017). SNC80 also produced decreases in immobility in the forced swim test and convulsions which are well established behavioral outputs generated by SNC80 (Broom *et al.,* 2002; Saito *et al.,* 2004; Dripps *et al.,* 2017). SNC80 failed to produce any of these behaviors in DOR homozygous knockout mice, further supporting the idea that these behaviors are specifically mediated by DOR. The potency of SNC80 to produce all three of these behaviors was significantly reduced in DOR heterozygous knockout mice, with a rank order of efficacy requirement: convulsions (1.7-fold) < antidepressant like effects (4.2-fold) < antihyperalgesia (7.1-fold). Consistent with the potency changes observed in DOR heterozygous knockout mice, a 30% reduction in DOR number was sufficient to decrease the potency of SNC80 to elicit antihyperalgesia and antidepressant-like effects, but failed to shift the convulsion dose response curve. The rank order was consistent with that observed in DOR transgenic mice. The minimal inhibition of SNC80-induced convulsions following significant reduction in DOR number suggests that convulsions have a large receptor reserve. A large receptor reserve indicates that few receptors need to be activated in order to produce convulsions. Therefore, SNC80-induced convulsions likely have a low efficacy requirement. Conversely, SNC80-induced antihyperalgesia was particularly sensitive to changes in DOR number suggesting a low receptor reserve and high efficacy requirement. The decrease in potency of SNC80-induced

antidepressant-like effects was moderate, suggesting an efficacy requirement between that for convulsions and antihyperalgesia.

Interestingly, the DOR competitive antagonist NTI differentially shifted the dose response curves of the observed DOR-mediated behaviors. A competitive antagonist needs to occupy 50% of receptor sites before its effects on an agonist can be noticed (Kenakin 2009). It is possible that the efficacy requirement for DOR-mediated convulsions is sufficiently low that a larger portion of receptors needs to be occupied to observe antagonism. This hypothesis is consistent with the previously discussed findings that a 30% loss in DOR (by 5'-NTII) does not alter SNC80-induced convulsions but a 60% loss (in DOR heterozygous knockouts) produces mild inhibition. This hypothesis is also consistent with the findings of Broom *et al.* (2002), who showed that a 75% reduction in DOR number abolished BW373U86-induced antinociception but still produced convulsions in a majority of NIH Swiss mice.

Although we did not test enough doses to perform a full pA_2 analysis, it is apparent from our data that the potencies with which NTI antagonizes DOR-mediated behaviors are distinct. For example, 1 mg/kg NTI was sufficient to completely block antihyperalgesia produced by 10 and 32 mg/kg SNC80 but did not affect convulsions produced by SNC80 at either of those doses. Discrepancies in the apparent potency of NTI could be due to differences in the times at which these behaviors can be observed. Because we measured the antihyperalgesic (30 min), antidepressant-like (60 min), and convulsive (0-30 min) effects of SNC80 at different times, the relative concentrations of SNC80 and NTI are likely different and this could impact the observed potency of NTI. However, different antagonist potencies across separate behavioral endpoints could suggest that different receptor populations mediate these behaviors. There are several possibilities regarding what these different receptor populations may represent. The putative DOR(1) and (2) subtypes have previously been implicated in mediating the behavioral effects of SNC80 (Pacheco et al. 2005; Rawls et al. p2005). The existence of delta-mu and delta-kappa receptor heterodimers that engage unique signaling mechanisms has been proposed, and activation of these heterodimers would presumably produce distinct behaviors relative to their monomeric counterparts (Jordan and Devi 1999; Rozenfeld and Devi 2007). Differences in the subcellular localization or internalizing properties of DORs could lead to differences in downstream signaling (Pradhan et al. 2009). Different DOR-mediated behaviors could also be generated by distinct brain regions with variations in G protein expression or coupling efficiency.

It should be noted that multiple DOR agonists have already been developed that do not produce convulsions when given systemically in large doses (Naidu *et al.*, 2007; Vergura *et al.*, 2008; Le Bourdonnec *et al.*, 2008; Saitoh *et al.*, 2011). If indeed the efficacy requirement for convulsions is low, it is critical to determine why these agonists do not produce convulsions. It is possible that these nonconvulsive DOR agonists are biased in such a way as to not produce convulsions. For example, these drugs may be unable to activate the DOR populations responsible for convulsions or may activate different intracellular signaling mechanisms. Alternatively, the pharmacokinetic properties of these drugs could inhibit their ability to produce convulsions. It has been shown that rapid intravenous infusion of SNC80 improves its potency to produce convulsions while slow (20 or 60 min) infusions of SNC80 greatly diminishes potency (Jutkiewicz *et al.,* 2005). Nonconvulsive DOR agonists could be absorbed more slowly, thus preventing the onset of a convulsion. Future studies will evaluate the pharmacokinetic properties of these different DOR agonists

In summary, these data suggest that DOR-mediated behaviors have distinct efficacy requirements with convulsions having the lowest efficacy requirement, followed by antidepressant-like effects and antihyperalgesia, respectively. Furthermore, these DOR-mediated behaviors are likely governed by distinct receptor populations as evidenced by the DOR competitive antagonist NTI attenuating DOR-mediated behaviors with different potencies. Future studies will investigate the nature of these distinct receptor populations and why some DOR agonists do not produce convulsions.

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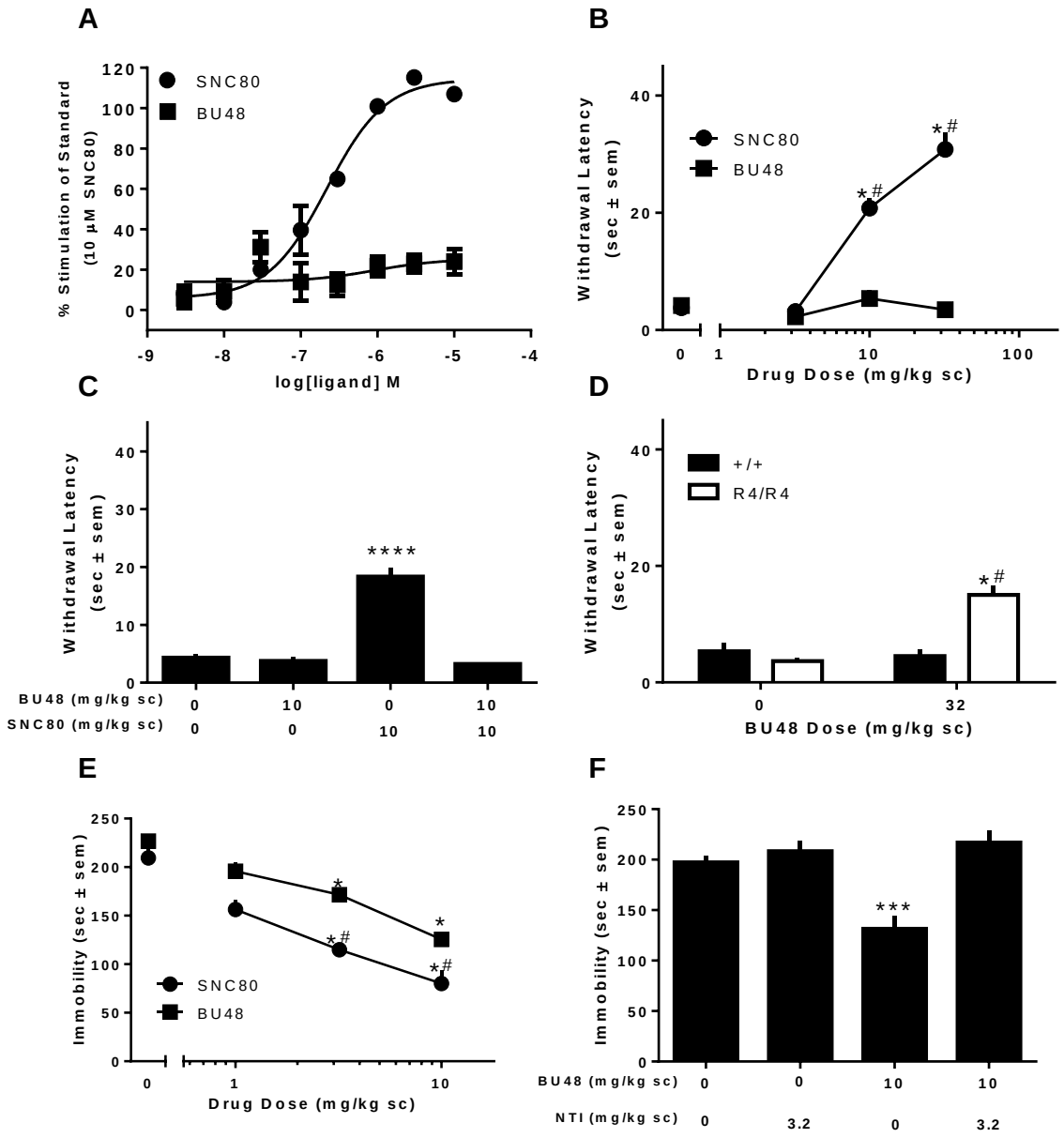

Figure 4.1. Characterization of BU48-induced behaviors. (A) Effect of increasing concentrations of BU48 or SNC80 on ^{35}[S]GTPγS binding in C57BL6 mouse forebrain tissue. Each point represents tissue from 1 mouse assayed in triplicate (n = 3) (B) Effects of different doses of BU48 or SNC80 on tail withdrawal latencies in NTG-treated mice. (C) Effect of 10 mg/kg SNC80 on tail withdrawal latencies in NTG-treated mice following pretreatment with 10 mg/kg BU48 or vehicle. (D) Effects of 32 mg/kg BU48 or vehicle on tail withdrawal latencies in NTG-treated RGS4 wild-type or knockout mice. (E) Effects of different doses of BU48 or SNC80 on immobility scores in the FST. (F) Effect of 10 mg/kg BU48 on immobility in the FST following pretreatment with 3.2 mg/kg NTI or vehicle. n = 6-8 mice per group for all behavior experiments. Data are shown as average per treatment condition with standard error of the mean (sem). * $p < 0.05$ compared to vehicle treatment, # $p < 0.05$ compared to wild-type mice with same BU48 dose. *** $p < 0.001$, **** $p < 0.0001$ compared to all other groups.

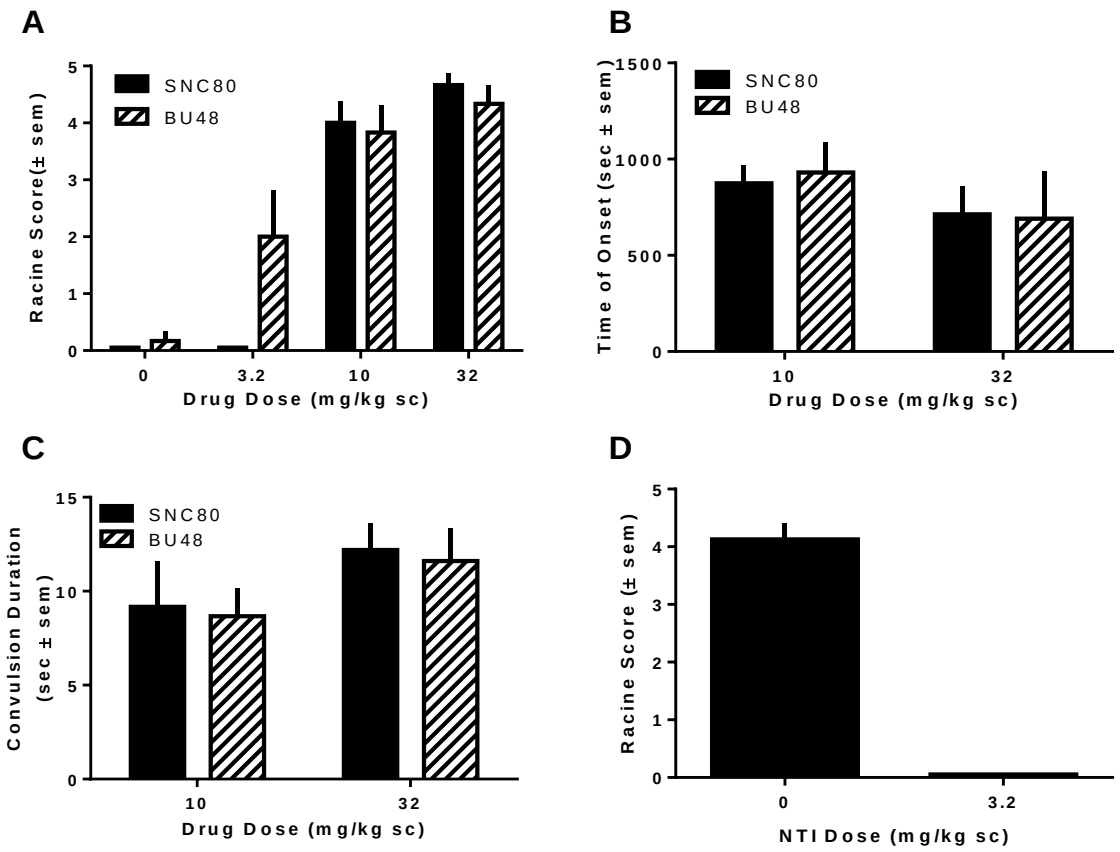

Figure 4.2. Comparison of BU48- and SNC80-induced convulsions. (A) Severity, (B) time of onset, and (C) duration of BU48- and SNC80-induced convulsions. (D) Effect of NTI on the severity of convulsions produced by 10 mg/kg BU48. n = 6-7 mice per group for all experiments. Data are shown as average per treatment condition with standard error of the mean (sem).

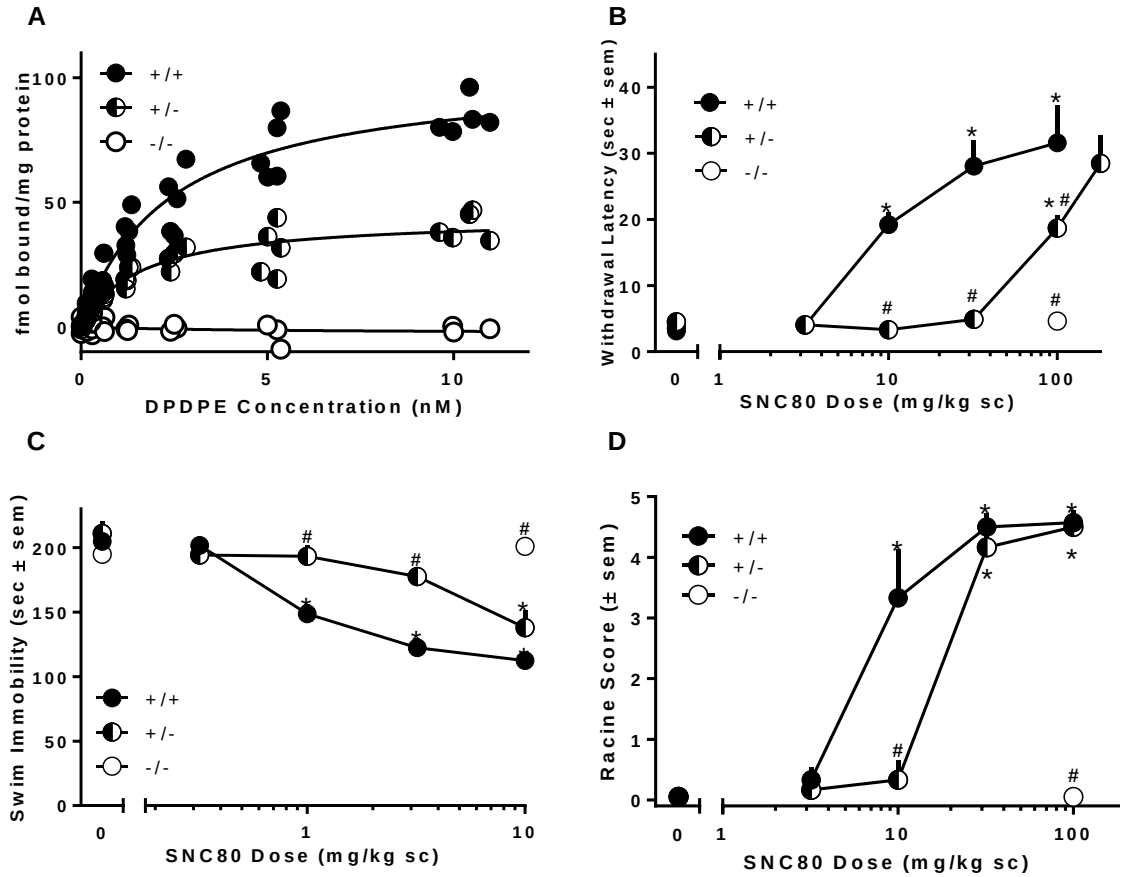

Figure 4.3. Role of receptor density in DOR-mediated behaviors. (A) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of DOR wild-type or mutant mice. Each point represents tissue from a single mouse assayed in triplicate (n = 5). (B) Effects of different doses of SNC80 on tail withdrawal latencies in NTG-treated DOR wild-type (+/+), heterozygous (+/-) and null mutant (-/-) mice. (C) Effects of different doses of SNC80 on immobility scores in the FST in DOR wild-type and mutant mice. (D) Severity of SNC80-induced convulsions in DOR wild-type and mutant mice as measured by a modified Racine scale. Data are shown as average per treatment condition with standard error of the mean (sem). n = 6-7 mice per group for all behavior experiments. * $p < 0.05$ compared to vehicle treatment in the same genotype, # $p < 0.05$ compared to wild-type mice with same drug dose.

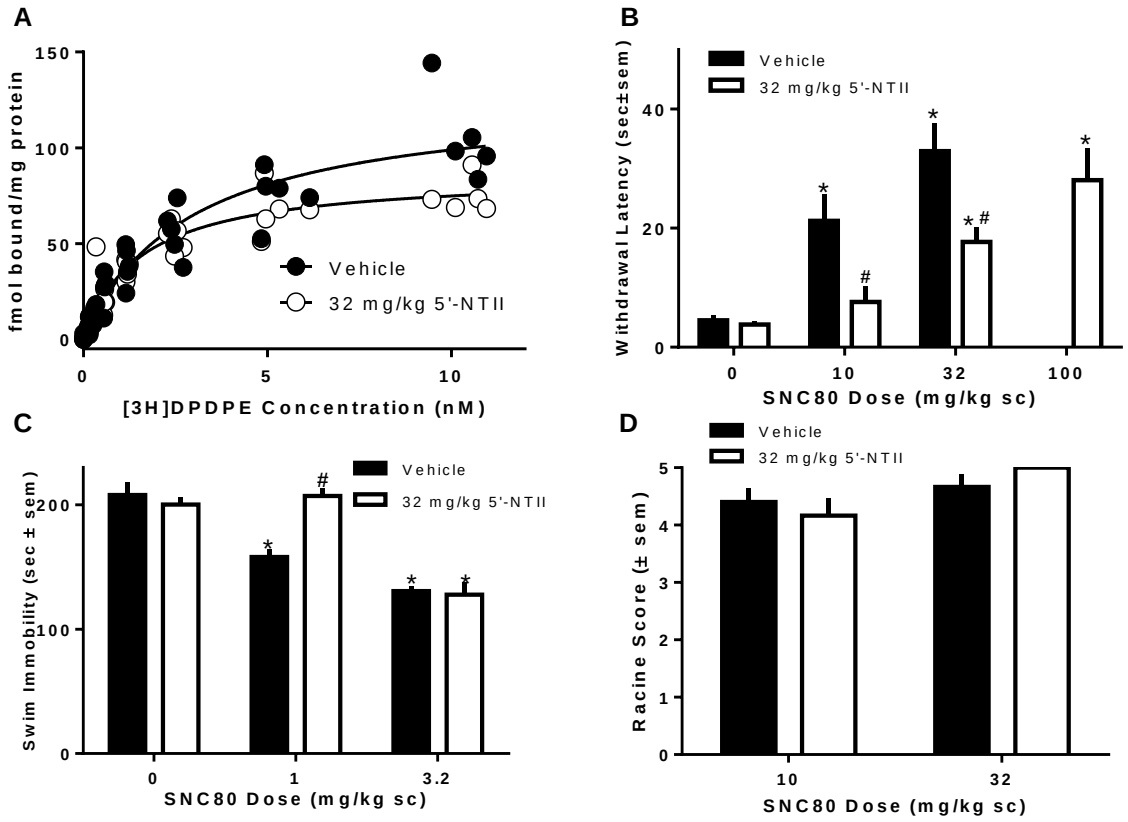

Figure 4.4. Effects of 5'-NTII on DOR density and DOR-mediated behaviors. (A) Saturation binding of [^{3}H]DPDPE to membranes prepared from forebrains of C57BL/6N mice 24 hrs after pretreatment with 32 mg/kg 5'-NTII or vehicle (10% DMSO). Each point represents tissue from a single mouse assayed in triplicate. (B) Effects of different doses of SNC80 on tail withdrawal latencies in NTG-treated mice 24 hrs after pretreatment with 32 mg/kg 5'-NTII or vehicle. (C) Effects of different doses of SNC80 on immobility scores in the FST in mice 24 hrs after pretreatment with 32 mg/kg 5'-NTII or vehicle. (D) Severity of SNC80-induced convulsions in mice as measured by a modified Racine scale 24 hrs after pretreatment with 32 mg/kg 5'-NTII or vehicle. Data are shown as average per treatment condition with standard error of the mean (sem). n = 5-6 mice per group for all experiments.* $p < 0.05$ compared to vehicle SNC80 treatment in the same pretreatment group, # $p < 0.05$ compared to vehicle pretreatment with same SNC80 dose.

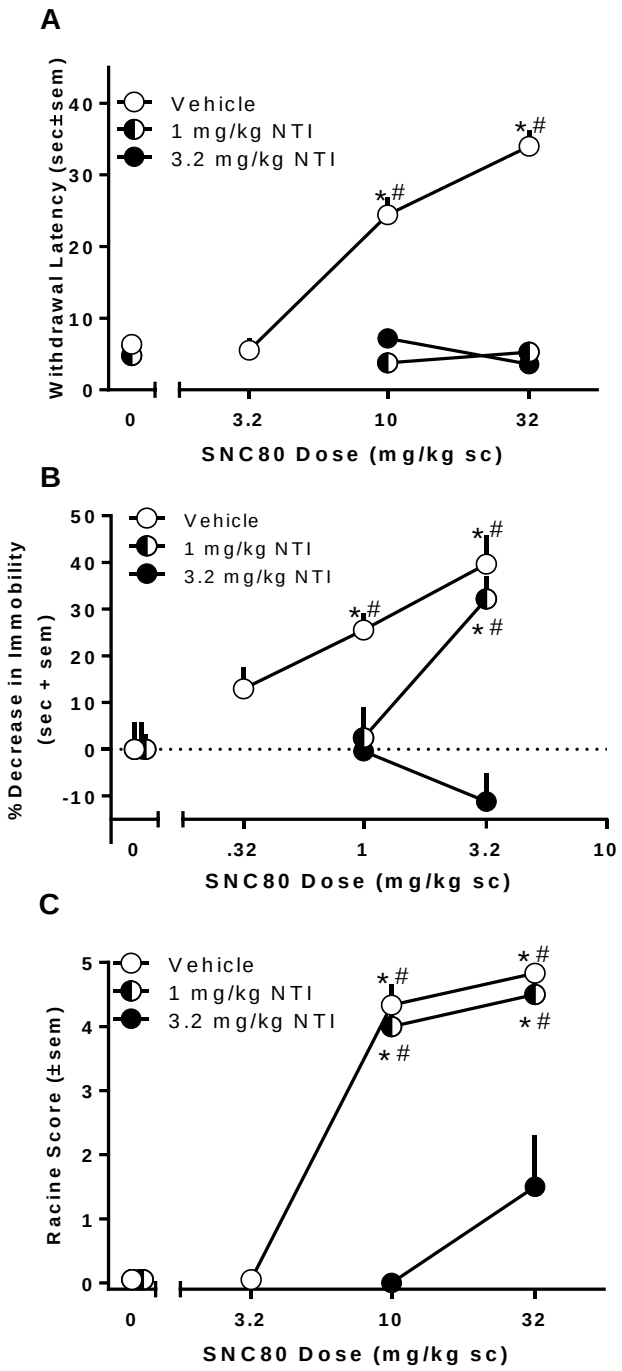

Figure 4.5. Potency of NTI to antagonize DOR-mediated behaviors. Effects of different doses of SNC80 following pretreatment with different doses of NTI on (A) tail withdrawal latencies in NTG-treated mice, (B) immobility scores in the FST, and (C) convulsion severity. Data are shown as average per treatment condition with standard error of the mean (sem). n = 6 mice per group for all experiments.* $p < 0.05$ compared to vehicle SNC80 treatment in the same pretreatment group, # $p < 0.05$ compared to 3.2 mg/kg NTI pretreatment with same SNC80 dose.

Table 4.1. DOR density and agonist affinity in DOR knockout and 5'-NTII treated mice

Group	B_{max} (fmol/mg ± sem)	[^{3}H] DPDPE K_d (nM ± sem)
DOR +/+	105 ± 7	2.3 ± 0.4
DOR +/-	42 ± 3	1.3 ± 0.3
DOR -/-	-3 ± 5	N/A
C57BL6 DMSO	127 ± 11	2.3 ± 0.4
C57BL6 NTII	86 ± 5	1.6 ± 0.3

Chapter V

General Discussion

The experiments described in this thesis sought to identify signaling proteins that regulate the behavioral effects of DOR agonists. Specifically, the antihyperalgesic, antidepressant-like, and convulsive effects of DOR agonists were examined in a number of mouse models. Based on the data presented here, the convulsive and therapeutic effects of DOR are likely governed by distinct signaling mechanisms.

Previous studies have shown that DOR agonists elicit a number of behavioral effects. They produce antinociception and antihyperalgesia in mice, rats, and monkeys (Hong et al. 1998; Gallantine and Meert 2005; Negus et al. 1998; Pradhan et al. 2014; Fraser et al. 2000; Allen et al. 2002), antidepressant-like effects in mice and rats (Saitoh et al. 2004; Broom et al. 2002a), and convulsions in mice, rats, and monkeys (Comer et al. 1993; Broom et al. 2002b; Dykstra et al. 1993). Consistent with these findings, the present studies confirm that SNC80 reliably and dose-dependently produces antinociception, antihyperalgesia, antidepressant-like effects, and convulsions in mice. These effects of SNC80 were all blocked by pretreatment with NTI and were absent in DOR homozygous knockout mice, further indicating that the therapeutic and convulsive effects of SNC80 are all mediated by DOR. Because convulsions are an on-target effect of DOR activation, simply using a more selective DOR agonist would likely not be sufficient to produce the therapeutic effects of DOR without convulsions. Therefore, we chose to investigate potential differences in the pharmacological mechanisms and intracellular signaling pathways that give rise to DOR agonist-induced behaviors.

Dissociating the Behavioral Effects of DOR Agonists: Functional Selectivity

One potential DOR signaling partner, RGS4, had been shown to negatively regulate DOR function *in vitro* (Leontiadis et al. 2009; Wang et al. 2009). *In vivo*, the antidepressant-like effects of a single 5 mg/kg dose of SNC80 were potentiated in RGS4 knockout mice (Stratinaki

et al. 2013). To better understand the role of RGS4 in regulating DOR-mediated behaviors, Chapter II of this thesis compared the ability of SNC80 to induce DOR-mediated antinociception, antihyperalgesia, antidepressant-like effects, and convulsions in wildtype and RGS4 knockout mice. These studies demonstrated that RGS4 differentially regulates DOR-mediated behaviors acting as a negative regulator of some, but not all, behavioral outcomes. Genetic loss of RGS4 or acute pharmacological inhibition of RGS4 with CCG-203769 increased the potency of SNC80 to produce antinociception and antihyperalgesia. The antinociceptive (acetic acid stretch assay), but not the antihyperalgesic effects of SNC80 were blocked by pretreatment with the peripherally-restricted opioid antagonist N-methylnaltrexone, suggesting peripheral DORs likely mediate the antinociceptive actions of SNC80 in the acetic acid stretch assay while DORs in the CNS mediate SNC80-induced antihyperalgesia. These observations suggest that DOR co-localizes with RGS4 within various pain pathways and neurocircuits of the peripheral and central nervous systems to modulate DOR signaling and DOR-induced pain relief *in vivo*. It has been proposed that activation of DOR produces analgesia by attenuating substance P release in the dorsal horn of the spinal cord (Kouchek et al. 2013). It would be interesting to evaluate whether inhibition of RGS regulation of DOR would potentiate DOR-mediated inhibition of substance P release.

RGS4 was shown to play a complex role in regulating DOR-mediated antidepressant-like effects, depending on the type of assay employed. In both the forced swim and tail suspension tests, SNC80 produced U-shaped dose effect curves, such that larger doses of SNC80 (10 mg/kg in TST, 32 mg/kg in FST) failed to produce significant antidepressant-like effects. In the forced swim test, elimination of RGS4 shifted the entire U-shaped function to the left, indicating that RGS4 acts as a negative regulator of DOR-mediated antidepressant-like effects and any possible competing behaviors in this assay. However, loss of RGS4 activity had no significant effect on SNC80-induced antidepressant-like effects in the tail suspension test. It is unlikely that differences in receptor occupancy or efficacy requirement could account for this discrepancy, as similar doses of SNC80 produced antidepressant-like effects in both assays. The behavioral effects of SNC80 in these two assays may originate from separate brain regions, behavioral mechanisms, and/or signaling pathways that are differentially dependent on RGS4 regulation.

In contrast to the role of RGS4 in antinociception, antihyperalegsia, and antidepressant-like effects in the FST, reductions in RGS4 did not alter the potency of SNC80 to induce

convulsions. Although DOR and RGS4 are both highly expressed in the hippocampus, the proposed origin site of convulsions (Simmons and Chavkin 1996; Chung et al. 2015), they may not be co-expressed in the same cells and, therefore, may not functionally interact. The fact that some potential therapeutic effects of DOR are significantly enhanced in heterozygote (+/R4) knockout mice while no changes to convulsion are observed even in homozygote (R4/R4) knockout mice, highlights the distinctiveness of the pathways that underlie these behaviors.

RGS proteins negatively regulate G protein signaling by binding Gα-GTP and accelerating Gα-mediated GTP hydrolysis which returns Gα to an inactive state. Loss of RGS function should prolong the lifetime of active Gα and increase downstream signaling. Consistent with this theory, this study demonstrated that loss of RGS4 potentiated DOR-mediated: 1) phosphorylation of ERK1/2 in mouse striatal tissue, 2) peripheral antinociception, 3) central antihyperalgesia, and 4) antidepressant-like effects measured in the FST, presumably due to prolongation of DOR-mediated G protein signaling and amplification of downstream second messengers. Therefore, it is likely that behaviors regulated by RGS4 are generated through a G protein signaling mechanism and that the convulsive effects of DOR agonists are not mediated by G protein signaling mechanisms.

To further explore regulation of DOR by G protein-dependent and -independent signaling mechanisms, Chapter III of this thesis evaluated the potency of SNC80 to produce antihyperalgesia, antidepressant-like effects, and convulsions in $G\alpha_o$ heterozygous knockout mice, $G\alpha_o$ RGS-insensitive heterozygous knock-in mice, as well as arrestin 2 and arrestin 3 knockout mice. We found that $G\alpha_o$ and arrestins differentially regulate the antihyperalgesia, antidepressant-like effects, and convulsions produced by the DOR agonist SNC80. The potency of SNC80 to produce antihyperalgesia and antidepressant-like effects in the forced swim test was significantly increased in the $G\alpha_o$ RGS-insensitive heterozygous mice. These data indicate DOR-mediated antihyperalgesia and antidepressant-like effects signal through $G\alpha_o$ and are negatively regulated by RGS proteins, consistent with observations from Chapter II that RGS4 negatively regulates these behaviors. Interestingly, the magnitude of these behavioral changes are similar in RGS4 knockout and $G\alpha_o$ RGS-insensitive heterozygous mice, suggesting that other RGS proteins may not play a significant role in regulating the antihyperalgesic and antidepressant-like effects of DOR. Furthermore, SNC80-induced antihyperalgesia was abolished in $G\alpha_o$ heterozygous knockout mice, suggesting that $G\alpha_o$ plays a critical role in mediating signaling

required for DOR-mediated antihyperalgesia. Conversely, decreased expression of $G\alpha_o$ did not affect DOR-mediated antidepressant-like effects in the forced swim test. It is possible that DOR can signal through other G proteins in order to produce antidepressant-like effects and compensate for the reduction in $G\alpha_o$ expression. Alternatively, the efficacy requirement for DOR-mediated antidepressant-like effects may be relatively low compared to that for DOR-mediated antihyperalgesia in which case one functional copy of *GNAO1* and approximately 50% of $G\alpha_o$ protein subunits (Lamberts et al. 2011) could be sufficient to produce a full response in the forced swim test. This hypothesis is consistent with results from experiments described in Chapter IV, which concluded that the efficacy requirement for DOR-mediated antidepressant-like effects is relatively lower than that for DOR-mediated antihyperalgesia. Taken together, these data suggest that DOR-mediated antihyperalgesia and antidepressant-like effects in the forced swim test signal through $G\alpha_o$ and are selectively regulated by RGS4. Future studies should evaluate the role of $G\alpha_o$ and other RGS proteins in regulating SNC80-induced antidepressant-like effects in the tail suspension test.

Because DOR-mediated convulsions were not altered in RGS4 knockout, $G\alpha_o$ heterozygote RGSi, or $G\alpha_o$ heterozygote knockout mice, we evaluated the hypothesis that convulsions are produced by a G protein-independent, arrestin-mediated signaling mechanism. No significant changes in DOR-mediated behaviors, including convulsions, were observed in arrestin 3 knockout mice. However, we did observe that the antinociceptive effects of morphine in the hot plate test were enhanced in arrestin 3 knockout mice, consistent with the findings of Bohn et al. (1999). Our observations with SNC80 are similar to previous reports showing that loss of arrestin 3 in mice did not alter the acute antihyperalgesic effects of DOR agonists in mice with CFA-induced inflammation and had no effect on the coupling of DOR to voltage-dependent calcium channels (Pradhan et al. 2013; Pradhan et al. 2016). These data are the result of acute administration of SNC80 and the possibility that arrestin 3 could play a role in regulating the effects of repeated doses of SNC80 should be explored in the future. Nevertheless, these findings indicate that arrestin 3 does not play a role in the acute behavioral effects of DOR.

In arrestin 2 knockout mice, we observed no changes in SNC80-induced antihyperalgesia or antidepressant-like effects; however, the potency of SNC80 to induce convulsions was enhanced, suggesting that arrestin 2 acts as a negative regulator of DOR-mediated convulsions. Interestingly, many arrestin 2 knockout mice convulsed multiple times in response to a single

dose of SNC80 despite the fact that under normal circumstances tolerance to the convulsive effects of DOR develops acutely and is long lasting (Comer et. al 1993; Hong et al. 1998). One possible explanation for this observation is that loss of arrestin 2 produces these behavioral changes by upregulating DOR trafficking to the cell membrane resulting in enhanced DOR signaling (Mittal et al. 2013). However, arrestin 2 and 3 expression is believed to be ubiquitous (Gurevich and Gurevich 2006), so if loss of arrestin 2 results in enhanced DOR membrane expression, all DOR-mediated behaviors would likely be affected and DOR-mediated antihyperalgesia and antidepressant-like effects were not significantly altered in arrestin 2 knockout mice. Alternatively, arrestin 2 could be necessary for the rapid desensitization and tolerance to the convulsive effects of SNC80. Thus, loss of arrestin 2 could allow signaling pathways that would normally be terminated to persist and produce multiple convulsive events. The behavioral effects of repeated dosing of SNC80 should be evaluated to assess the role of arrestin 2 in the development of tolerance to the effects of DOR agonists. A third possibility, related to the second, is that arrestin 3 is not required for the initiation of a convulsion but is involved in signaling events that sustain convulsive activity. Although class A GPCRs preferentially interact with arrestin 3 (Oakley et al. 2000), arrestin 2 expression in neurons has been shown to be up to 20 times greater than arrestin 3 expression (Gurevich et al. 2004). It is possible that arrestin 2 exerts a protective role in neurons and prevents multiple convulsive events by outcompeting arrestin 3 for binding to DOR. Thus, when arrestin 2 is lost, arrestin 3 dominates and allows for the production of multiple convulsive events. This hypothesis could be evaluated by determining if overexpression of arrestin 3 mimics the effects produced by a loss of arrestin 2.

Overall, these studies demonstrate a role for RGS4 and $G\alpha_o$, but not arrestins, in regulating the acute antihyperalgesic and antidepressant-like effects of DOR. However, DOR-mediated convulsions appear to be negatively regulated by arrestin 2 and are not altered by manipulations to RGS4 or $G\alpha_o$ function. Taken together, these findings suggest that different signaling pathways underlie the convulsive effects of DOR relative to the antihyperalgesic and antidepressant-like effects. This apparent functional selectivity in the DOR system may explain the existence of DOR agonists that do not produce convulsions at doses far exceeding those needed to produce antinociception and antidepressant-like effects (Le Bourdonnec et al. 2008; Saitoh et al. 2011; Chung et al. 2015). These agonists may be biased in such a way as to

preferentially activate signaling pathways regulating the so-called therapeutic effects of DOR. However, the properties of DOR agonists that determine their convulsive nature remain unclear. Future studies should continue to investigate the signaling mechanisms responsible for the behavioral effects of DOR agonists.

Dissociating the Behavioral Effects of DOR Agonists: Efficacy and Receptor Populations

Chapter IV of this thesis explored the hypothesis that differences in the regulation of DOR-mediated behaviors by intracellular signaling partners is due to different efficacy requirements and/or receptor populations. We began exploring the pharmacological characteristics differentiating the behavioral effects of DOR agonists by evaluating the potency and effectiveness of the DOR partial agonist BU48 to produce DOR-mediated antihyperalgesia, antidepressant-like effects and convulsions. BU48 has previously been shown to elicit DOR-mediated convulsions, but not DOR-mediated antinociception in NIH Swiss mice (Broom *et al.*, 2000). In C57BL6 mice, BU48 produced dose-dependent increases in convulsion severity with similar potency and efficacy to SNC80. Unlike with SNC80, mice treated with 3.2 mg/kg BU48 exhibited some preconvulsive behavior, such as head twitches and brief myoclonic jerks.

BU48 also produced antidepressant-like effects in the forced swim test with reduced potency relative to SNC80. The apparent difference in observed efficacy in these behavioral tests between BU48 and SNC80 may be due to differences in intrinsic efficacy at DOR and/or the combination of DOR agonism with some KOR agonist properties of BU48. It is somewhat surprising that BU48 produces antidepressant-like effects despite being equipotent at DOR and KOR and more efficacious at KOR *in vitro* (Broom et al. 2000). Perhaps BU48 could be a useful pharmacological tool for evaluating the relative efficacy requirements for DOR-mediated antidepressant-like effects and KOR-mediated prodepressant-like effects. *Ex vivo,* BU48 did not significantly stimulate ^{35}[S]GTPγS binding in forebrain tissue. This finding suggests that BU48 is a low efficacy agonist, at least at the level of G protein activation, or engages G protein-independent signaling pathways.

In the NTG-induced thermal hyperalgesia assay, BU48 not only failed to produce antihyperalgesia in wild-type mice, it antagonized SNC80-induced antihyperalgesia. Although it is possible that BU48 is a biased agonist incapable of activating the intracellular signaling

mechanisms needed to produce antihyperalgesia, BU48 did produce mild antihyperalgesia in RGS4 knockout mice. Because RGS4 acts as a negative regulator of $G\alpha_{i/o}$ signaling this observation suggests that the inability of BU48 to produce antihyperalgesia is due to insufficient efficacy and that BU48 is capable of producing antihyperalgesia via a SNC80-like mechanism. Taken together, these data strongly suggest that BU48 is a DOR partial agonist. A partial agonist should more readily produce low efficacy-requiring behaviors as compared to behaviors with higher efficacy requirements. Therefore, the ability of BU48 to produce DOR-mediated convulsions with potency comparable to a full agonist is consistent with convulsions having a low efficacy requirement. Furthermore, BU48 producing antidepressant-like effects with inferior potency and failing to generate antihyperalgesia on its own indicates that these behaviors have higher efficacy requirements relative to convulsive effects.

To further evaluate the role of efficacy requirements and receptor reserve in DOR-mediated behaviors, the potency of SNC80 to produce antihyperalgesia, antidepressant-like effects, and convulsions was evaluated in DOR heterozygous knockout mice (Receptor densities— +/+: 105 ± 7 fmol/mg; +/-: 42 ± 3 fmol/mg). The potency of SNC80 to elicit all three of these behaviors was significantly reduced in DOR heterozygous knockout mice, with the following rank order of efficacy requirement: convulsive effects < antidepressant-like effects < antihyperalgesia. SNC80 did not produce any of these behaviors in DOR homozygous knockout mice, further validating the idea that these behaviors are specifically mediated by DOR.

The effect of changes in DOR number on DOR-mediated behaviors was also investigated using the irreversible antagonist 5'-NTII. Consistent with the potency changes observed in DOR heterozygous knockout mice, a 30% reduction in DOR number was sufficient to decrease the potency of SNC80 to produce antihyperalgesia and antidepressant-like effects. However, this small decrease in DOR number failed to shift the convulsion dose response curve. The minimal inhibition of SNC80-induced convulsions following reduction in DOR number of either 30% or 60% suggests that convulsions have a large receptor reserve. Therefore, few receptors would need to be activated in order to produce convulsions. Consequently, DOR-mediated convulsions likely have a low efficacy requirement. Conversely, SNC80-induced antihyperalgesia was quite sensitive to reductions in DOR number suggesting a low receptor reserve and high efficacy requirement. The decrease in potency of SNC80-induced antidepressant-like effects was moderate, suggesting an efficacy requirement between that for convulsions and antihyperalgesia.

Chapters II and II of this thesis demonstrated that DOR-mediated behaviors are differentially regulated by $G\alpha_o$, RGS4, and arrestin 2. Specifically, SNC80-induced antihyperalgesia and antidepressant-like effects were potentiated in RGS4 knockout mice and in $G\alpha_o$ RGS-insensitive knock-in mice. In addition, SNC80-induced antihyperalgesia was abolished $G\alpha_o$ heterozygous knockout mice, but antidepressant-like effects were unaffected. However, SNC80-induced convulsions were unaltered in those transgenic mouse strains and potentiated in arrestin 2 knockout mice. We hypothesized that these observations could reflect functional selectivity within the DOR system and that convulsions may be mediated by distinct signaling pathways relative to the antihyperalgesic and antidepressant-like effects.

The results described in Chapters II and III of this thesis are also consistent with the alternative hypothesis presented in Chapter IV that differential regulation of DOR-mediated behaviors is due to differences in the efficacy requirements of those behaviors. For example, the efficacy requirement for DOR-mediated convulsions may be so low that they are unaffected by a loss of RGS regulation or a 50% reduction in $G\alpha_o$. The efficacy requirement for antidepressant-like effects may be high enough so as to be under regulation of RGS proteins but still low enough that 50% of $G\alpha_o$ expression is sufficient to produce a normal response. Finally, the efficacy requirement for antihyperalgesia would be so high that it is affected by both RGS regulation and a 50% loss of $G\alpha_o$.

Interestingly, the DOR competitive antagonist NTI shifted the dose response curves of DOR-mediated behaviors to different degrees. Although we did not test enough doses to perform a full pA_2 analysis, our data still suggest that NTI antagonizes DOR-mediated behaviors with distinct potencies. For example, 1 mg/kg NTI was sufficient to completely block antihyperalgesia produced by 10 and 32 mg/kg SNC80 but did not affect convulsions produced by SNC80 at either of those doses. These differences in antagonist potencies across separate behavioral endpoints suggest that different receptor populations mediate these behaviors. There are several possibilities regarding what these different receptor populations represent. The putative DOR(1) and (2) subtypes may mediate different behavioral effects of SNC80 (Pacheco et al. 2005; Rawls et al. 2005). Delta-mu and delta-kappa receptor heterodimers that engage unique signaling mechanisms to produce distinct behaviors relative to their monomeric counterparts have been proposed (Jordan and Devi 1999; Rozenfeld and Devi 2007). Differences in the subcellular localization or internalizing properties of DORs could lead to differences in

downstream signaling (Pradhan et al. 2009). It is well accepted that different DOR-mediated behaviors are generated by distinct brain regions. For example, DOR-mediated analgesia is governed by the dorsal horn (Cahill et al. 2003) and PAG (Ossipov et al. 1995) while DOR-mediated convulsions originate in the hippocampus (Simmons and Chavkin 1996). However, DOR subpopulations may exist within these distinct brain regions with variations in G protein expression, subtypes, or coupling efficiency. Alternatively, these distinct receptor populations could also represent differences in the times at which these behaviors can be observed. If these distinct DOR populations are identified, it would be interesting to investigate in future studies whether they are differentially regulated by proteins such as RGS4, $G\alpha_o$, and arrestins.

In summary, these data suggest that DOR-mediated behaviors have distinct efficacy requirements with convulsions having the lowest efficacy requirement, followed by antidepressant-like effects and antihyperalgesia, respectively. Furthermore, these DOR-mediated behaviors are likely governed by distinct receptor populations as evidenced by the DOR competitive antagonist NTI attenuating DOR-mediated behaviors with different potencies.

Dissociating the Behavioral Effects of DOR Agonists: Nonconvulsive Delta Agonists

As described in the introduction of this thesis, multiple DOR agonists have already been developed that do not produce convulsions when given systemically in large doses (Naidu *et al.,* 2007; Vergura *et al.,* 2008; Le Bourdonnec *et al.,* 2008; Saitoh *et al.,* 2011). If indeed the efficacy requirement for convulsions is low, it is critical to determine why these agonists do not produce convulsions. It is possible that these nonconvulsive DOR agonists are biased in such a way as to not produce convulsions. For example, these drugs may be unable to activate the DOR populations responsible for convulsions or may selectively activate different intracellular signaling mechanisms downstream of DORs. Alternatively, the pharmacokinetic properties of these drugs could inhibit their ability to produce convulsions. It has been shown that rapid intravenous infusion of SNC80 improves its potency to produce convulsions while slow (20 or 60 min) infusions of SNC80 greatly diminishes potency (Jutkiewicz *et al.,* 2005). Nonconvulsive DOR agonists could be absorbed more slowly, thus preventing the onset of a convulsion.

To begin to address these possibilities, we evaluated the behavioral effects of the nonpeptidic DOR agonist KNT-127 (See Appendix). In C57BL6 wild-type mice, KNT-127

produced DOR-mediated antihyperalgesia and DOR-mediated antidepressant-like effects with similar potency to SNC80 (Figures A.1-A.4). Consistent with previous reports, KNT-127 did not produce convulsions at doses of at least 32 mg/kg sc (Figure A.5; Saitoh et al. 2011). KNT-127 also did not produce convulsions when administered intravenously (iv) at doses up to 10 mg/kg, suggesting that the discrepancies in the convulsive properties of KNT-127 and SNC80 are likely not due to differences in absorption or distribution. It is possible that KNT-127 could produce convulsions if given directly into the brain, however we were unable to produce convulsions in mice given intracerebroventricular injections of SNC80 (data not shown).

To explore the hypothesis that KNT-127 is biased in such a way that it does not activate the signaling mechanisms needed to produce convulsive effects, we examined the effects of KNT-127 pretreatment on SNC80-induced convulsions. If KNT-127 engages the same receptor populations and receptor binding sites as SNC80 but is biased against convulsive effects, it should antagonize SNC80-induced convulsions. However, pretreatment with 10 mg/kg KNT-127 did not block convulsions produced by 10 mg/kg SNC80 (Figure A.6). It is possible that larger doses of KNT-127 are needed to inhibit SNC80-induced convulsions and this should be evaluated in the future. Another possibility is that KNT-127 does not interact with the DOR population responsible for convulsions. However, KNT-127 did reduce the dose of the chemical convulsant PTZ needed to produce convulsions (Figure A.7). Although these data suggest that KNT-127 does have some convulsive properties, the DORs responsible for enhancing PTZ-induced convulsions may be distinct from those that mediate SNC80-induced convulsions. In support of this hypothesis, it was recently shown that loss of DOR expression in GABAergic forebrain neurons was sufficient to prevent SNC80-induced convulsions and EEG disturbances, however PTZ produced similar convulsive effects in these transgenic mice and wild-type controls (Chung et al. 2015). Taken together, these data may suggest that KNT-127 does not produce convulsions on its own because it does not activate the receptor population responsible for DOR-mediated convulsions. This hypothesis could be tested by comparing the potencies of SNC80 and KNT-127 to activate G protein across different brain regions using GTPγS autoradiography. Future studies should further evaluate the nature of these distinct receptor populations and why some DOR agonists do not produce convulsions.

Conclusions

DOR agonists produce a number of behavioral effects, including antihyperalgesia, antidepressant-like effects, and convulsions. These behavioral effects are differentially regulated by multiple signaling molecules such as RGS4, $G\alpha_o$, and arrestin2. These behavioral effects also display the following rank order of efficacy requirement: convulsions < antidepressant-like effects < antihyperalgesia. Distinct populations of DORs may mediate these different behavioral effects and it is possible that DOR agonists differentially activate these receptor populations. Future studies should continue to examine the nature of these distinct DOR populations. Future work should also investigate where the signaling molecules that differentially regulate DOR-mediated behavior exert their effects. For example, it would be interesting to investigate whether loss of RGS regulation of DOR or $G\alpha_o$ expression specifically in the dorsal horn and/or PAG affected DOR-mediated analgesia. In further exploring the mechanisms responsible for DOR-mediated antidepressant-like effects, the effect of RGS4 and $G\alpha_o$ function on BDNF and glutamate release should be examined. Overall, the work presented in this thesis should benefit the production of safer DOR agonists with improved clinical utility. The possibility of pharmacologically targeting receptor populations or signaling pathways responsible for DOR-mediated analgesic and antidepressant-like effects without activating receptors that mediated convulsions would greatly aid the clinical viability of DOR agonists.

Appendix

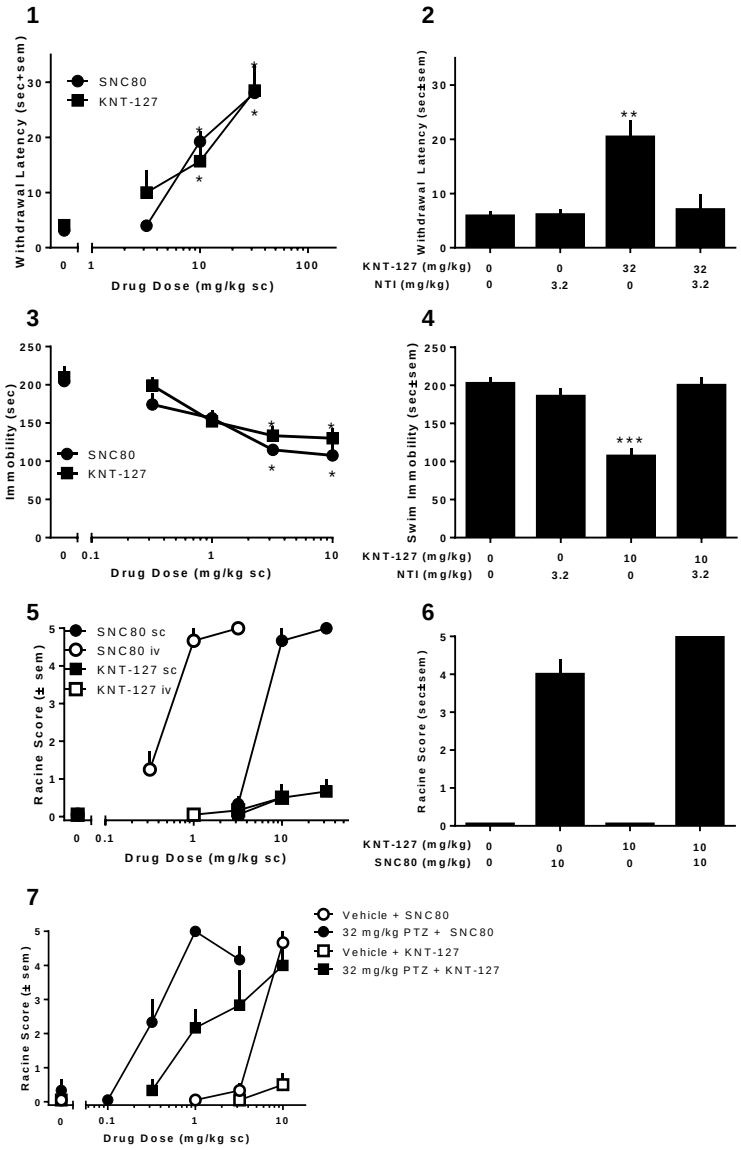

Figure A. Characterization of KNT-127-induced behaviors in C57BL6 wild-type mice. (1) Effects of different doses of KNT-127 or SNC80 on tail withdrawal latencies in NTG-treated mice. (2) Effect of NTI on KNT-127-induced increases in tail withdrawal latency in NTG-treated mice. (3) Effects of different doses of KNT-127 or SNC80 on immobility scores in the forced swim test. (4) Effect of NTI on KNT-127-induced decreases in immobility in the forced swim test. (5) Effects of route of administration on KNT-127- and SNC80-induced convulsive effects (6) Effects of KNT-127 on SNC80-induced convulsions (7) Effects of different doses of KNT-127 or SNC80 on PTZ-induced convulsions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to vehicle treatment.

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