Role of Regulator of G-Protein Signaling Proteins in Serotonin and Opioid Mediated Behaviors

by

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Analysis of variance (ANOVA)

artificial cerebrospinal fluid (aCSF)

Bicinchoninic acid (BCA)

cAMP response element-bindinG-protein (CREB)

Central nervous system (CNS)

Complete Freund's adjuvant (CFA)

Cornu Ammonis 1 (CA1)

Cyclic adenosine monophosphate (cAMP)

Delta opioid receptor (DOPR)

Dorsal raphe nucleus (DRN)

Dorsal root ganglion (DRG)

Fibroblast growth factor 2 (FGF2)

G-protein-coupled receptor (GPCR)

G-protein-coupled receptor kinase (GRK)

G-protein-coupled inwardly-rectifying potassium channel (GIRK)

G-protein gamma-like (GGL)

Gamma-Aminobutyric acid (GABA)

Glycogen synthase kinase 3 beta (GSK3β)

GTPase activatinG-protein (GAP)

Guanosine diphosphate (GDP)

Guanosine triphosphate (GTP)

Input resistance (IR)

Intracerebroventricular (i.c.v.)

Intraperitoneal (i.p.)

Intraplanter (i.pl.)

Kappa opioid receptor (KOPR)

Major depressive disorder (MDD)

Messenger ribonucleic acid (mRNA)

Mitogen-activated protein kinase (MAPK)

Monoamine oxidase inhibitor (MAOI)

Mu opioid receptor (MOPR)

N-methyl-D-aspartate (NMDA)

Nicotinamide adenine dinucleotide phosphate (NADPH)

Nociceptin receptor (NOPR)

Nociceptin/Orphanin FQ (N/OFQ)

Periaqueductal gray (PAG)

Regulator of G-protein signaling (RGS)

Resting membrane potential (RMP)

Rho guanine nucleotide exchange factor (RhoGEF)

RGS homology (RH)

RGS insensitive (RGSi)

Selective serotonin reuptake inhibitors (SSRI)

Serotonin (5-HT)

Serotonin 1A receptor (5-HT1AR)

Serotonin norepinephrine reuptake inhibitor (SNRI)

Single nucleotide polymorphism (SNP)

Sodium dodecyl sulfate (SDS)

Standard error of measurement (SEM)

Subcutaneous (s.c.)

Tail suspension test (TST)

Triton-X 100 (TTX100)

Triton-X 114 (TTX114)

Wild type (WT)

ABSTRACT

Multiple classes of drugs, including antidepressants as well as opioid analgesics, exert their therapeutic effects at least in part by direct or indirect actions at G-protein-coupled receptors (GPCR's). This class of receptor propagates a signal inside the cell by activating heterotrimeric G-proteins (comprised of Gα and Gβγ subunits). Following receptor activation GDP bound to the Gα subunit of the heterotrimer is exchanged for GTP, followed by separation of the Gα and Gβγ subunits. Both Gα and Gβγ then activate downstream signaling pathways inside the cell. The regulator of G-protein signaling (RGS) proteins are a class of intracellular regulatory proteins that serve as negative modulators of GPCR signaling. They exert their actions by binding to active Gα-GTP subunits and accelerating the hydrolysis of GTP to GDP with subsequent reformation of the Gα/βγ heterotrimer, thus terminating signaling.

In this work, I describe the use of mice expressing Gα subunits of one of two types (either $G\alpha_{i2}$ or $G\alpha_{o}$) that do not bind to RGS proteins and so are RGS insensitive (RGSi) to gain a better understanding of how RGS proteins regulate both antidepressant-like and antinociceptive behaviors. Site-specific microinjections of a serotonin 1A receptor (5HT1AR) antagonist and agonist are used to show that mice expressing RGSi $G\alpha_{i2}$ have a robust antidepressant-like phenotype dependent on hippocampal 5-HT1AR activity. *Ex vivo* recording from hippocampal tissue confirms that a 5-HT1AR agonist inhibits cellular activity more effectively in mice expressing RGSi $G\alpha_{i2}$. Furthermore, I demonstrate that hippocampal administration of an RGS4/19 inhibitor (CCG203769) produces antidepressant-like effects. I show that mice expressing RGSi $G\alpha_{o}$ have a complex phenotype including increased and decreased sensitivity to noxious stimuli consistent with alterations in both nociceptin receptor (NOPR) and mu opioid receptor (MOPR) activity. The balance between the antinociceptive MOPR and pronociceptive NOPR systems is disturbed in the RGSi $G\alpha_{o}$ mice and in wild type mice during inflammatory pain. This work expands upon previous findings showing profound differences between mice

with enhanced signaling downstream of different $G\alpha_{i/o}$ proteins due to the loss of RGS control, and provides novel information regarding the receptor systems and brain regions involved, with implications for both the treatment of depressive disorders and pain. This work is significant because it provides a greater understanding of the G-proteins involved in the perception of pain and depression, as well as the receptor systems and RGS proteins that control their activity.

INTRODUCTION

G-protein-coupled receptors (GPCRs) play an integral role in therapeutics as approximately 40% of all available prescription pharmaceuticals target GPCRs (Filmore 2004). While there is considerable structural variability between GPCRs, this family of proteins exhibit a conserved heptahelical transmembrane structure (Lagerstrom and Schioth 2008) that leads to the alternate nomenclature '7-transmembrane domain receptors.' The mu-opioid receptor (Fig. 0.1) is a typical example. There are over 800 GPCRs expressed in the human genome (Gloriam *et al.*, 2007), and drugs targeting these receptors are indicated for conditions including chronic pain, neuropsychiatric disorders, cancer and cardiac dysfunction among many others (Filmore 2004). Understanding the signaling processes that GPCRs regulate is therefore of critical importance not only for the development of new pharmaceuticals, but also to gain a better understanding of how

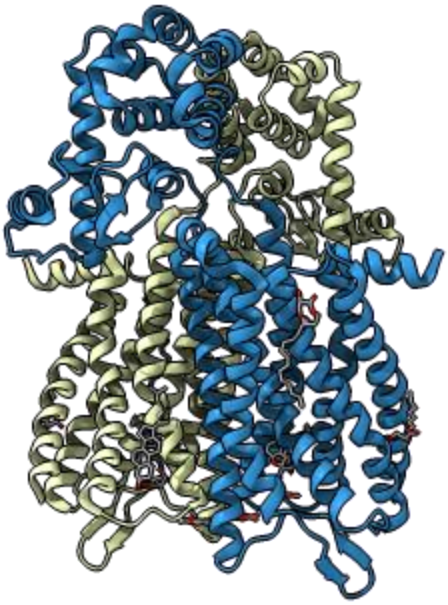

Fig. 0.1: Ribbon diagram of the mu-opioid receptor crystal structure bound to a morphinan antagonist.

Adapted with permission from Manglik *et al.*, 2012.

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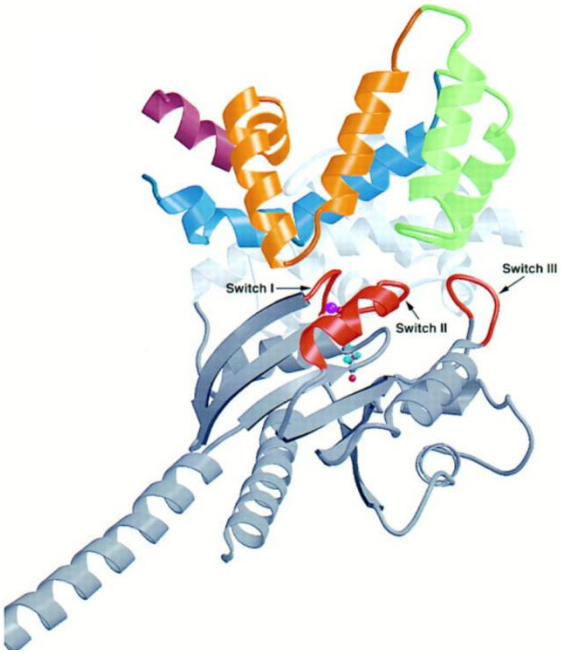

Fig. 0.2: Ribbon diagram of RGS4 (top) in contact with $G\alpha_{i1}$ (bottom) generated from a AlF_4-stabilized crystal structure.

From Tesmer *et al.*, 1997. Reprinted with permission from AAAS

existing drugs that target GPCRs function. The regulator of G-protein signaling (RGS) proteins (Fig. 0.2) are a family of intracellular regulatory proteins that have received increasing attention due to their ability to regulate GPCR activity (Fig. 0.3). These proteins express a conserved RGS homology (RH) domain which provides the ability for RGS proteins to act as GTPase activating proteins (GAPs). This GAP activity allows RGS proteins to inhibit signaling downstream of GPCRs by facilitating the conversion of GTP to GDP in active Gα subunits. There is evidence that RGS proteins have selectivity for different Gα subunits (Table 0.1) and receptors. The specifics of RGS protein regulation of GPCR signaling in relation to antidepressant action is discussed at length in chapter 1 and in relation to opioid analgesic action in chapter 3.

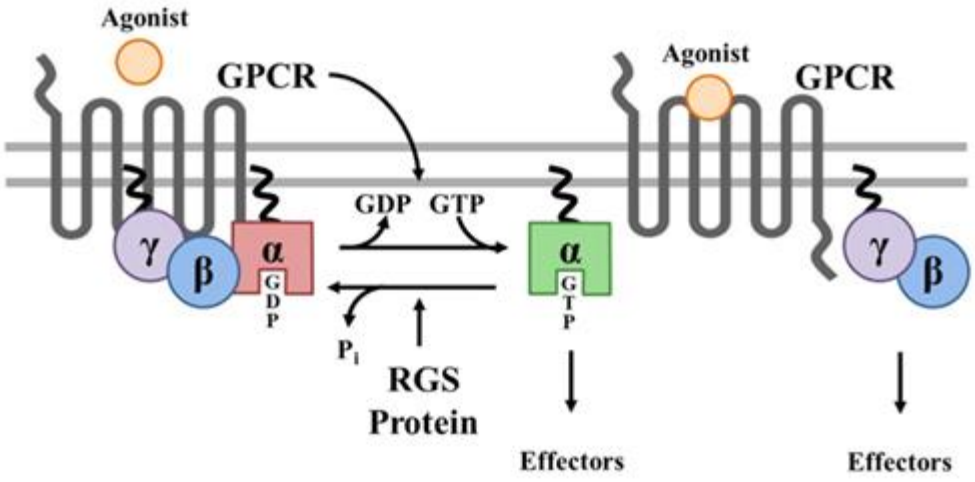

Fig. 0.3: Depiction of GPCR cycle with regulation by RGS proteins. RGS proteins interact with the active Gα subunit and facilitate its inactivation, allowing reformation of the heterotrimeric G-protein complex and association with the receptor, thereby terminating signaling.

Adapted with permission from Stewart *et al.*, 2012. Originally published in Frontiers in Physiology

The work presented in chapters 2 and 4 will describe the use of knock-in mouse models expressing modified Gα proteins that are insensitive to the negative modulatory effects of the RGS proteins. These mouse models are used to explore the intracellular signaling processes downstream of the mu opioid (MOPR), nociceptin (NOPR) and serotonin 1A (5-HT1A) receptors that contribute to both antidepressant-like (chapter 2) and antinociceptive effects (chapter 4). The loss of RGS control at these modified G-proteins depends on a glycine to serine

mutation at position 184 in either $G\alpha_{i2}$ or $G\alpha_{o}$. The resulting gain of function G-protein variant are known as RGS insensitive (RGSi) G-proteins. Further background on these RGSi G-proteins is provided in chapters 1 and 3.

Table 1. Mammalian RGS Proteins

RGS family	Domains present	RGS	G protein specificity
Classical RGS – known Gα binding, GAP activity, and functional inhibition			
RZ	Cysteine-string	RGS19 (GAIP)	Gi Gz Gq
		RGS17 (RGS Z2)	Gi Gz Gq
		RGS20 (RGS Z1)	Gi, Gz
R4	N-terminal amphipathic sequence	RGS1	Gi Gq
		RGS4	Gi Gq
		RGS2	Gq»Gi
		RGS3	Gi Gq
		RGS5	Gi Gq
		RGS8	Gi Gq
		RGS13	ND
		RGS16	Gi Gq
		RGS18	Gi Gq
		RGS21	Gi Gq
R7	GGL–(Gβ5) DEP–(R9AP, R7BP)	RGS9	Go
		RGS7	Goα > Giα2 > Giα1
		RGS11	Go
		RGS6	Go
R12	GoLoco–(Gα-GDP) RBD–(rap) PDZ	RGS12	Gi
		RGS14	Gi
		RGS10	Gi

Table 0.1: RGS protein families, with conserved domains and G-protein specificity highlighted.

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CHAPTER 1

The Role of RGS and G-proteins in the Regulation of Depressive Disorders

Major depressive disorder (MDD) is one of the most prevalent psychiatric disorders with over 16% of adults in the US experiencing a depressive event within their lifetime, and over half of these events leading to severe or very severe role impairment (García-Velázquez *et al.*, 2017; Kessler *et al.*, 2003). While a multitude of antidepressant drugs are now available, no one treatment is fully effective in all patients, and any individuals fail to respond to even 2nd or 3rd line treatment options (Insel and Wang 2009). This high rate of treatment failure combined with the high prevalence of depressive disorders demonstrates the need not only for improved treatment options, but also for a better understanding of the factors that determine whether a given treatment will succeed or fail.

GPCRs in Depression

The vast majority of drugs prescribed for depressive disorders either interact directly with G-protein-coupled receptors (GPCRs) (e.g. buspirone with 5-HT1A or aripiprazole with a multitude of monoaminergic GPCRs) or indirectly regulate GPCR function by affecting endogenous neurotransmitter levels (e.g. selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine and monoamine oxidase inhibitors (MAOIs) such as selegiline; Kantor *et al.*, 2015). Additionally, many GPCRs have been associated with the development of depression. Aberrations in both alpha- and beta-adrenergic receptor signaling have been found in depressed patients (Matussek *et al.*, 1980; Ebstein *et al.*, 1988) and the brains of suicide victims consistently have alterations in 5-HT1A receptor expression (and various nuclear receptors) resembling the alterations produced by chronic stress in animal models (Lopez *et al.*, 1998). In contrast, study of 5-HT2A and 5-HT2C receptor expression levels has produced inconsistent

results (see Stockmeier 2003 for review). Nonetheless, a 5-HT2C receptor polymorphism in the N-terminal extracellular domain has been associated with MDD in a large population study (Lerer *et al.,* 2001). Preclinical models also implicate the 5-HT1B receptor both in the genesis of depressive states and in antidepressant action (Svenningsson *et al.,* 2006). Polymorphisms in both the dopamine D3 and D4 receptors have been correlated with the development of major depressive disorder (Dikeos *et al.,* 1999; Lopez Leon *et al.,* 2005), while D1 and D2 receptors have instead been linked to bipolar disorder (Dmitrzak-Weglarz *et al.,* 2006; Massat *et al.,* 2002). Interestingly the GABA-B receptor agonist baclofen produces a transient depressive state in some patients (Post *et al.,* 1991) suggesting this receptor may play a role in major depressive disorder. A corticotropin-releasing hormone receptor 1 antagonist has also been found to have antidepressant activity (Zobel *et al.,* 2000) in agreement with predictions from preclinical studies (Mansbach *et al.,* 1997).

In the inactive state GPCRs form a multi-protein complex with the intracellular α, β and γ type G-protein subunits (see Figure 0.1). Following GPCR activation by endogenous neurotransmitters or exogenous agonists both the Gα subunit and the Gβγ complex will dissociate from the receptor and go on to stimulate or inhibit a range of intracellular effectors. The Gα activation process involves a loss of bound GDP (inactive form) in exchange for GTP (active form). Signaling is terminated by the hydrolysis of the bound GTP back to GDP by the GTPase activity of the Gα subunit. A regulator of G-protein signaling (RGS) protein then binds to active Gα and facilitates its inactivation through an intrinsic GTPase accelerating protein (GAP) activity. This enhances the Gα subunits' innate GTPase activity, allowing the return to an inactive state through hydrolysis of Gα bound GTP. The inactive GDP-bound Gα subunit can then recouple with both the receptor and Gβγ complex until the receptor is again activated and the cycle repeats. For a review of GPCR signaling see Oldham and Hamm (2008).

While this general process is well understood, determining specific RGS and G-proteins involved in antidepressant action may facilitate future antidepressant drug development. Furthermore, studying dysfunction of these systems in the depressed brain may provide insight into the etiology of depression. Therefore, this chapter will highlight findings that provide insight into how specific RGS and G-proteins contribute to depressive disorders and regulate antidepressant action.

G-Protein Subunits Involved in Depression and Antidepressant Action

In preclinical studies, central nervous system (CNS) G-protein expression levels do not appear to change consistently as a result of antidepressant drug treatment. $G\alpha_s$, $G\alpha_o$ and $G\alpha_i$ mRNA expression in the rat hippocampus remained constant following chronic treatment with the tricyclic antidepressant imipramine (Lasoń *et al.,* 1993). Similarly, chronic treatment with the dual serotonin norepinephrine reuptake inhibitor (SNRI) amitriptyline, the tricyclic antidepressant desipramine, the MAOI tranylcypromine or electroconvulsive shock did not affect protein levels of $G\alpha_s$, $G\alpha_o$, $G\alpha_i$ or $G\beta$ in the rat cerebral cortex (Chen and Rasenick 1995b; Emamghoreishi *et al.,* 1996; Dwivedi and Pandey 1997). In contrast, sub-chronic treatment with the MAOI antidepressant phenelzine increased $G\alpha_{i2}$ protein expression in the rat cortex and hippocampus without affecting $G\alpha_s$, $G\alpha_o$, $G\alpha_q$ or $G\alpha_{i1}$ expression in any brain region tested (Dwivedi and Pandey 1997) consistent with the involvement of $G\alpha_{i2}$ in the actions of serotonergic antidepressants observed in mouse models (Talbot *et al.,* 2010). However, this does not appear to be a conserved effect for all MAOI antidepressants on $G\alpha_{i2}$, as chronic treatment with tranylcypromine did not affect cortical $G\alpha_{i2}$ levels while chronic clorgiline instead produced a small decrease (Lesch *et al.,* 1991; Emamghoreishi *et al.,* 1996). Three-week treatment with various tricyclic antidepressants (imipramine, desipramine, or chlomipramine) produced slight increases of brain $G\alpha_o$ and decreases of $G\alpha_s$ and $G\alpha_i$, although the magnitude of these changes (~10-30% from baseline) may not be great enough to produce functional consequences (Lesch *et al.,* 1991). Furthermore, tricyclics such as desipramine and amitriptyline did not produce this effect in other studies (Chen and Rasenick 1995b; Emamghoreishi *et al.,* 1996).

A series of post-mortem studies examining the involvement of G-proteins in depressive states contrasts with the conclusions from pre-clinical studies discussed above. Post-mortem studies indicate that a downregulation of $G\alpha_o$ and $G\alpha_{i2}$ protein levels and mRNA co-occurs with an upregulation of $G\alpha_s$ protein levels and mRNA in the prefrontal cortex of adult suicide victims (Dwivedi *et al.,* 2002). However, only teenage subjects with a diagnosed history of mental illness showed such abnormalities with decreased mRNA for $G\alpha_o$ and $G\alpha_{i2}$ and increased $G\alpha_s$ mRNA in the frontal cortex, without changes in protein levels (Dwivedi *et al.,* 2002). This fits with data from $G\alpha_{i2}$ knock-out mice which show that loss of $G\alpha_{i2}$ contributes to depressive behaviors (Talbot *et al.,* 2010), suggesting that the observed alterations of $G\alpha_{i2}$ in these subjects

may have contributed to their pathology. Overall there is no consistent effect on G-protein expression in the brain following chronic antidepressant treatment, the effects that have been seen are not consistent between antidepressant drugs with similar pharmacology, and any changes that have been found are of relatively small magnitude.

Despite the lack of any clear effect on G-protein expression levels, chronic but not acute antidepressant drug treatment (including amitriptyline, desipramine and iprindole) increases cAMP concentrations in a $G\alpha_s$ dependent manner in the rat brain, but not other tissues (Menkes *et al.,* 1983; Ozawa and Rasenick 1989; De Montis *et al.,* 1990). In addition to antidepressant drug treatments, chronic electroconvulsive treatment increases coupling between $G\alpha_s$ and adenylyl cyclase in the brain but not peripheral tissues (Ozawa and Rasenick 1991). In agreement with this increased cyclase activity, increased activity of cAMP dependent kinases have also been observed in the rat brain following chronic antidepressant treatment. These changes occurred with chronic but not acute treatment with desmethylimipramine or fluoxetine, and were seen in the cerebral cortex but not hippocampus, striatum or cerebellum (Perez *et al.,* 1989). This suggests a more general role for brain $G\alpha_s$/adenylyl cyclase coupling in antidepressant action downstream of their better characterized direct effects on transporters and GPCRs.

In order to understand how antidepressants affect G-protein signaling, it is necessary to consider not only the expression levels of these proteins and their binding partners, but also their subcellular localization in microdomains. Within the plasma membrane bilayer there are lipid raft membrane microdomains which contain an increased proportion of both cholesterol and sphingomyelin (Simons *et al.,* 2000). G-proteins are known to accumulate in these lipid raft domains, with $G\alpha_s$, $G\alpha_q$ and $G\alpha_{i/o}$ subunits all found at higher concentrations in these regions (Allen *et al.,* 2005; Pesanová *et al.,* 1999; Dunphy *et al.,* 2001). These microdomains can affect G-protein dependent signaling, with either faciliatory or inhibitory effects on signaling depending on the G-protein. For example, localization to raft regions inhibits the ability of $G\alpha_s$ proteins to increase cAMP levels through adenylyl cyclase activation while raft localization of $G\alpha_q$ greatly enhances signaling downstream of 5-HT2A receptor activation (Rybin *et al.,* 2000; Bhatnagar *et al.,* 2004; Ostrom and Insel 2004 for review). In addition to an upregulation of $G\alpha_s$ protein expression, a shift in $G\alpha_s$ subcellular membrane localization also occurs in the brains of

depressed individuals. Compared to non-psychiatric control subjects there was approximately a two-fold increase in the localization of $G\alpha_s$ to lipid raft domains (see Figure 1.1; Donati *et al.*, 2008). As these lipid raft domains are known to inhibit signaling downstream of $G\alpha_s$, including coupling to adenylyl cyclase, this increased lipid raft localization likely indicates decreased $G\alpha_s$ signaling in the brain of depressed individuals as well (Head *et al.*, 2006). In fact, a $G\alpha_s$ dependent adenylyl cyclase dysfunction in the brains of suicide victims is supported by an impairment in the ability of forskolin to stimulate adenylyl cyclase activity in post-mortem tissue from individuals who committed suicide (Cowburn *et al.*, 1994). This loss of cyclase activity is associated with decreased expression and activity of the cAMP dependent kinase (PKA) in the frontal cortex but not hippocampus of suicide victims (Pandey *et al.*, 2005).

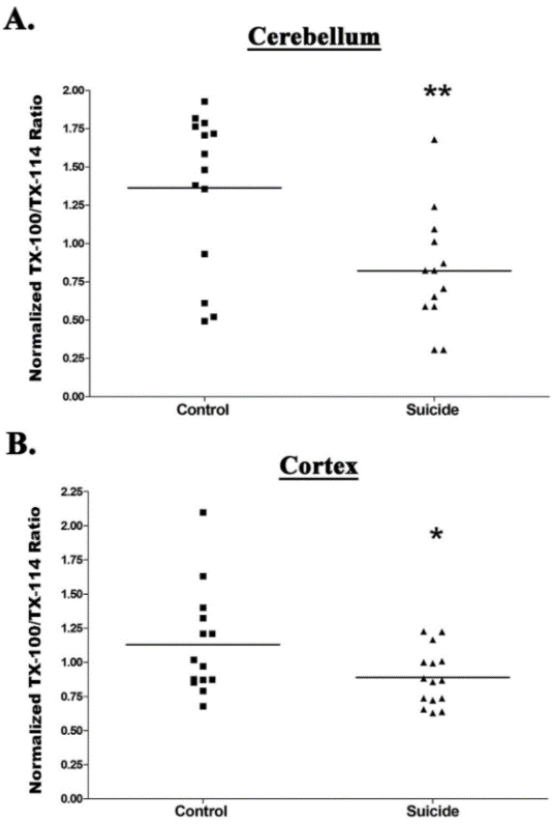

Fig. 1.1: Ratio of non-lipid raft vs. lipid-raft associated $G\alpha_s$ protein expression in post-mortem tissue from suicide victims vs. controls. The reduction in TTX-100/TTX-114 detergent soluble ratio indicates an increased accumulation of $G\alpha_s$ in lipid raft regions in the brains of suicide victims.

Adapted with permission from Donati *et al.*, 2008.

Preclinical models suggest that antidepressants have both a stimulatory effect on adenylyl cyclase activity and reduce the amount of $G\alpha_s$ localized in lipid rafts (Toki *et al.*, 1999; Donati and Rasenick 2005; Chen and Rasenick 1995a; Chen and Rasenick 1995b). Both of these effects would in theory directly counteract changes detected in post-mortem brain tissue from suicide victims, namely impaired adenylyl cyclase activity and increased accumulation of $G\alpha_s$ in lipid rafts (Cowburn *et al.*, 1994; Donati *et al.*, 2008). While these results predict that antidepressant treatment should correct these deficits observed in the depressed brain this hypothesis has not yet been fully tested.

In cellular models, multiple G-protein subtypes accumulate in lipid rafts and antidepressant drug treatment reduces the amount of lipid raft associated $G\alpha_s$ without changing the abundance of other G-proteins in the rafts (Toki *et al.,* 1999; Donati and Rasenick 2005). This reduction in lipid raft $G\alpha_s$ content occurs without changes in the overall expression level of $G\alpha_s$ protein, or in the expression of other G-proteins including $G\alpha_i$, $G\alpha_o$ or $G\beta$ (Chen and Rasenick 1995a). The shift of $G\alpha_s$ from lipid raft regions to non-lipid raft regions coincides with increased coupling between $G\alpha_s$ and adenylyl cyclase as well as increased adenylyl cyclase activation and cAMP accumulation (Chen and Rasenick 1995a; Chen and Rasenick 1995b). These effects occur with tricyclic antidepressants, SSRIs and atypical antidepressants suggesting a potential conserved antidepressant mechanism independent of primary site of action.

Importantly the transfer of $G\alpha_s$ out of lipid raft domains occurs following chronic treatment with the antidepressant (S)-stereoisomer of the SSRI citalopram, but not the (R)-stereoisomer which lacks antidepressant effects (Zhang and Rasenick 2010). This stereospecific effect of citalopram occurs in C6 cells lacking the serotonin transporter, suggesting that $G\alpha_s$ translocation out of lipid rafts occurs due to interaction of the antidepressant with some other protein target. Furthermore, antidepressant drugs with diverse mechanisms of action (including desipramine, reboxetine and fluoxetine) themselves accumulate in these lipid raft domains over time (Eisensamer *et al.,* 2005). While together these data suggest that a specific binding site for antidepressants within lipid rafts may exist, a suitable candidate site has yet to be identified.

Therefore, in general antidepressant drugs liberate $G\alpha_s$ from the inhibitory effects of lipid raft localization allowing this subunit to signal more effectively through downstream effectors including adenylyl cyclase (Head *et al.,* 2006). These effects are specific to the brain as peripheral tissues do not show the same response, and even within the brain there is regional specificity as brain regions other than the cerebral cortex show reduced $G\alpha_s$ translocation if any (Dwivedi *et al.,* 2002). This provides a plausible mechanism for the long-recognized ability of antidepressant drugs to increase the coupling between $G\alpha_s$ and adenylyl cyclase (Menkes *et al.,* 1983). Importantly this enhancement occurs only following extended antidepressant treatment, consistent with the hysteresis observed between the initiation of antidepressant treatment and the onset of therapeutic effects (Chen and Rasenick 1995b).

Multiple serotonin receptors likely contribute to the antidepressant effects of serotonergic antidepressants; however, a wealth of data suggests that the 5-HT1A receptor plays an important role. 5-HT1A is a typical $G\alpha_{i/o}$ coupled receptor, and many lines of evidence suggest it couples to $G\alpha_{i3}$ and $G\alpha_{i2}$ with considerable selectivity compared to $G\alpha_{i1}$ and $G\alpha_{o}$ (Bertin *et al.,* 1992; Clawges *et al.,* 1997; Garnovskaya *et al.,* 1997). Unfortunately, all of these studies used the prototypical 5-HT1A agonist 8-OH-DPAT, while other agonists at this receptor may have distinct selectivity profiles of receptor/G-protein coupling. This agonist-dependent G-protein selectivity has been shown at other receptors including the D2 dopamine receptor and the M1 muscarinic acetylcholine receptor among others (Masuho *et al.,* 2015; Melancon *et al.,* 2013) and reveals a potential advantage of drugs that interact directly with receptors instead of indirectly modulating neurotransmitter levels such as the SSRIs. Regardless of which SSRI is employed, the therapeutic effects arise from serotonin interacting with serotonin receptors, precluding the possibility for an agonist-dependent G-protein selectivity. While some direct 5-HT1A agonists such as buspirone show promise for treating depression, a detailed analysis of G-protein selectivity for these agonists has not been performed.

Mice lacking $G\alpha_{i2}$ exhibit a spontaneous pro-depressant phenotype (Talbot *et al.,* 2010). Conversely mice expressing a gain of function knock-in variant of $G\alpha_{i2}$ exhibit spontaneous antidepressant-like behaviors (Talbot *et al.,* 2010). As these behaviors are reversible by pretreatment with a 5-HT1A antagonist this suggests that signaling through the 5-HT1A/$G\alpha_{i2}$ signaling complex promotes antidepressant-like effects while disruption of this complex may contribute to depressive behaviors. Therefore, disruption of $G\alpha_{i2}$, either due to genetic variation or by other means, may have detrimental neuropsychiatric effects. Conversely 5-HT1A agonists with preference for $G\alpha_{i2}$ signaling compared to other $G\alpha_{i/o}$ proteins could have superior effects compared to a 5-HT1A agonist without G-protein selectivity.

Together these data suggest a dysregulation of G-protein signaling in the brains of depressed individuals. The dysregulation likely involves changes in overall G-protein expression, as well as translocation of $G\alpha_{s}$ into a more restrictive membrane microenvironment where signaling to downstream effectors is inhibited, with apparent consequences for cyclase dependent signaling pathways including PKA. It remains unclear whether these changes contribute to suicidal behavior and depression, or are simply correlated with the expression of these states without a

causative effect. In either case the existence of these changes may allow for new strategies to diagnose and potentially treat depressive disorders.

RGS Protein Regulation of G-protein Activity

RGS proteins (see Table 0.1 for general information on these proteins) have some degree of specificity for which Gα subunits they can regulate. For example, RGS4 and RGS8 potently inhibit signaling downstream of $G\alpha_{i2}$ in vitro while RGS7 has no effect (Talbot *et al.,* 2009). RGS-PX1 exclusively regulates signaling downstream of $G\alpha_s$ (Zheng *et al.,* 2001), while RGS2 preferentially modulates $G\alpha_q$ signaling (Heximer *et al.,* 1997). RGS proteins also appear to have specificity in terms of which GPCRs they regulate, even when those GPCRs signal through the same type of G-proteins. The dopamine D2 receptor and 5-HT1A receptors are both $G\alpha_{i/o}$ coupled GPCRs, however RGS4, RGS10 and RGSZ1 reduced 5-HT1A-mediated signaling in vitro but did not affect D2-mediated signaling (Ghavami *et al.,* 2003). It remains unclear whether RGS proteins achieve this specificity through direct interaction with certain GPCRs or by interactions with other intracellular binding partners. This level of specificity has been studied more thoroughly with RGS/opioid receptor interactions and is discussed further in chapter 3.

Membrane anchoring can also affect how RGS proteins regulate G-protein action. For example, the loss of two available palmitoylation sites on RGS16 can prevent it from negatively regulating $G\alpha_i$ and $G\alpha_q$ signaling (Hiol *et al.,* 2003). RGS proteins in complex with a Gα subunit can also directly affect signaling to downstream effectors, independent of the RGS GAP function. This level of regulation also depends on the specific RGS protein involved, for instance RGS2 in complex with $G\alpha_q$ can prevent $G\alpha_q$ from binding to the downstream effectors p63 RhoGEF and GRK2, while RGS4 in complex with $G\alpha_q$ has little effect on binding to these effectors (Shankaranarayanan *et al.,* 2008).

Therefore, while different RGS proteins have classically been thought of as having largely redundant actions, current evidence suggests that different family members have considerable variation in G-protein preference, receptor selectivity, and scaffolding functions towards

downstream effectors. This highlights the need for a better understanding of what role the RGS proteins play in neuropsychiatric disorders and treatments.

RGS Proteins Involved in Depression and Antidepressant Action

RGS2

Changes in individual RGS protein expression may have the potential to produce pro-depressant effects. For example, mice lacking RGS2 showed a baseline increase in anxious and depressive-like behaviors (Lifschytz *et al.,* 2012; Oliveira-dos-Sanos *et al.,* 2000). These behavioral alterations occurred alongside decreased raphe 5-HT1A receptor expression and function suggesting that these receptors may play a role in the observed behavioral phenotype (Lifschytz *et al.,* 2012). In contrast, a genetic manipulation which specifically increases raphe 5-HT1A expression in mice increases vulnerability to stress and decreases response to antidepressants (Richardson-Jones *et al.,* 2010), suggesting that reductions in raphe 5-HT1A following disruption of RGS2 may be a compensatory change rather than a causative factor of the pro-depressant behaviors. Nonetheless, these data demonstrate that RGS2 may have a protective effect against anxiety and depression, and that RGS2 disruption may have detrimental neuropsychiatric effects.

In line with these pre-clinical results, individuals expressing any of several single nucleotide polymorphisms (SNPs) in the RGS2 gene experience anxiety disorders and suicidal ideations at an increased rate (Amstadter *et al.,* 2009; Leygraf *et al.,* 2006; Smoller *et al.,* 2008). In addition, an increase in RGS2 immunoreactivity was found in post-mortem tissue from both the prefrontal cortex and amygdala of individuals who committed suicide (Cui *et al.,* 2008). Together these findings provide solid evidence that genetic alterations in the RGS2 gene can influence the development or expression of affective disorders in human populations, in line with findings from RGS2 knockout mice.

While it remains difficult to study antidepressant action in vitro, a number of studies have provided evidence on how RGS2 can affect cellular processes in ways that could modify antidepressant action. An increase in hippocampal synaptic plasticity typically occurs following antidepressant treatment, while depressive states cause decreased plasticity (Kozisek *et al.,* 2008;

Nissen *et al.,* 2010). Loss of RGS2 produces a similar loss of synaptic plasticity in mouse hippocampal tissue potentially by altering $G\alpha_{i/o}$-mediated inhibition of hippocampal Ca^{2+} channels (Han *et al.,* 2006). This suggests that a loss of neural plasticity due to genetic variation in RGS2 could have detrimental neuropsychiatric effects and might impair the function of antidepressant drugs.

RGS4

Studies in rodent models have consistently shown that 5-HT1A agonists such as 8-OH-DPAT cause a reduction in extracellular serotonin levels in the brain by activating 5-HT1A autoreceptors in the dorsal raphe nucleus (DRN) (Casanovas and Artigas 1996; Adell and Artigas 1998; Celada *et al.,* 2001). It is thought that this 5-HT1A dependent reduction in central serotonin levels may delay the beneficial effects of SSRI antidepressants, and strategies to limit 5-HT1A autoreceptor activity during SSRI treatment, such as co-administration of 5-HT1A antagonists or weak partial agonists, have shown promising results (Artigas *et al.,* 1994; Perez *et al.,* 1997; Tome *et al.,* 1997; Maes *et al.,* 1999). Although RGS4 mRNA is not normally expressed in the DRN (Gold *et al.,* 1997), RGS4 overexpression in the DRN attenuates the ability of 5-HT1A receptors to reduce central serotonin levels (Beyer *et al.,* 2004). Based on these results, overexpression of RGS4 in brain regions containing 5-HT1A autoreceptors should have beneficial effects on antidepressant drug action, similar to the results obtained by combining 5-HT1A antagonists with traditional antidepressants (Artigas *et al.,* 1994).

Delta opioid receptor (DOPR) agonists produce antidepressant-like behavioral effects in rodent models including the tail suspension and forced swim tests (Broom *et al.,* 2002; Naidu *et al.,* 2007). RGS4 knockout mice show an enhanced antidepressant-like response to DOPR agonists in the forced swim test, but not tail suspension test, suggesting that these antidepressant-like behaviors may depend on distinct signaling intermediates downstream of DOPR activation (Stratinaki *et al.,* 2013; Dripps *et al.,* 2017). This effect on forced swim test behavior appears to depend on nucleus accumbens RGS4 expression, as specific RGS4 knockdown in this region produces similar effects as global RGS4 knockout (Stratinaki *et al.,* 2013). RGS proteins capable of modulating DOPR-mediated tail suspension test behavior have not yet been identified.

In addition to inhibiting the antidepressant-like effects of DOPR agonists, loss of RGS4 appears to inhibit the antidepressant-like effects of SSRIs, norepinephrine reuptake inhibitors and the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine as well (Stratinaki *et al.,* 2013). Acute treatment with either a DOPR agonist or ketamine decreases frontal cortex RGS4 expression yet only chronic treatment with a DOPR agonist increases RGS4 expression in the nucleus accumbens (Stratinaki *et al.,* 2013). While this would suggest that nucleus accumbens and frontal cortex RGS4 may have opposing effects on antidepressant action, this has yet to be conclusively demonstrated.

Although RGS4 expression in the brain does not appear to differ between post-mortem tissue from depressed and healthy individual, an upregulation of RGS4 has been observed in post-mortem nucleus accumbens tissue from depressed individuals undergoing antidepressant treatment compared to untreated depressed individuals (Stratinaki *et al.,* 2013). Coupled with findings from rodent models showing increased antidepressant effectiveness in animals with overexpression of RGS4 in this brain region, it appears possible that nucleus accumbens RGS4 has a facilitatory effect on antidepressant treatment.

RGS6

Mice lacking RGS6 display antidepressant-like and anxiolytic behaviors at baseline, including in the elevated plus maze and novelty induced hyponeophagia test (Stewart *et al.,* 2014). This behavioral phenotype is fully reversible by 5-HT1A antagonist pretreatment, and by direct activation of adenylyl cyclase with forskolin (Stewart *et al.,* 2014). Loss of RGS6 did not affect mitogen-activated protein kinase (MAPK) or glycogen synthase kinase 3 beta (GSK3β) signaling (Stewart *et al.,* 2014), changes that have been detected in mice with a 5-HT1A dependent antidepressant-like phenotype due to loss of RGS control at $G\alpha_{i2}$ (Talbot *et al.,* 2010). Instead the RGS6 knockout mouse phenotype appears to depend on increased phospho-CREB in the hippocampus and cortex, areas with high 5-HT1A receptor expression (Stewart *et al.,* 2006). These findings suggest that RGS6 may normally limit the actions of serotonergic antidepressants by reducing adenylyl cyclase inhibition downstream of 5-HT1A receptor activation, and that strategies to limit RGS6 activity may have beneficial effects for antidepressant treatment.

RGS16

Palmitoylation causes the accumulation of RGS16 in lipid raft domains, a subcellular membrane compartment known to generally promote signaling downstream of $G\alpha_q$, which RGS16 regulates (Rybin *et al.,* 2000; Hiol *et al.,* 2003). This localization places RGS16 at or near a putative, but not yet positively identified binding site for antidepressant drugs within lipid rafts, as discussed earlier (Eisensamer *et al.,* 2005). This localization may provide RGS16 with an increased ability to regulate antidepressant drug action, although this prediction has yet to be tested.

RGS19

RGS19 has been shown to regulate 5-HT1A signaling in both C6 and SH-SY5Y cells (Wang *et al.,* 2014), while RGS4 knockdown did not significantly affect signaling. RGS19 knockdown facilitated 5-HT1A agonist induced activation of MAPK and inhibition of adenylyl cyclase (Wang *et al.,* 2014). The effect of RGS19 knockdown was magnified when the cells were co-treated with fibroblast growth factor 2 (FGF2), a factor known to act synergistically with 5-HT1A receptor activity in the hippocampus to facilitate synaptic plasticity (Wang *et al.,* 2014; Borroto-Escuela *et al.,* 2012). The enhancement of 5-HT1A agonist stimulated MAPK activity following RGS19 knockdown seen in cellular models was replicated in mouse primary hippocampal neurons, including the synergistic enhancement by co-treatment with FGF2 and a 5-HT1A receptor agonist (Wang *et al.,* 2014). This suggests that reducing RGS19 activity may facilitate the action of serotonergic antidepressants due to disinhibition of hippocampal 5-HT1A receptor activity.

RGSZ1

Chronic estradiol treatments both desensitize hypothalamic 5-HT1A receptors and cause an increase in RGSZ1 expression (Carrasco *et al.,* 2004). Increased RGSZ1 would be expected to reduce signaling downstream of 5-HT1A and may therefore contribute to the observed 5-HT1A receptor desensitization, although this interaction has not been conclusively demonstrated (Carrasco *et al.,* 2004). 5-HT1A receptor desensitization in the DRN, not the hypothalamus, is generally considered a critical step in antidepressant action. However, RGSZ1 expression in the DRN has not been assessed following chronic antidepressant treatment, so it remains possible that a similar process contributes to 5-HT1A receptor desensitization in this brain region.

RGS Insensitive G-Proteins

When an individual RGS protein is knocked out or genetically modified, other available RGS proteins can at least partially compensate. Similarly, when a G-protein is lost a GPCR may be able to continue signaling through other available G-proteins. In order to overcome these difficulties a series of modified G-proteins were developed which are totally insensitive to the negative regulatory effects of RGS proteins.

The first known RGS insensitive (RGSi) G-protein was found in a strain of saccharomyces cerevisiae yeast as a naturally occurring allele of the endogenous Gα protein subunit (DiBello *et al.,* 1998). This mutant G-protein was insensitive to the negative regulatory effects of RGS proteins, although its signaling was not altered otherwise (DiBello *et al.,* 1998). This profound loss of RGS sensitivity occurred due to a single missense mutation (gly-to-ser at position 302) in the switch 1 region of the Gα subunit. This glycine is conserved across Gα subtypes and is located in a region where RGS proteins and G-protein make direct contact (see Figure 0.2; Tesmer *et al.,* 1997).

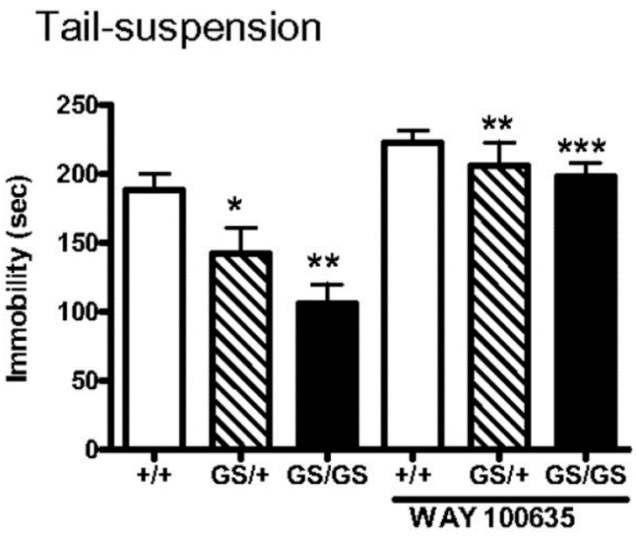

Fig. 1.2: Antidepressant-like phenotype of heterozygous (GS/+) and homozygous (GS/GS) RGS insensitive $G\alpha_{i2}$ knock-in mice compared to wild type (+/+) littermates. Pretreatment with the 5-HT1A receptor antagonist WAY-100635 reversed this behavior to wild type levels.

Adapted with permission from Talbot *et al,* 2010.

The potential utility of this RGSi G-protein was quickly recognized and a series of novel mammalian RGSi G-proteins were created including $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_q$ among others (DiBello *et al.,* 1998; Lan *et al.,* 1998). Like the originally identified yeast RGSi G-protein these mutations did not affect the kinetics of GDP release, GTP hydrolysis, Gβγ binding, or interaction with the receptor, but produced up to 100-fold loss of affinity for RGS proteins (Day *et al.,* 2004; Fu *et al.,* 2004). These properties allowed investigators to probe the effects of removing all RGS

control at a specific G-protein without affecting G-protein signaling otherwise, avoiding the difficulties posed by RGS protein redundancy.

Mice expressing an RGSi knock-in variant of $G\alpha_{i2}$ display a profound antidepressant-like phenotype across a number of behavioral tests including tail suspension, forced swim, elevated plus maze and novelty induced hypophagia (see Figure 1.2; Talbot *et al.*, 2010). These mice also have antidepressant-like signaling changes in the hippocampus and frontal cortex, including an inhibition of GSK3β activity (Talbot *et al.,* 2010). GSK3β inhibition produces neurogenesis in the adult hippocampus, and this neurogenic effect may be a critical component of antidepressant action (Malberg *et al.,* 2000; Li *et al.,* 2004; Tsai *et al.,* 2008).

Both the changes in hippocampal GSK3β and antidepressant-like behaviors observed in RGSi $G\alpha_{i2}$ knock-in mice are fully reversed by pretreatment with a 5-HT1A antagonist (Talbot *et al.,* 2010). Coupled with the fact that 5-HT1A agonists produce more potent antidepressant-like effects in these animals, it appears that the loss of RGS control at $G\alpha_{i2}$ promotes 5-HT1A receptor signaling leading to robust antidepressant-like effects.

Although activating post-synaptic 5-HT1A heteroreceptors is generally considered beneficial for antidepressant action, pre-synaptic 5-HT1A autoreceptor activation can limit antidepressant action and may contribute to the hysteresis observed between initiation of treatment and the onset of therapeutic effects (Artigas *et al.,* 1994; Hjorth and Sharp 1993; Le Poul *et al.,* 1995; Matsuda *et al.,* 1995). Interestingly RGSi $G\alpha_{i2}$ knock-in mice display enhancements of responses known to depend on 5-HT1A heteroreceptor activity (e.g. antidepressant-like behaviors, hippocampal GSK3β inhibition) but not responses dependent on 5-HT1A autoreceptor activity (e.g. hypothermia; Hillegaart 1991; Matsuda *et al.,* 1995; Li *et al.,* 2004; Talbot *et al.,* 2010). This suggests that disrupting RGS control of $G\alpha_{i2}$ may represent a novel strategy to selectively enhance the antidepressant effects of 5-HT1A receptor activation without promoting the detrimental effects of autoreceptor activation.

Unfortunately, specific RGS proteins involved in the RGSi $G\alpha_{i2}$ antidepressant-like phenotype are not currently known. While RGS19 has received attention for its ability to strongly regulate 5-HT1A receptor function in vitro, it remains unclear whether this effect involves $G\alpha_{i2}$ or another $G\alpha_{i/o}$ protein (Wang *et al.,* 2014). RGS6 has also been proposed as a possible mediator of the RGSi $G\alpha_{i2}$ knock-in mouse phenotype due to similarities between these mice and RGS6

knock-out mice, including a 5-HT1A reversible antidepressant-like phenotype (Stewart *et al.*, 2014). Substantial mechanistic differences exist between these mouse models however, including a lack of 5-HT1A reversible GSK3β inhibition in RGS6 knock-out mice. This calls into question whether RGS6 is the primary mediator of the RGSi $G\alpha_{i2}$ mouse phenotype, although it may be one of several RGS proteins involved.

Conclusions

RGS and G-proteins likely play important roles in the development of depressive states, and also influence the effectiveness of antidepressant therapies. Changes in expression level of these proteins can have dramatic effects on these complex disorders, while even more subtle alterations (such as G-protein translocation between subcellular microdomains) can profoundly regulate antidepressant action. Although preclinical studies provide a plethora of hypotheses for how these proteins behave in depressed populations in the clinic, only a handful of these theories have been adequately explored at the patient level. Considering how alterations in RGS or G-proteins differentially affect responses to different antidepressant treatments, it appears reasonable that a better understanding of these proteins could aid in the tailoring of personalized treatment strategies for depressed individuals. Screening for changes in RGS or G-proteins could also provide new insight into susceptibility towards depressive disorders at an individual level. In addition, direct RGS-inhibiting compounds have been proposed as novel treatment options for a variety of indications, and selective small molecule RGS inhibitors have already been identified (Zhong and Neubig 2001; reviewed in Roman *et al.*, 2007). A more complete understanding of how these families of proteins interact with antidepressant therapies and the development of depressive states is therefore badly needed.

The following chapter will build on the work described here by testing the hypothesis that the 5-HT1A receptor in the hippocampus is the key driver of the antidepressant-like phenotype in the RGSi $G\alpha_{i2}$ knock-in mouse. Studies using intra-hippocampal delivery of an RGS4/19 inhibitor (CCG-203769) to produce antidepressant-like effects are also highlighted. This work will allow a greater understanding of how RGS proteins can be modulated to produce antidepressant-like behaviors with implications for research on depression and antidepressant drug action.

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CHAPTER 2

Loss of RGS Control at $G\alpha_{i2}$ Produces Antidepressant-Like Behavior by an Action at 5-HT1A Receptors in the Hippocampus

Abstract

A single base mutation in the $G\alpha_{i2}$ protein (G184S) renders this Gα subunit insensitive to the negative modulatory effects of regulator of G-protein signaling (RGS) proteins. Mice expressing this RGS insensitive (RGSi) variant of $G\alpha_{i2}$ (RGSi $G\alpha_{i2}$) display a spontaneous antidepressant-like phenotype that is reversible by pretreatment with the 5-HT1A receptor (5-HT1AR) antagonist WAY100635. Here we test the hypothesis that increased activity of 5-HT1ARs in the hippocampus of RGSi $G\alpha_{i2}$ knock-in mice is responsible for the expression of the observed antidepressant-like behavior. We administered the 5-HT1AR antagonist WAY100635 or the agonist 8-OH-DPAT via bilateral intra-hippocampal infusion cannulae and tested for antidepressant-like behavior using the tail suspension test (TST). WAY100635 reversed the antidepressant-like phenotype of the RGSi $G\alpha_{i2}$ knock-in mice and 8-OH-DPAT produced an antidepressant-like response in wild type mice that was blocked by systemic WAY100635. Furthermore, intra-hippocampal infusion of the RGS4/19 inhibitor CCG-203769 produced an antidepressant-like effect in female mice. Ex-vivo slice recording confirmed an enhancement of the 5-HT1AR-mediated decrease in hippocampal CA1 pyramidal neuron excitability of the RGSi $G\alpha_{i2}$ knock-in mice. There was no change in hippocampal 5-HT1AR expression as measured by ligand binding or in the ability of 8-OH-DPAT to activate G-protein as measured in the [^{35}S]GTPγS assay. These findings suggest that RGS proteins highly expressed in the hippocampus should be investigated as targets for novel antidepressant therapies.

Introduction

Although selective serotonin reuptake inhibitors (SSRIs) are widely used in psychiatric treatments, this class of drugs suffers from serious drawbacks including limited clinical efficacy and a long delay between the initiation of treatment and the onset of therapeutic effects. This lag period is thought to be caused by the need to activate and subsequently desensitize serotonin 1A (5-HT1A) autoreceptors located predominantly in the raphe nucleus (Hjorth *et al.,* 2000). However, a substantial body of research has identified 5-HT1A receptors (5-HT1ARs) on postsynaptic sites (i.e. heteroreceptors) in the frontal cortex and hippocampus as potential mediators of the beneficial effects of serotonergic antidepressants (Celada *et al.,* 2013). The involvement of 5-HT1ARs in both the therapeutic and negative effects of SSRIs has hindered the development of antidepressant therapies which maintain the beneficial effects of SSRIs while avoiding their drawbacks.

The 5-HT1AR is a typical 7-transmembrane domain G-protein-coupled receptor (GPCR) with high expression throughout the brain (Ito *et al.,* 1999). The 5-HT1AR couples to heterotrimeric $G\alpha_{i/o}$ proteins comprised of Gα and βγ subunits and as such its signaling is moderated by the regulators of G-protein signaling (RGS) proteins (Beyer *et al.,* 2004; Ghavami *et al.,* 2004; Wang *et al.,* 2014). RGS proteins are a family of intracellular proteins that regulate G-protein function by directly interacting with and inactivating heterotrimeric G-proteins (Berman *et al.,* 1996). RGS proteins have GTPase accelerating activity which promotes the hydrolysis of active Gα-GTP to form inactive Gα-GDP. This allows for reformation of the inactive heterotrimer, thus halting the downstream signaling activity of both the Gα and βγ subunits (see Fig 0.3; Stewart *et al.,* 2012).

The high degree of functional redundancy between individual RGS proteins has often provided a significant hurdle to understanding the specific function of individual RGS proteins (Dong *et al.,* 2000; Doupnik *et al.,* 2001; Chen *et al.,* 2010). In order to overcome this issue a series of RGS insensitive (RGSi) Gα protein variants have been developed (Lan *et al.,* 1998; Huang *et al.,* 2006). These mutant Gα proteins have a single base mutation (Gly to Ser) at the site where RGS proteins normally interact with the Gα subunit (see Figure 0.2; Tesmer *et al.,* 1997). For $G\alpha_{i2}$ this is Gly184. The mutation prevents interaction of the Gα protein with all RGS proteins while maintaining normal enzyme kinetics and interactions with receptor and downstream effectors (Fu

et al., 2004; Clark *et al.,* 2003) and so provides the opportunity to determine the effect of removing RGS control of a specific Gα protein.

Homozygous mice expressing the RGSi $G\alpha_{i2}$ protein ($G\alpha_{i2}^{GS/GS}$) display a baseline antidepressant-like phenotype which is fully reversible by 5-HT1AR antagonist pretreatment (Talbot *et al.,* 2010), suggesting an important role for $G\alpha_{i2}$ and RGS proteins downstream of the 5-HT1AR. However, only a subset of 5-HT1AR dependent effects appear to be enhanced in these mice. Hypothermia, an effect traditionally associated with 5-HT1A autoreceptor activation, is not affected by the mutation suggesting that 5-HT1A heteroreceptors may be the important mediators of the behavioral phenotype. Identifying the brain locus responsible for the antidepressant-like phenotype in the $G\alpha_{i2}^{GS/GS}$ mice would be an important step forward and allow us to study individual RGS proteins expressed in this region of the brain that regulate $G\alpha_{i2}$ downstream of the 5-HT1AR.

The hippocampus and frontal cortex of the $G\alpha_{i2}^{GS/GS}$ mice have increased levels of the Ser-9 phosphorylated version of glycogen synthase kinase-3 beta (GSK3β), although pretreatment with the 5-HT1AR antagonist WAY100635 reverses this change fully only in the hippocampus (Talbot et al., 2010). GSK3β is a neurogenic factor that is phosphorylated by antidepressant drugs and may contribute to their therapeutic effects (Malberg *et al.,* 2000; Tsai *et al.,* 2008). The 5-HT1A antagonist reversible increase of hippocampal phospho-GSK3β in the $G\alpha_{i2}^{GS/GS}$ mice suggests the hippocampus as a potential critical site of their 5-HT1AR dependent antidepressant-like behavior. To test this hypothesis, we delivered drugs directly to the hippocampus and measured antidepressant-like behavior using the tail suspension test (TST). We find that hippocampal microinjection of a 5-HT1AR antagonist fully reverses the $G\alpha_{i2}^{GS/GS}$ antidepressant-like phenotype, while hippocampal injection of a 5-HT1AR agonist to wild type animals produces effects consistent with the RGSi $G\alpha_{i2}^{GS/GS}$ behavioral phenotype. We also show that 5-HT1AR agonists have enhanced inhibitory effects on the intrinsic excitability of CA1 hippocampal neurons from heterozygous $G\alpha_{i2}^{+/GS}$ mice. There are no observed changes in 5-HT1AR density in the hippocampus of the RGSi $G\alpha_{i2}$ knock-in mice, suggesting that the phenotypic differences occur due to signaling changes downstream of the receptor. Finally, we demonstrate that inhibiting an RGS protein highly expressed in the hippocampus can produce an antidepressant-like effect in female wild type mice.

Materials and Methods

RGSi $G\alpha_{i2}$ knock-in mice. Wild type ($G\alpha_{i2}{}^{+/+}$), heterozygous ($G\alpha_{i2}{}^{+/GS}$) and homozygous ($G\alpha_{i2}{}^{GS/GS}$) RGSi $G\alpha_{i2}$ knock-in mice were derived from heterozygous breeding as described previously (Huang *et al.,* 2006). Animals were backcrossed onto the C57BL/6J background strain for four generations before heterozygous breeding.

RGSi Gαo knock-in mice. Wild type (+/+) and heterozygous (+/GS) RGSi $G\alpha_o$ knock-in mice were generated as described previously on a 129S1/SvIMJ background strain (Fu *et al.,* 2004; Fu *et al.,* 2006; Huang *et al.,* 2006; Goldenstein *et al.,* 2009). As mice on the 129S1/SvIMJ background typically produce small, inconsistent litters (unpublished observation), heterozygous female RGSi $G\alpha_o$ knock-in mice on a 129S1/SvIMJ were bred with male wild type C57BL/6J mice. The resulting F1 C57/129 cross were used for experiments involving RGSi $G\alpha_o$.

All animals were between 8 and 16 wk of age at time of testing, and animals were age and sex matched in each experiment. In experiments where sex is not specified a pilot experiment was performed to identify potential sex differences. If no difference was observed in this pilot then results from male and female animals were pooled in further experiments. Mice were group housed with up to five same-sex littermates per cage. The vivarium was maintained on a 12 hr light/dark cycle with lights on at 7:00 AM. All testing occurred during the light phase. Drugs were typically administered i.p. 30 min before testing unless otherwise indicated. All experimental procedures were approved by the local Institutional Animal Care and Use Committee and followed the National Institute of Health guidelines outlined in "Using Animals in Intramural Research."

Tail suspension test. A piece of adhesive tape was affixed to the distal end of the mouse' tail and attached to a metal bar elevated 30 cm above the table surface (Steru *et al.,* 1985). Behavior was recorded for 6 min and later scored for immobility time. Immobility was defined as any period without continuous movement. Isolated head movements, and swinging without other movement were also defined as immobile.

Intra-hippocampal cannulation. Mice were anesthetized with a combination of ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Carprofen (5 mg/kg s.c.) was administered before and 24 hr following surgery as an analgesic. Mice were placed into the stereotax (Kopf Instruments

Model 902 Dual Small Animal Stereotaxic Instrument) and a midline incision made over the top of the skull. Bregma and lambda were located and marked to determine implant position. Bilateral implant coordinates were 1.5 mm posterior, 1.0 mm ventral and 1.0 mm distal from the midline on each side. Bilateral guide cannulae (C235GS-5-2.0/SPC) were custom ordered from Plastics One Inc. with a center-to-center distance of 2.0mm between each cannula and 2.0 mm cannula cut length. To prevent blockage within the guide cannula a bilateral dummy cannula was kept in the guide cannula at all times following surgery except during intra-hippocampal infusions. Animals were allowed to recover for at least seven days following surgery before any experimental testing took place. Any animals that showed signs of distress during this recovery period were removed from the experiment and euthanized. Following experimental testing a solution containing Fast Green FCF dye was infused through the cannula. Brains were then dissected and rapidly frozen before sectioning. When staining indicated a misplaced cannula, data from this animal were excluded from the experiment.

Intra-hippocampal infusions. Immediately before infusion animals were placed in a drop jar containing isoflurane and breathing was monitored until rate reached approximately one breath per second. A bilateral injection cannula attached by flexible plastic tubing to two Hamilton syringes (Hamilton #86274 syringe) was then inserted through the guide cannula. A 500 nl infusion was then delivered to each side at a rate of 250 nl per min using a syringe pump. Following infusion, the injection cannula remained in place for a further two min to prevent backflow away from the infusion site. Drugs were administered 30 min before testing unless otherwise indicated. For experiments involving repeated intra-hippocampal infusions this process was repeated once every 24 hr for three days, and experimentation occurred 30 min after the final infusion.

Drugs. (R)-(+)-8-OH-DPAT hydrobromide ((R)-(+)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide) and WAY100635 maleate salt (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt) were purchased from Sigma-Aldrich Co. LLC. CCG-203769 was generously donated by the lab of Dr. Richard Neubig.

Ex-vivo hippocampal cell recordings. Whole cell patch clamp recordings of hippocampal CA1 neurons were made from wild type and heterozygous RGSi $G\alpha_{i2}$ knock-in mice (5-8 wk of age).

Mice were anesthetized with isoflurane and brains were rapidly removed and placed in ice-cold oxygenated (95% O_2-5% CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): 200 sucrose, 25 $NaHCO_3$, 12.5 glucose, 1.25 NaH_2PO_4, 3.5 KCl, 1L-ascorbic acid, 0.5 $CaCl_2$, 3 $MgCl_2$, 305 mOsm, pH 7.4. Coronal slices (300 μm) containing the hippocampus were made using a Leica VT1200 vibratory microtome (Leica Biosystems, Buffalo Grove, IL, USA) and allowed to rest in oxygenated aCSF for at least 40 min before recording. For the recording aCSF, $CaCl_2$ was increased to 2.5 mM and $MgCl_2$ was decreased to 1 mM. Patch pipettes were pulled from 1.5 mm borosilicate glass capillaries (WPI, Sarasota, FL) to a resistance of 3–7 MΩ with a horizontal puller (Model P97, Sutter Instruments, Novato, CA, USA) and filled with a solution containing (in mM): 130 K-methanesulfonate, 10 KCl, 0.4 EGTA, 2 $MgCl_2$, 2 Mg^{2+}-ATP, 0.25 Na^{3+}-GTP, and 10 HEPES, pH 7.3, 285 mOsm when performing current clamp experiments. CA1 hippocampal neurons were identified based on their response to current injection (−200 to 140 pA, 10 pA increments, 500 ms). Neuronal excitability was determined by measuring the number of action potentials elicited by each depolarizing current injection. Input resistance (IR) was determined by the change in voltage from 0 pA to -170 pA current injections. Rheobase is defined as the minimum amount of current injection to elicit an action potential.

Hippocampal membrane preparation for binding assays. Immediately before tissue preparation mice were sacrificed by cervical dislocation followed by decapitation and the brain was removed. The hippocampus was then microdissected and isolated. The hippocampi from 6-8 mice matched for age, gender and genotype were pooled to obtain enough tissue for experiment. Hippocampi were homogenized in ice cold 50 mM tris buffer pH 7.4 then prepared as described previously (Lester and Traynor 2006). Protein concentration was then determined with a BCA assay kit (Thermo Scientific, Rockford, IL).

[^{3}H]8-OH-DPAT saturation binding assay. Membrane homogenates containing hippocampal tissue (100 μg/well) were incubated in 50 mM pH 7.4 Tris buffer with [^{3}H]8-OH-DPAT ranging in concentration from 0.156 nM up to 20 nM increasing in 2-fold steps. Non-specific binding was determined at each point with the addition of 10 μM WAY-100635. Each condition was performed in triplicate and each experiment was independently replicated three times. After the addition of all components each assay incubated for 60 min at room temperature before filtration through a Whatman GF/C tilter using a MLR-24 Brandel harvester. Bound radioactivity was

then determined by scintillation counting with a Wallac 1450 Microbeta counter (Perkin Elmer). Further methodological details have been published previously (Lamberts *et al.*, 2013).

Western blotting. Hippocampal homogenates containing 20 μg of protein were mixed with sample buffer (63 mM Tris, pH 6.8, with 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.008% bromophenol blue, and 50 mM dithiothreitol) and 50 mM pH 7.4 Tris buffer to a total volume of 25 μl. Samples were then separated by SDS-PAGE using polyacrylamide gels and transferred to a nitrocellulose membrane (Pierce). Membranes were probed with primary antibodies against each Gα subtype ($G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, Gβγ(1-6) and RGS19; Santa Cruz Biotechnology). Each membrane was also stripped and re-probed with a primary antibody specific for α-Tubulin as a loading control (Sigma-Aldrich). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were used for chemiluminescent detection in combination with SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific). Signal intensity was determined using ImageJ software (http://rsbweb.nih.gov/ij/index.html).

8-OH-DPAT induced hypothermia. Two hours before experiment mice were moved from group housing to individual cages for testing. Free access to food and water was maintained during this time. Baseline temperatures were taken once every 10 min for 30 min before experimental treatment. The final baseline recording was used as the reported baseline measurement. Temperatures were determined using a Tcat 2df controller rectal thermometer (Physitemp Clifton, NJ) inserted to a 20 mm probe depth. All animals in this study received both an i.p. and an intra-hippocampal injection, with the i.p. injection immediately following the third baseline measurement and intra-hippocampal infusion immediately following i.p. injection. Drug effects were determined 10, 20 and 30 min after injection for each animal.

Statistical analysis. GraphPad Prism 7.0 (GraphPad; LaJolla, CA) was used to analyze all reported data. 2-way analysis of variance (2-way ANOVA) followed by Tukey's post-hoc test for multiple comparisons was used to analyze data involving two independent drug treatments as well as data involving a genetic variable and a drug treatment. If statistics for a main effect, interaction or post-hoc comparison are not detailed in the results these effects did not reach significance. Experiments involving only one independent variable were analyzed by student's t-test. Threshold for significance was $p<0.05$ for all experiments. In saturation binding

experiments Kd and Bmax were obtained using a one-site saturation binding curve with Hill slope set to 1 as described previously (Lamberts *et al.*, 2013).

Results

Effects of intra-hippocampal WAY100635 in wild type and $G\alpha_{i2}$ $^{GS/GS}$ mice

Homozygous $G\alpha_{i2}$ $^{GS/GS}$ knock-in mice given saline bilaterally into the hippocampus exhibited less immobility in the TST than their wild type littermates (Figure 2.1), confirming that the intra-hippocampal microinjection procedure does not disrupt the previously described antidepressant-like phenotype in these mice. Bilateral intra-hippocampal administration of WAY100635 (3 μg each side) fully reversed this baseline reduction in immobility back to levels seen in wild type littermates. WAY100635 similarly administered to wild type (WT) littermates had no effect (Figure 2.1). Two-way ANOVA revealed significant main effects of genotype ($F_{(1,22)}$=7.053,

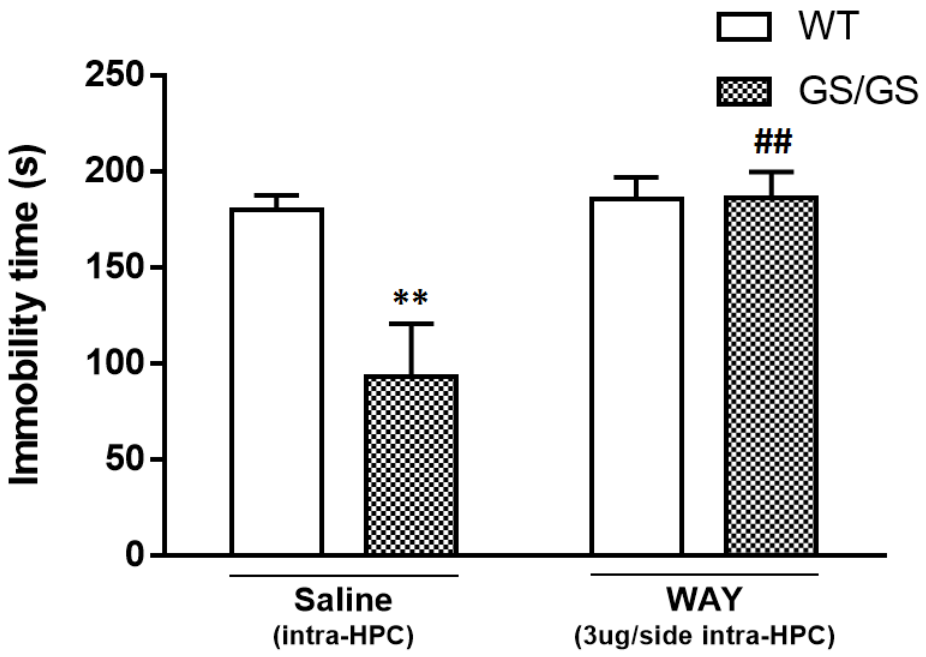


Fig. 2.1: Effects of the 5-HT1A antagonist WAY100635 on spontaneous antidepressant-like behavior in homozygous RGSi $G\alpha_{i2}$ expressing mice (GS/GS). Antidepressant-like behavior in RGSi $G\alpha_{i2}$ expressing mice in the tail suspension test is reversed by intra-hippocampal WAY-100635 (3 μg/side). Each column depicts the mean immobility score +/- SEM of 6-7 mice (2-way ANOVA followed by Tukey's post-hoc test; significant main effects of genotype, treatment, and interaction; ** indicates a difference from saline treated wild type condition ($p<.01$); ## indicates a difference from saline treated RGSi $G\alpha_{i2}$ expressing mice.

p=0.0144), treatment ($F_{(1,22)}$=9.452, p=0.0055), and genotype x treatment interaction ($F_{(1,22)}$=7.338, p=0.0128).

Effects of intra-hippocampal 8-OH-DPAT on tail suspension test immobility and hypothermia in wild type mice

The complete reversal of the $G\alpha_{i2}$ $^{GS/GS}$ behavioral phenotype by intra-hippocampal WAY100635 suggests the hippocampus as the site of the increased 5-HT1AR signaling and thus of the antidepressant-like phenotype. Therefore, we sought to test if we could mimic this behavioral phenotype by administering the 5-HT1AR agonist 8-OH-DPAT directly into the hippocampus of wild type $G\alpha_{i2}$ $^{+/+}$ mice. Intra-hippocampal 8-OH-DPAT (3 μg) bilaterally into the hippocampus

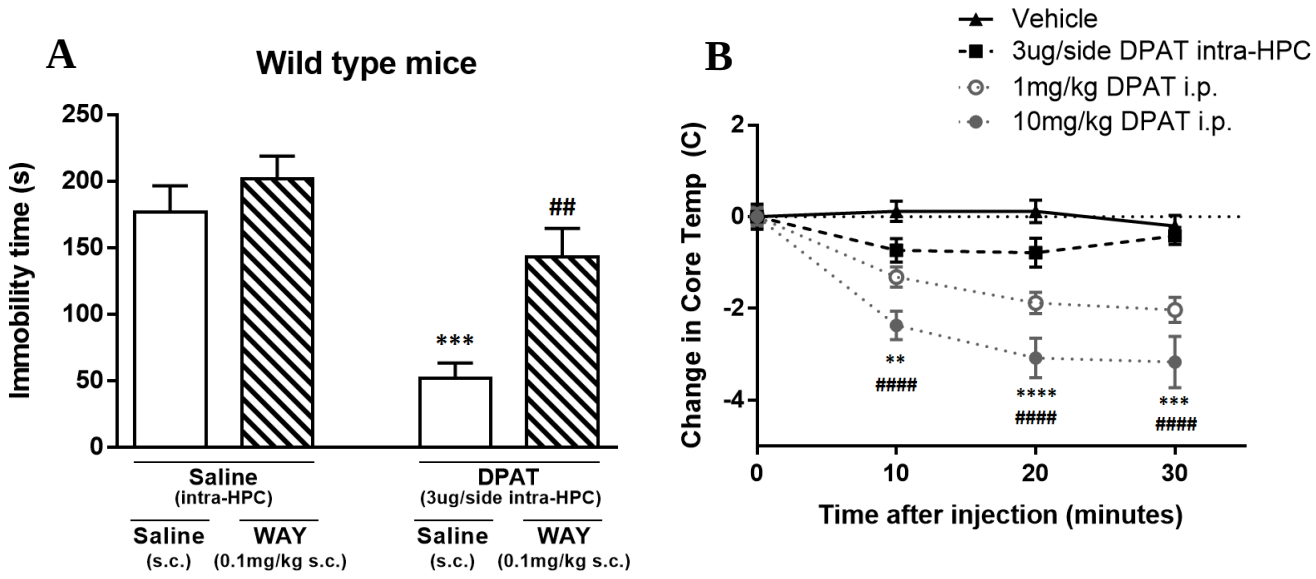


Fig. 2.2: Effects of intrahippocampal 8-OH-DPAT administration in wild type mice. (A) Intra-HPC 8-OH-DPAT produces a WAY-100635 reversible antidepressant-like effect in the tail suspension test in wild type mice. All animals received both an intra-hippocampal infusion and s.c. injection. Each column depicts the mean immobility score +/- SEM of 6 mice (2-way ANOVA followed by Tukey's post-hoc test; significant main effects of WAY and 8-OH-DPAT treatments; *** indicates a difference from saline/saline treated condition (p<.001); ## indicates a difference from mice receiving intra-HPC 8-OH-DPAT and s.c. saline (p<.01)). **(B)** Intrahippocampal 8-OH-DPAT does not produce a hypothermic effect at doses capable of producing antidepressant-like effects. All animals received both an intra-hippocampal infusion and i.p. injection. Each line represents the mean temperature +/- SEM recorded from 6-7 mice at multiple timepoints (2-way ANOVA followed by Tukey's post-hoc test; significant main effects of treatment, time and interaction; **, *** and **** indicate a difference between 1 mg/kg 8-OH- DPAT i.p. and vehicle treated animals at that timepoint (p<.01, p<.001 and p<.0001 respectively); #### indicates a difference between 10 mg/kg DPAT i.p. and vehicle treated animals at that timepoint (p<.0001)). Animals receiving 3 μg/side DPAT intra-HPC were not significantly different from vehicle treated animals at any timepoint.

of $G\alpha_{i2}^{+/+}$ mice reduced immobility. This action of 8-OH-DPAT was significantly attenuated by systemic (s.c) administration of 0.1 mg/kg WAY100635 (Figure 2.2A). This dose of WAY100635 did not affect the behavior of animals given an intra-hippocampal saline infusion (Figure 2.2A). Two-way ANOVA revealed significant main effects of 8-OH-DPAT treatment ($F_{(1,20)}$=10.57, p=0.0040), and WAY100635 treatment ($F_{(1,20)}$=43.55, p<0.0001).

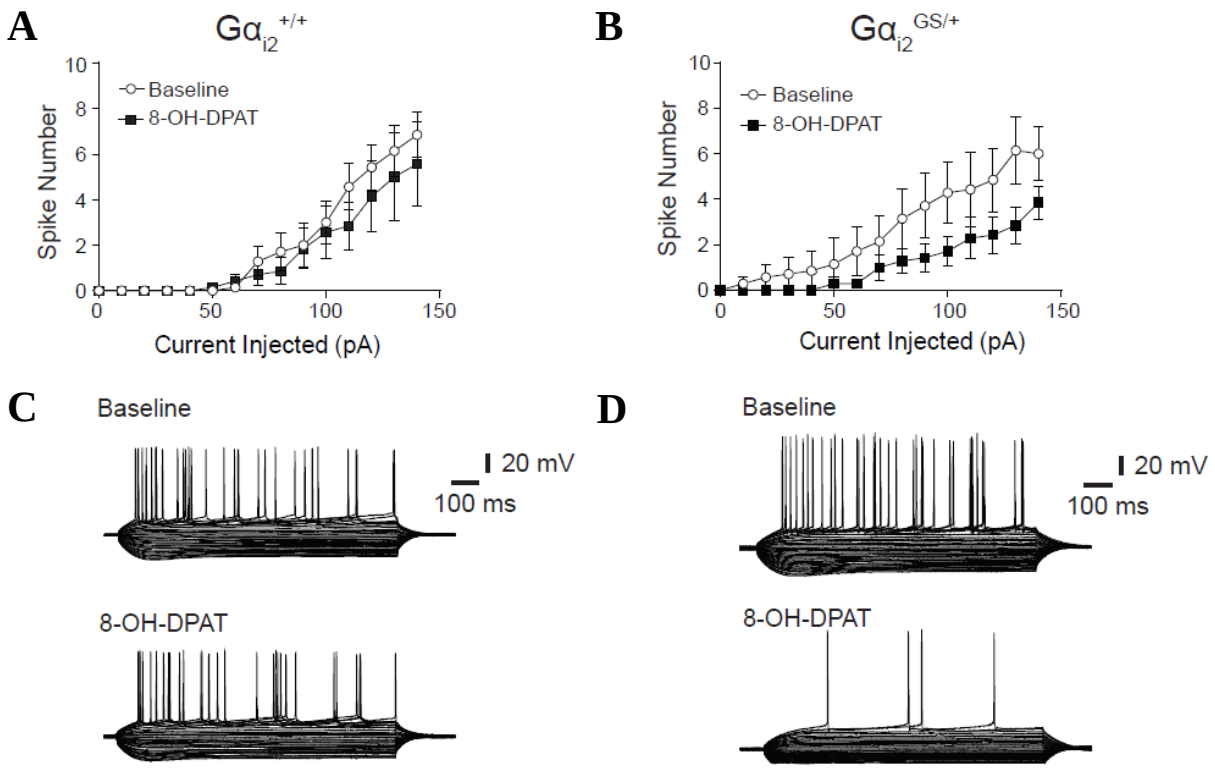
Activation of 5-HT1ARs in the raphe nuclei modulates body temperature (Hillegaart 1991). Doses of 8-OH-DPAT which produce an antidepressant-like effect when administered peripherally (1 mg/kg and 10 mg/kg, i.p., see Figure 2.5B and Talbot *et al.,* 2010) produced a lasting hypothermic effect in $G\alpha_{i2}^{+/+}$ animals (Figure 2.2B). In contrast doses of 8-OH-DPAT which produce an antidepressant-like effect in the TST when administered into the hippocampus (3 μg/side) did not affect body temperature (Figure 2.2B). Two-way ANOVA revealed significant main effects of treatment ($F_{(3,84)}$=39.86, p<0.0001), time ($F_{3,84)}$=20.24, p<0.0001), and treatment x time interaction ($F_{(9,84)}$=5.011, p<0.0001).

Recording of hippocampal slices from wild type and RGSi $G\alpha_{i2}$ knock-in mice in the presence of 8-OH-DPAT

Both 5-HT1AR and $G\alpha_{i2}$ (see Figure 2.5B) are expressed in mouse hippocampus, in agreement with published results (Laporte *et al.,* 1994; Allen Brain Atlas experiment numbers 79556616 and 2263). Based on the above results and our previous findings (Talbot *et al.,* 2010) we predicted that the decreased immobility in the $G\alpha_{i2}^{GS/GS}$ mice is due to increased activation of hippocampal 5-HT1ARs coupled to $G\alpha_{i2}$. Activation of 5-HT1A receptors reduces membrane excitability by altering intrinsic membrane properties. For example, 5-HT1AR activation alters potassium currents and hyperpolarization-activated currents to regulate cell excitability (Ko *et al.,* 2016; Andrade *et al.,* 1987; Colino *et al.,* 1987; Oleskevich *et al.,* 1995). Therefore, to test for altered 5-HT1AR activity, we compared the effect of 5 μM 8-OH-DPAT between heterozygous $G\alpha_{i2}^{GS/+}$ and $G\alpha_{i2}^{+/+}$ littermates on excitability of CA1 hippocampal neurons. Heterozygous mice were used because the homozygous animals already show a maximal antidepressant-like effect in the tail suspension test (Talbot *et al.,* 2010) and we have previously shown that this 5-HT1AR agonist has increased potency in the heterozygotes (Talbot *et al.,* 2010). We recorded the responses of CA1 hippocampal neurons to current injection from -200 pA to +140 pA at +10 pA intervals before and after 5 μM 8-OH-DPAT application in $G\alpha_{i2}^{+/+}$

and $G\alpha_{i2}^{GS/+}$ littermates ($G\alpha_{i2}^{GS/+}$ n=7 cells from 3 mice; $G\alpha_{i2}^{+/+}$ n=9 cells from 5 mice for all measures). Application of 8-OH-DPAT did not affect neuronal excitability in $G\alpha_{i2}^{+/+}$ mice (Figure 2.3A: 2-way RM ANOVA, treatment x current injection interaction: $F_{(14,112)}=0.8026$, p=0.6647); example traces shown in Figure 2.3C. In contrast, the same 8-OH-DPAT treatment significantly decreased excitability in the $G\alpha_{i2}^{GS/+}$ mice (Fig 2.3B: 2-way RM ANOVA, treatment x current injection interaction: $F_{(14,84)}=2.632$, p=0.0033); example traces shown in Fig 2.3D. Thus, consistent with behavioral data above, 5 μM 8-OH-DPAT decreased membrane excitability in hippocampal CA1 neurons from $G\alpha_{i2}^{GS/+}$ mice, but not in neurons from $G\alpha_{i2}^{+/+}$ littermates. Higher concentrations of 8-OH-DPAT produce similar effects in wild type animals (Czyrak *et al.,* 2002) suggesting that 5-HT1A agonist potency is increased in $G\alpha_{i2}^{GS/+}$ mice.

In addition, bath application of 8-OH-DPAT produced a significant decrease in resting membrane potential in cells from the $G\alpha_{i2}^{GS/+}$ mice (Figure 2.3F; $t_6=6.9$; $p<0.001$), but not their $G\alpha_{i2}^{+/+}$ littermates (Figure 2.3E; $t8=1.02$, p=0.34) and the minimum amount of current needed to reach the firing threshold (i.e. the rheobase) was significantly increased by 8-OH-DPAT in cells from the $G\alpha_{i2}^{GS/+}$ mice (Figure 2.3E; $t_6=4.25$, $p<0.01$), but not in cells from $G\alpha_{i2}^{+/+}$ mice (Figure 2.3F; $t_8=1.31$, p=0.23). Overall, the results demonstrate that 5 μM 8-OH-DPAT application caused a decrease in membrane excitability in hippocampal CA1 neurons only from $G\alpha_{i2}^{GS/+}$ mice, but this concentration was ineffective in neurons from $G\alpha_{i2}^{+/+}$ littermates, showing increased activity of the 5-HT1AR agonist in the absence of RGS activity.



E

Gαi2 +/+	**Baseline**	**5µM DPAT**	**Sig.**
Rheobase, pA	84 +/- 15	100 +/- 23	No
RMP, mV	-74 +/- 1.1	-76 +/- 2.2	No
IR, MΩ	160 +/- 19	150 +/- 18	No

F

Gαi2 GS/+	**Baseline**	**5µM DPAT**	**Sig.**
Rheobase, pA	71 +/- 17	96 +/- 21	**
RMP, mV	-72 +/- 2.0	-76 +/- 2.1	***
IR, MΩ	180 +/- 19	170 +/- 15	No

Fig. 2.3: Recording from hippocampal slices in the presence of 8-OH-DPAT from wild type and RGSi Gαi2 knock-in mice. (A) Application of 8-OH-DPAT did not affect neuronal excitability in wild type mice (Gαi2 $^{+/+}$; 2-way RM ANOVA, treatment x current injection interaction: $F_{(14,112)}$=0.8026, p=0.6647). **(B)** Application of 8-OH-DPAT decreased neuronal excitability in heterozygous RGSi $G\alpha_{i2}$ mice ($G\alpha_{i2}$ $^{GS/+}$; 2-way RM ANOVA, treatment x current injection interaction: $F_{(14,84)}$=2.632, p=0.0033). **(C)** Example traces of data quantified in 2.3A. **(D)** Example traces of data quantified in 2.3B. **(E)** Bath application of 8-OH-DPAT produced no significant changes in measured currents in cells from wild time mice (Gαi2 $^{+/+}$ mice; t-test performed between baseline and 5 µM 8-OH-DPAT measurements, ** = p<.01). **(F)** Bath application of 8-OH-DPAT produced a significant decrease in resting membrane potential (RMP) and increased rheobase in cells from the heterozygous RGSi $G\alpha_{i2}$ mice ($G\alpha_{i2}$ $^{GS/+}$), but did not affect input resistance (IR) (t-test performed between baseline and 5 µM 8-OH-DPAT measurements, ** = p<.01, *** = p<.001; n=9 cells recorded for each measure, data expressed as mean +/- SEM; 0 indicates terminal significant figure when otherwise ambiguous).

Effects of an RGS inhibitor (CCG-203769) on tail suspension test immobility in male and female wild type mice

We have previously suggested that RGS19 acts as a negative modulator of 5-HT1AR signaling in mouse hippocampal neurons in vitro (Wang *et al.,* 2014). To examine if this is a critical component of the 5-HT1AR signaling pathway in the hippocampus in vivo we used the RGS4/RGS19 inhibitor CCG-203769 (Blazer *et al.,* 2015). A single intra-hippocampal administration of CCG-203769 (3 μg/side) produced a non-significant trend towards decreased immobility in the tail suspension test (data not shown). However, CCG-203769 is an irreversible inhibitor of RGS4 and RGS19. Therefore, to further inhibit RGS4/19 activity we gave three infusions each separated by 24h. After this treatment, female wild type mice showed a significant reduction in immobility compared to vehicle treated controls, while there was no significant effect in male animals (Figure 2.4A). Two-way ANOVA revealed significant main effects of sex ($F_{(1,21)}$=15.68, p=0.0007), treatment ($F_{(1,21)}$=4.354, p=0.0493), and sex x treatment interaction ($F_{(1,21)}$=6.361, p=0.0198). In contrast, there was no significant difference in the potency of 8-OH-DPAT to produce antidepressant-like effects between male and female wild type mice (Figure 2.4B). Two-way ANOVA revealed a significant main effect of treatment ($F_{(4,51)}$=9.058, p<0.0001), but not sex ($F_{(1,51)}$=0.1785, p=0.6745) or sex x treatment interaction ($F_{(4,51)}$=1.706, p=0.1630). Hippocampal homogenates from male and female wild type mice did not differ in RGS19 protein expression as determined by western blot (Figure 2.4C).

Hippocampal G-protein expression and [^{3}H]8-OH-DPAT binding in wild type and RGSi $G\alpha_{i2}$ knock-in mice

In order to determine whether the electrophysiological and behavioral changes observed in the RGSi $G\alpha_{i2}$ mice could be explained by compensatory changes in 5-HT1AR expression, we characterized 5-HT1AR ligand binding in the mouse hippocampus. Saturation binding with [^{3}H]8-OH-DPAT was performed in hippocampal membrane homogenates from wild type and heterozygous (+/GS) RGSi $G\alpha_{i2}$ mice (Figure 2.5A). Neither the maximal receptor expression (Bmax) nor the affinity (Kd) of [^{3}H]8-OH-DPAT binding were significantly different between RGSi $G\alpha_{i2}$ and wild type mice (Table 2.1).

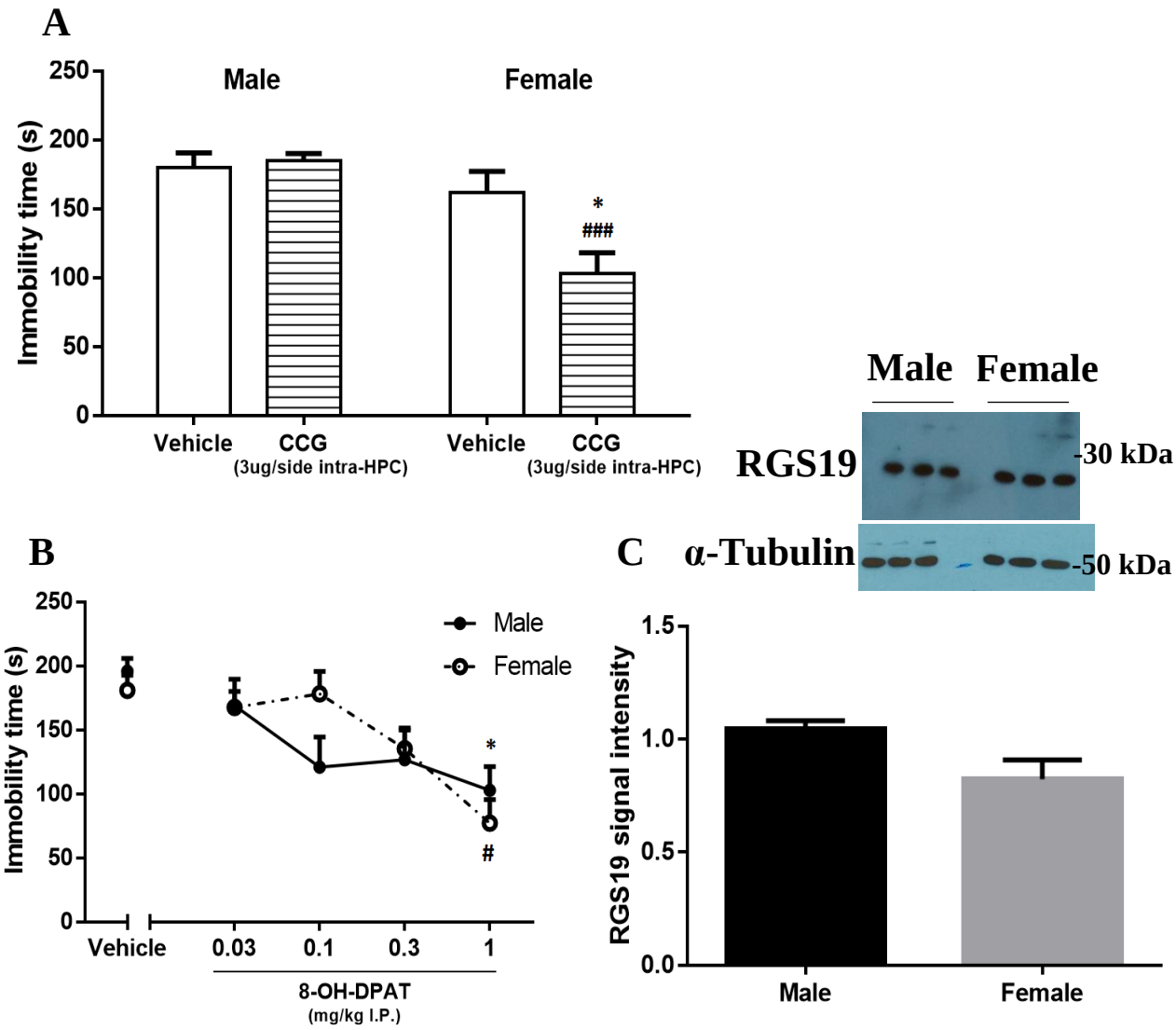


Fig. 2.4: Intra-hippocampal CCG-203769 administration produces antidepressant-like effects in female mice. (A) Administered once daily for three days intra-hippocampal CCG-203769 (CCG; 3 μg/side) produces an antidepressant-like effect in female but not male wild type mice. Each column depicts the mean immobility score +/- SEM of 6-7 mice (2-way ANOVA followed by Tukey's post-hoc test; significant main effects of sex, treatment and interaction; * indicates a difference from vehicle treated female mice ($p<.05$); ### indicates a difference from CCG-203769 treated male mice ($p<.001$)). **(B)** This sex difference cannot be explained by differential sensitivity to the antidepressant-like effects of 5-HT1A activation. Each point in (B) represents the mean immobility score +/- SEM of 6-7 mice (2-way ANOVA followed by Tukey's post-hoc test; significant main effect of treatment; * indicates a difference between 1 mg/kg 8-OH-DPAT and vehicle treated male animals ($p<.05$); # indicates a difference between 1 mg/kg DPAT and vehicle treated female animals ($p<.05$). **(C)** Hippocampal RGS19 expression. RGS19 signal intensity was normalized to α-Tubulin expression for each sample, and ratio of G-protein/α-Tubulin expression was averaged across three independent experiments +/- SEM (RGS19 signal intensity was compared between male and female animals with unpaired t-test, no significant difference). Representative blot shows RGS19 bands detected at ~25 kDa for hippocampal homogenates from female mice (lanes 1-3) and male mice (lanes 4-6).

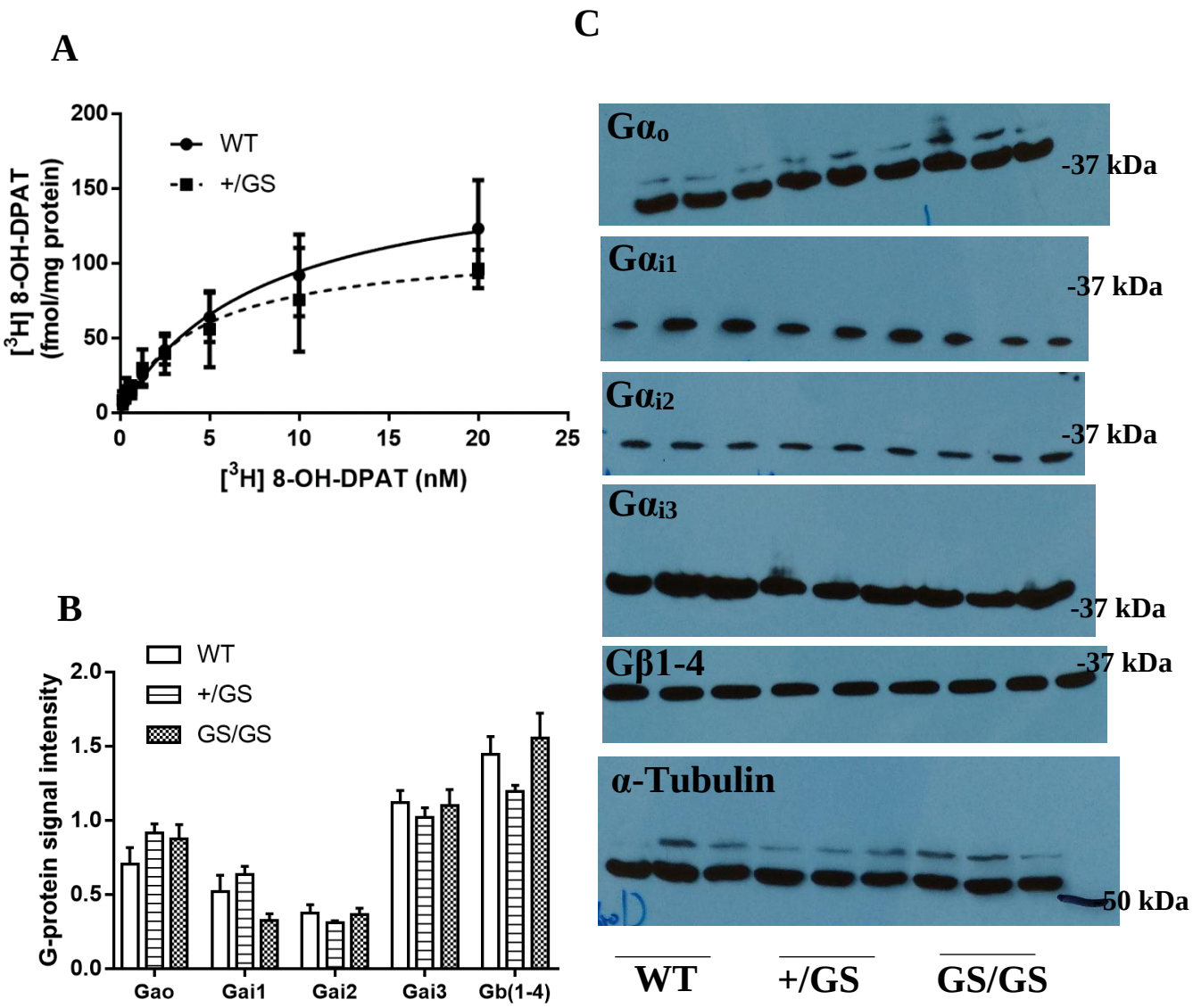


Fig. 2.5: Hippocampal [^{3}H]8-OH-DPAT binding and G-protein expression is unaltered in heterozygous RGSi Gα_{i2} knock-in mice. (A) Specific binding of 8-OH-DPAT: Each point represents the mean specific binding +/- SEM from three independent experiments (best fit lines for Kd and Bmax calculation were determined using a one-site saturation binding fit with Hill slopes set to 1 and fitted to mean results averaged across experiments; Bmax and Kd compared between WT and +/GS using unpaired t-test, no significant differences). **(B)** G-protein signal intensity was normalized to α-Tubulin expression for each sample, and mean G-protein/α-Tubulin expression +/- SEM was compared for three animals at each G-protein subunit (expression of each protein target for +/GS and GS/GS animals compared to WT expression using unpaired t-test, no significant differences). **(C)** In each blot, the first three lanes show hippocampal samples from wild type (WT) mice, lanes 4-6 show hippocampal samples from heterozygous RGSi Gα_{i2} mice (+/GS), and lanes 7-9 show hippocampal samples from homozygous RGSi Gα_{i2} mice (GS/GS).

Hippocampal homogenates from wild type and RGSi $G\alpha_{i2}$ knock-in mice were assayed by western blotting. Primary antibodies against $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\beta\gamma$(1-6) were used to screen for expression differences of these proteins in the hippocampus (Figure 2.5B). There were no significant differences in protein expression between RGSi $G\alpha_{i2}$ mice and wild type controls.

	Bmax +/- SEM (fmol/mg)	**Kd +/- SEM (nM)**
WT	140 +/- 30	6.4 +/-3.0
+/GS	110 +/- 17	3.8 +/- 1.6

Table 2.1: Bmax and Kd of hippocampal [^{3}H]8-OH-DPAT binding. Bmax and Kd were compared between WT and heterozygous RGSi Gαi2 mice (+/GS) using unpaired t-test, no significant differences; 0 indicates terminal significant figure when otherwise ambiguous.

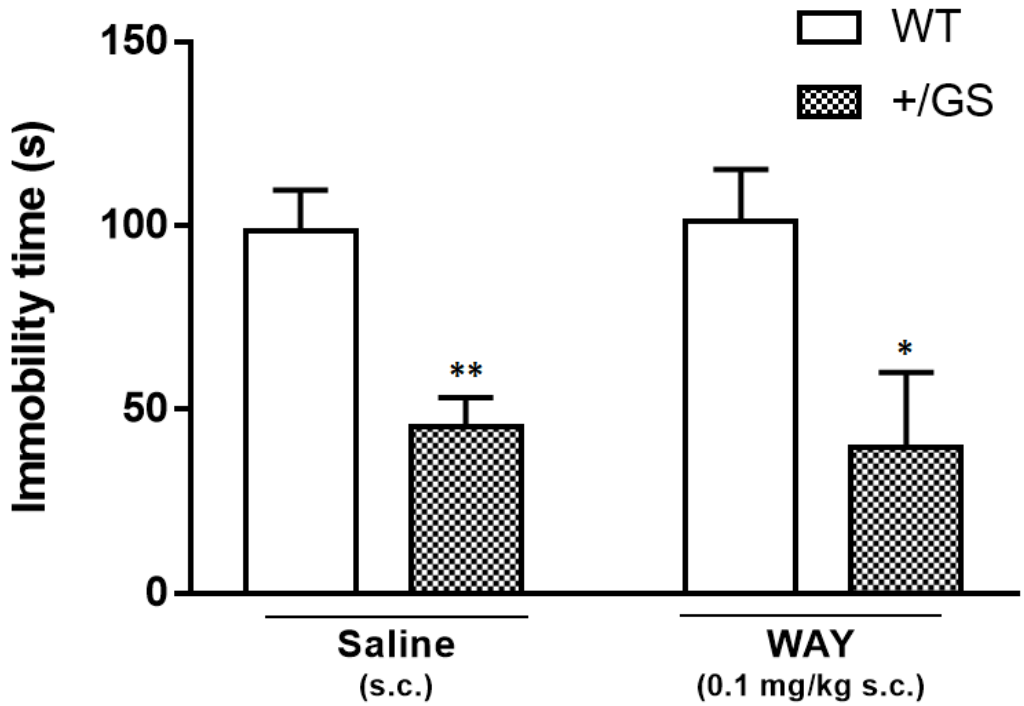


Fig. 2.6: Spontaneous antidepressant-like behavior in mice expressing RGSi $G\alpha_o$ is not reversed by the 5HT1A antagonist WAY100635. Antidepressant-like behavior in heterozygous RGSi $G\alpha_o$ (+/GS) expressing mice in the tail suspension test is not reversed by s.c. WAY-100635. Each column depicts the mean immobility score +/- SEM of 4-13 mice (2-way ANOVA followed by Tukey's post-hoc test; significant main effect of genotype; ** and * indicate a difference from saline treated wild type condition, $p<.01$ and $p<.05$ respectively).

Effects of peripheral WAY100635 on tail suspension test immobility in wild type and heterozygous RGSi $G\alpha_o$ knock-in mice

$G\alpha_{i2}$ appears to play a critical role in regulating antidepressant-like behavior via the 5-HT1AR as RGSi $G\alpha_{i2}$ knock-in mice have an antidepressant-like phenotype, while $G\alpha_{i2}$ knockout mice exhibit pro-depressant behaviors (Talbot *et al.,* 2010). However, 5-HT1ARs also couple to $G\alpha_o$ especially in the frontal cortex and hippocampus (La Cour *et al.,* 2006). To examine whether loss of RGS control of $G\alpha_o$ similarly affects behavior, we studied mice expressing RGSi $G\alpha_o$ proteins (Fu *et al.,* 2006). The homozygous knock-in mice are not viable so we used heterozygotes. These mice also showed a reduction in immobility compared to wild type littermates in the TST (Figure 2.6). However, unlike the RGS-$G\alpha_{i2}$ mice systemic (s.c.) injection of 0.1 mg/kg WAY100635 did not affect immobility time (Figure 2.6). Two-way ANOVA revealed a significant main effect of genotype ($F_{(1,30)}$=16.2, p=0.0004), but not treatment ($F_{(1,30)}$=0.0191, p=0.9138) or genotype x treatment interaction ($F_{(1,30)}$=0.08618, p=0.7711).

Discussion

The current results show that the antidepressant-like behavioral phenotype observed on the tail suspension test in mice expressing an RGSi knock-in variant of $G\alpha_{i2}$ ($G\alpha_{i2}^{GS/GS}$) was fully reversed by administration of the 5-HT1AR antagonist WAY100635 locally to the hippocampus. Moreover, this behavioral phenotype was mimicked by hippocampal administration of the 5-HT1AR agonist 8-OH-DPAT to mice expressing wild type $G\alpha_{i2}$ ($G\alpha_{i2}^{+/+}$) that was in turn fully blocked by systemic WAY100635. The behavioral phenotype of the RGSi $G\alpha_{i2}$ mice was accompanied by increased activity of the agonist 8-OH-DPAT on hippocampal slices in the heterozygotes ($G\alpha_{i2}^{GS/+}$) such that a concentration of 8-OH-DPAT that was ineffective in slices from wild-type mice caused hyperpolarization in slices from the mutant mice. The lack of pretreatment changes in neuronal excitability suggest that the observed behavioral changes were not due to increased constitutive activity of hippocampal 5-HT1ARs, but were instead due to enhanced signaling of endogenous serotonin at this site. There were no changes in hippocampal 5-HT1ARs or their ability to activate G-proteins, nor any significant changes in hippocampal heterotrimeric G-protein subunit expression. These data suggest that promoting signaling through the 5-HT1AR/$G\alpha_{i2}$ complex in the hippocampus selectively enhances the antidepressant-

like effects of 5-HT1AR agonism. We did not study the 5-HT1AR-rich frontal cortex but our finding that intra-hippocampal WAY100635 fully reverses the behavioral phenotype indicates that activation of hippocampal 5-HT1ARs appears to be necessary and sufficient to explain the antidepressant-like behavior in mice expressing RGSi $G\alpha_{i2}$.

While it remains difficult to develop drugs that will target a specific GPCR bound to a particular G-protein subunit, targeting RGS proteins may provide an additional level of selectivity. RGS family members 2, 7, 8, 10, 14 and 19 are expressed in the hippocampus at high levels, while 4, 5, 11 and 13 are expressed only moderately, and 3, 6, 9 and 16 are expressed at very low levels or are absent (Gold *et al.,* 1997; Grafstein-Dunn *et al.,* 2001). RGS19 has especially high expression in the hippocampus compared to other brain regions (Grafstein-Dunn *et al.,* 2001). Furthermore, RGS19 readily regulates 5-HT1AR function in isolated hippocampal neurons compared to other RGS proteins tested (Wang *et al.,* 2014). These properties make RGS19 an attractive target to selectively enhance hippocampal 5-HT1AR function for potential antidepressant effects while avoiding the drawbacks of activating all 5-HT1ARs expressed in the CNS.

Due to the lack of highly selective RGS19 small molecule inhibitors we tested the effects of CCG-203769, an RGS4 and RGS19 dual inhibitor (Blazer *et al.,* 2015). CCG-203769 forms a disulfide bridge with these RGS proteins and permanently inactivates them (Turner *et al.,* 2011). Repeated (3-day) administration of CCG-203769 bilaterally into the hippocampus was necessary to produce an anti-depressant-like effect, but surprisingly this was only seen in female mice. This sex difference was not explained by differences in hippocampal RGS19 protein expression as levels were similar between male and female mice. In addition, there was no sex difference in the antidepressant-like effects of 8-OH-DPAT suggesting that a differential response to 5-HT1AR activation is not the primary cause. The disparity may be due to a lower potency of CCG-203769 in males compared to females rather than an all-or-none difference. However, higher doses were not thoroughly tested in male mice because intra-hippocampal infusion of more than 3 μg/side CCG-203769 produced motor suppression and catatonia in both male and female mice (unpublished observation), although this motor suppressant effect was not quantified.

Although RGS19 inhibition is a promising candidate mechanism for the antidepressant-like action in the $G\alpha_{i2}$ $^{GS/GS}$ mice and for CCG-203769's antidepressant-like effects, inhibition of other RGS proteins may play a role. Out of all the RGS proteins tested with CCG-203769 only RGS4 is inhibited more potently than RGS19 (Blazer *et al.*, 2015). On the other hand, overexpressed RGS4 does inhibit 5-HT1AR signaling in the raphe nuclei (Beyer *et al.*, 2004), and genetic knockdown of RGS4 did not affect baseline antidepressant-like behaviors and decreased the potency of serotonergic antidepressants (Stratinaki *et al.*, 2013) suggesting that RGS4 inhibition likely cannot explain the antidepressant-like effects seen in the $G\alpha_{i2}$ $^{GS/GS}$ mice or after CCG-203769 treatment. In contract, RGS6 knockout (RGS6 $^{-/-}$) mice do show a 5-HT1AR-mediated baseline antidepressant-like phenotype (Stewart *et al.*, 2014). Whereas this behavioral response is consistent with antidepressant-like effects seen in the $G\alpha_{i2}$ $^{GS/GS}$ mice there is no evidence that CCG-203769 interacts with RGS6. RGS6 is a member of the R7 family of RGS proteins that includes RGS6, 7, 9 and 11 (Hollinger and Helper, 2002). CCG-203769 has over 1000-fold selectivity for RGS19 compared to other R7 RGS family members (Blazer *et al.*, 2015) and RGS6 lacks an available cysteine residue within the RGS box region to form a covalent interaction with CCG-203769. In addition, the increased phospho-GSK3β levels seen in the $G\alpha_{i2}$ $^{GS/GS}$ mice (Talbot *et al.*, 2010) are not seen in the RGS6$^{-/-}$ mice which instead show an increase in phospho-CREB (Stewart *et al.*, 2014). Thus, the mechanisms underlying the 5-HT1AR-mediated phenotype in the RGS6$^{-/-}$ and Gαi2 $^{GS/GS}$ mice appear to be different.

Nonetheless, the above discussion suggests that the complex of 5-HT1AR/$G\alpha_{i2}$ with a specific RGS protein, possibly RGS19, might provide a suitable target for antidepressant drug therapy. Furthermore, this could offer an explanation for the selectivity of compounds developed by Newman-Tancredi *et al* which show preference for frontal cortex 5-HT1A heteroreceptors compared to raphe nuclei 5-HT1A autoreceptors (e.g. F15599; Newman Tancredi *et al.*, 2009) or selectivity for 5-HT1A autoreceptors compared to other 5-HT1ARs (e.g. F13640 and F13714; Buritova *et al.*, 2009). The heteroreceptor selective F15599 also stimulates 5-HT1ARs coupled to $G\alpha_i$ more potently and efficaciously than 5-HT1ARs coupled to $G\alpha_o$, while serotonin shows no G-protein preference (Newman Tancredi *et al.*, 2009), supporting the notion that a small molecule agonist can achieve selectivity.

Surprisingly we found that heterozygous RGSi $G\alpha_o$ knock-in mice also have an antidepressant-like phenotype. However, this behavioral phenotype was not reversed by the 5-HT1AR antagonist WAY100635, suggesting that the antidepressant-like behaviors displayed by the RGSi $G\alpha_{i2}$ and RGSi $G\alpha_o$ mice are driven by distinct mechanisms. Studies in E. coli suggest that the 5-HT1AR couples more efficiently to $G\alpha_{i2}$ than $G\alpha_o$ proteins (Bertin *et al.,* 1992), so the lack of a 5-HT1AR dependent phenotype in the RGSi $G\alpha_o$ mice is not surprising. Although 5-HT1AR do couple to other Gα proteins the close association between 5-HT1AR and $G\alpha_{i2}$ is supported by a 5-HT1AR dependent antidepressant-like phenotype in the $G\alpha_{i2}^{GS/GS}$ mice and a prodepressant phenotype in $G\alpha_{i2}^{-/-}$ mice (Talbot *et al.,* 2010).

When both the therapeutic and detrimental effects of a drug are mediated by the same molecular target, developing new therapies is particularly challenging. The 5-HT1A receptor has long been recognized as one such target, where activation of autoreceptors on serotonergic cells is generally considered detrimental while activating heteroreceptors expressed on cells downstream of the serotonin neurons produces beneficial effects (Artigas 1993; Blier and Abbott 2001). A considerable amount of effort has thus been spent on identifying strategies to block the 5-HT1A autoreceptors without affecting heteroreceptor activity, or conversely activating heteroreceptors without stimulating autoreceptor activity (Blier *et al.,* 1993; Romero *et al.,* 1996; Rabiner *et al.,* 2000; Newman Tancredi *et al.,* 2009). The RGSi $G\alpha_{i2}$ mutation appear to accomplish this as evidenced by a promotion of 5-HT1AR dependent antidepressant-like behaviors, but not hypothermic effects. A therapeutic that can selectively enhance signaling through 5-HT1A/$G\alpha_{i2}$ complexes, or alternatively selectively inhibit RGS proteins acting at $G\alpha_{i2}$, may dissociate the therapeutic and detrimental effects of 5-HT1A agonism with potential benefits for the neuropsychiatric treatment of depression.

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CHAPTER 3

RGS Regulation of Opioid Receptor Signaling and Antinociception

Uncontrolled pain is a significant problem with as many as 20% of all adults experiencing chronic pain (Brattberg *et al.,* 1989), and at least 10% of Americans experiencing severe pain in 2015 (https://nccih.nih.gov/news/press/08112015). Opioids have been used therapeutically to control pain for over 200 years (Weiser *et al.,* 1956) and to this day remain as the most commonly used analgesic medications (Okie 2010). Although non-opioid analgesics are available and effective for certain types of pain (e.g. COX-2 inhibitors for arthritic/inflammatory pain (Lee *et al.,* 2005) or pregabalin for neuropathic pain (Verma *et al.,* 2014), for many patients opioid analgesics remain the best available option. This fact, along with an increased focus on controlling pain among physicians, has led to opioid prescriptions increasing by a factor of 10 since just 1990 (Okie 2010). The huge amount of opioid prescriptions has led to a dramatic increase in unintentional overdose deaths, with over a fourfold increase in the number of deaths since the early 1970's (Okie 2010). These drugs also have significant abuse liability, and a history of substance abuse disorders dramatically increases the risk of accidental overdose death for those prescribed opioid analgesics (Bohnert *et al.,* 2011).

Four types of opioid receptors have been identified: the mu opioid receptor (MOPR), delta opioid receptor (DOPR), kappa opioid receptor (KOPR) and nociceptin receptor (NOPR) (Goldstein and Naidu 1989; Bunzow *et al.,* 1994). While each of these receptors has the ability to regulate the perception of pain, all currently available opioid medications exert their analgesic effects by binding to and activating MOPR (Gunther *et al.,* 2017). Nonetheless, ligands acting at the other opioid receptors have been proposed as putative analgesic medications with improved side effect profiles compared to the current generation of opioid analgesics (Gunther *et al.,* 2017). In addition, strategies to reduce the side effects of drugs acting at MOPR have been

suggested, including the use of allosteric modulators of MOPR (Livingston and Traynor 2017) and biased agonists that can selectively stimulate analgesic pathways (Raehal *et al.,* 2011; Siuda *et al.,* 2017). However much remains unknown about how these medications propagate their signal within the cell, including how various intracellular regulatory proteins affect this process.

One such class of proteins known to affect opioid receptor signaling and resultant analgesia are the regulators of G-protein signaling (RGS) proteins. These proteins are a 20+ member family of intracellular regulatory proteins characterized by an 'RGS box' domain (see Table 0.1; Hollinger and Hepler 2002). This domain makes direct contact with activated GTP-bound Gα proteins and facilitates their inactivation through an intrinsic GTPase activating (GAP) activity (see Figure 0.2; Tesmer *et al.,* 1997; Lan *et al.,* 2000). This terminates G-protein signaling and allows the receptor to reassociate with the heterotrimeric G-protein complex. This inhibitory effect on G-proteins allows RGS proteins to modulate the downstream effects of many GPCRs that are targeted by common pharmaceuticals including dopamine receptors (Wani *et al.,* 2012), serotonin receptors (Ghavami *et al.,* 2004) and opioid receptors (Clark *et al.,* 2003) among many others.

RGS proteins are extensively expressed throughout the central nervous system (CNS) and broadly overlap with opioid receptor expression (Gold *et al.,* 1997; Peckys and Landwehrmeyer 1999; Grafstein-Dunn *et al.,* 2001; Traynor and Neubig 2005). They display selectivity for certain G-protein subtypes over others (Posner *et al.,* 1999; Lan *et al.,* 2000) as well as selectivity for certain receptors (Xu *et al.,* 1999; Wang *et al.,* 2009). Their selectivity and expression pattern make RGS proteins attractive drug targets, and small molecule RGS inhibitors have now been identified (Roof *et al.,* 2006; Roman *et al.,* 2007). Furthermore, RGS proteins regulate the effects of drugs acting at the opioid receptors in ways that are not yet entirely clear. Therefore, this chapter will focus on the interaction between opioid receptors and RGS proteins and the implications for analgesic treatment. Table 3.1 provides a general summary of findings discussed below relating to RGS protein regulation of MOPR, DOPR, KOPR and NOPR.

R4 RGS family regulation of MOPR signaling and antinociception

The regulation of MOPR signaling by RGS4 is one of the most well characterized RGS/receptor pairings. RGS4 has widespread distribution throughout the CNS where it can regulate the analgesic effects of MOPR agonists (see Traynor and Neubig 2005 for review). RGS4 is

	Influence on Receptor Mediated Effects			
	MOPR	**DOPR**	**KOPR**	**NOPR**
R4 Family				
RGS2	Increase			
RGS3	Increase			
RGS4	Reduction	Reduction	Reduction	
RGS8	Reduction			
RGS16	Reduction			
R7 Family				
RGS6	Minor reduction	No effect		
RGS7	Minor reduction	No effect		
RGS9-1	No effect	No effect		
RGS9-2	Reduction	No effect		
RGS11	Reduction	No effect		
R12 Family				
RGS12	Reduction			
RGS14	Reduction			
RZ Family				
RGS19	Reduction	Reduction/ No effect		No effect
RGSZ-1	Reduction	No effect		
RGSZ-2	Reduction	No effect		

Table 3.1: General overview of findings described in this chapter showing RGS protein regulation of MOPR, DOPR, KOPR and NOPR-mediated effects. Blank entries indicate insufficient data to determine effect of RGS/receptor interaction.

proposed to interact directly with MOPR via an interaction between the fourth-intracellular loop of MOPR (residues 329–355) and RGS4's N-terminal domain (Leontiadis *et al.,* 2009). Removal of the RGS4 N-terminal domain not only reduces receptor/RGS4 interactions but also eliminates receptor selectivity seen with the endogenous RGS4 protein (Zeng *et al.,* 1998; Leontiadis *et al.,* 2009). In a resting state RGS4 binds weakly to $G\alpha_{i/o}$ proteins, but this interaction is greatly enhanced by MOPR activation (Leontiadis *et al.,* 2009). When overexpressed in HEK293 cells RGS4 has a diffuse localization throughout the cytosol, nucleus and plasma membrane, however following application of the MOPR agonist DAMGO expression shifts to the plasma membrane such that RGS4 is colocalized with MOPR (Leontiadis *et al.,* 2009). In contrast, in SH-SY5Y cells that endogenously express RGS4 and MOPR,

knockdown of RGS4 did not affect responses to the MOPR agonist morphine (Wang *et al.,* 2009) suggesting that the ability of RGS4 to regulate MOPR may be cell type dependent.

Following partial sciatic nerve ligation rats become hyperalgesic and MOPR agonists decrease in potency (Garnier *et al.,* 2003). This coincides with an upregulation of RGS4 mRNA expression in the dorsal horn of the spinal cord, but no change in the mRNA of other RGS proteins tested (RGS6/7/8/9/11/12/14/17/19; Garnier *et al.,* 2003). The possibility that this compensatory change in RGS4 contributes to the loss of MOPR agonist potency is bolstered by the fact that overexpression of RGS4 reduces the ability of MOPR agonists to inhibit adenylyl cyclase *in vitro,* and that RGS4 and MOPR show significant expression overlap within the dorsal horn (Peckys and Langwehrmeyer 1999; Garnier *et al.,* 2003). In dorsal root ganglion primary sensory neurons however, RGS4 mRNA decreases following sciatic nerve injury (Costigan *et al.,* 2003), suggesting a cell type dependent regulation of this transcript between the dorsal root ganglion and dorsal horn of the spinal cord.

In support of changes dependent on duration of exposure, acute administration of morphine causes an upregulation of RGS4 mRNA levels in the dorsal central gray and nucleus accumbens, but reduces RGS4 mRNA in the reticulotegmental pontine nucleus and locus coeruleus (Bishop *et al.,* 2002). Both the dorsal central gray (Guimaraes and Prado 1994; Helmstetter and Tershner 1994) and nucleus accumbens (Han *et al.,* 2010) are known to contribute to the antinociceptive effects of opioids. RGS4 upregulation in these regions would therefore be expected to attenuate the analgesic effects of opioids and may be involved in the development of opioid tolerance following repeated administration (Bishop *et al.,* 2002). Locus coeruleus RGS4 mRNA was not altered during chronic morphine exposure, but increased 2 to 3-fold following naltrexone precipitated withdrawal (Gold *et al.,* 2003). Interestingly RGS4 mRNA also increases in the locus coeruleus following naltrexone administration to opioid naïve animals, suggesting that endogenous opioids may tonically inhibit locus coeruleus RGS4 expression (Gold *et al.,* 2003).

In PC12 cells that endogenously express RGS4 the MOPR agonists morphine and DAMGO increase RGS4 mRNA expression transiently over the course of 8-12 hours before mRNA levels return to basal levels (Nakagawa *et al.,* 2001). This time course reflects the results observed in the locus coeruleus where acute morphine treatment increases RGS4 mRNA (Bishop *et al.,* 2002) while RGS4 mRNA levels have returned to normal following chronic morphine treatment

(Gold *et al.,* 2003). On the other hand, RGS4 protein levels increase during chronic morphine treatment (Gold *et al.,* 2003), although it remains unclear whether these changes contribute to either the development of opioid tolerance or the withdrawal syndrome following chronic opioid exposure.

Intracellular application of purified RGS4 to locus coeruleus neurons attenuated the electrophysiological response to acute morphine, confirming that increased levels of RGS4 in this brain region can counteract opioid effects (Gold *et al.,* 2003). Overexpression of RGS4 also attenuated the ability of MOPR agonists to inhibit adenylyl cyclase activity and stimulate MAPK in HEK293 cells (Leontiadis *et al.,* 2009) and activate GIRK in Xenopus oocytes (Ippolito *et al.,* 2002). On the other hand, the MOPR agonist DAMGO inhibited firing of locus coeruleus neurons to the same extent in wild type and RGS4 knockout mice (Han *et al.,* 2010) and in SH-SY5Y cells RGS4 knockdown had no effect on morphine stimulated MAPK or adenylyl cyclase activity (Wang *et al.,* 2009). One possible explanation for this discrepancy is that in knockdown models other RGS proteins are able to fully compensate for the loss of RGS4, while increasing RGS4 expression further inhibits opioid activity, although this hypothesis has not been confirmed. However, even the results of RGS4 overexpression studies are not fully consistent, as RGS4 overexpression in xenopus oocytes has been reported alternatively to attenuate MOPR signaling (Ippolito *et al.,* 2002) or have no effect (Potenza *et al.,* 1999) although this may result from the use of different agonists (morphine and DAMGO respectively). Indeed, RGS4 regulation of MOPR agonist-induced antinociception is strongly agonist-dependent, as RGS4 knockout mice are less sensitive to the antinociceptive effects of both fentanyl and methadone, while the antinociceptive effect of morphine is not affected (Han *et al.,* 2010). This lack of an RGS4 effect with morphine is not consistent across studies however, as mice administered antisense-DNA against RGS4 had a greater response to intracerebroventricular (i.c.v.) morphine compared to controls (Garzon *et al.,* 2001), although different routes of administration between these two studies (i.c.v. vs. subcutaneous injection) may contribute to this discrepancy.

The ability of RGS4 to regulate fentanyl antinociception appears to depend on nucleus accumbens RGS4 expression, as specific knockdown in this brain region also reduces fentanyl antinociception (Han *et al.,* 2010). While the upregulation of nucleus accumbens RGS4 mRNA following acute morphine exposure in the rat may therefore contribute to the reduction in

morphine's antinociceptive effects after repeated administration (Bishop *et al.,* 2002), RGS4 protein levels in the nucleus accumbens are not significantly elevated following repeated morphine exposure in mice (Narita *et al.,* 2002). However, in this study morphine was only administered once every 72 hr (Narita *et al.,* 2002) whereas studies showing effects on brain RGS4 protein level administered morphine continuously over 5 days (Gold *et al.,* 2003). Therefore, the possibility remains that opioid effects on brain RGS4 protein expression may require more frequent dosing.

The injection of formalin into a mouse hind paw produces a biphasic hyperalgesic behavior with an early phase and a late phase that are differentially regulated by RGS4 (Yoon *et al.,* 2015). RGS4 knockout mice spend less time licking (i.e. are less hyperalgesic) during the late phase of formalin hyperalgesia, but behave similar to wild type controls during the early phase (Yoon *et al.,* 2015). The antihyperalgesic effect of the MOPR agonist DAMGO was left-shifted over 10-fold in RGS4 knockout animals compared to wild type, confirming the ability of RGS4 to limit antihyperalgesic effects of exogenous opioids (Yoon *et al.,* 2015). Similarly, intrathecal injection of the small molecule RGS4 inhibitor CCG50014 (Roman *et al.,* 2007) to wild type mice produced a dose dependent reduction in licking during the late phase without affecting early phase behavior (Yoon *et al.,* 2015). This effect of CCG50014 was completely blocked by naloxone pretreatment, confirming the involvement of opioid receptors in this response (Yoon *et al.,* 2015). Despite these antihyperalgesic effects of RGS4 inhibition, healthy RGS4 knockout mice do not display alterations in pain sensitivity when tested on the tail flick, hot plate or shock threshold tests, either at baseline or following morphine administration (Grillet *et al.,* 2005). This suggests that RGS4 may regulate the antihyperalgesic effects of opioids rather selectively compared to their antinociceptive effects in healthy animals.

Less is known about how R4 RGS family members other than RGS4 (e.g. RGS1/2/3/5/8/13/16/18; Hollinger and Helpler 2002) regulate MOPR signaling. Locus coeruleus RGS2 showed a very similar change in expression to RGS4 following morphine exposure, with an increase in protein level during chronic morphine treatment and an increase in mRNA level following naltrexone precipitated withdrawal (Gold *et al.,* 2003). Out of the RGS proteins known to be expressed in the locus coeruleus (RGS2/3/4/5/7/8/11) only RGS2 and RGS4 were affected in this manner (Gold *et al.,* 2003). However, RGS8 is capable of

modulating MOPR signaling as GTP hydrolysis stimulated by DAMGO in C6 cells is inhibited by the addition of purified RGS8 (Clark *et al.,* 2003; Talbot *et al.,* 2010), so in the locus coeruleus RGS8 may not be expressed in the appropriate cell type or at sufficient levels to regulate MOPR signaling. In a melanophore based assay overexpression of RGS2, but not RGS1, RGS3 or RGS4 potentiated morphine's effects (Potenza *et al.,* 1999), so despite similar regulation of RGS2 and RGS4 in the locus coeruleus, these proteins appear to regulate MOPR signaling differently in certain cell types.

In agreement with the findings of Potenza *et al* (1999) knockdown of RGS2 and RGS3 had no effect on baseline antinociception but paradoxically inhibited the antinociceptive response to morphine and beta-endorphin on the tail flick test (Garzon *et al.,* 2001). In contrast RGS16 knockdown increased the antinociceptive response to morphine without changing baseline behavior, a profile similar to the behavior of RGS4 knockdown animals (Garzon *et al.,* 2001). The basis of these opposing effects of different R4 family members has not been adequately explored, although it has been suggested that distinct Gα interaction profiles between the different RGS proteins may be responsible (Garzon *et al.,* 2000; Garzon *et al.,* 2001).

R7 RGS family regulation of MOPR signaling and antinociception

The R7 family of RGS proteins, including RGS6, RGS7, RGS9-1, RGS9-2 and RGS11 (Hollinger and Hepler 2002), have also been extensively studied in terms of their ability to regulate MOPR signaling. Cells transfected with RGS9-2 (but not the retina specific variant RGS9-1) had an attenuated response to morphine in a melanophore based assay (Rahman *et al.,* 1999). Although mice administered antisense-DNA against RGS7 or RGS9-2 i.c.v. had no baseline change in antinociception, knockdown of either protein resulted in greater responses to morphine, DAMGO and beta-endorphin on the tail flick test (Sanchez-Blazquez *et al.,* 2003; Garzon *et al.,* 2001). Knockdown of RGS9-2 or RGS11 more effectively enhanced DAMGO induced antinociception than RGS6 or RGS7 knockdown (Garzon *et al.,* 2003). Together these data show selectivity within the R7 family for control of MOPR signaling, with RGS9-2 and RGS11 producing greater effects than RGS6 and RGS7, while the retinal specific variant RGS9-1 is incapable of regulating MOPR signaling. One mechanism of R7 modulation of MOPR signaling involves inhibition of G-protein-coupled inwardly-rectifying potassium channel

(GIRK) signaling, as elimination of the R7 RGS binding protein (R7BP) disinhibits MOPR agonist stimulated GIRK activity (Zhou *et al.,* 2012).

In permeabilized C6 cells expressing MOPR and $G\alpha_{i2}$, addition of the RGS box region of RGS7 did not affect DAMGO-induced inhibition of cAMP accumulation (Talbot *et al.,* 2010). When $G\alpha_o$ was expressed instead of $G\alpha_{i2}$, the addition of RGS7 box region effectively inhibited DAMGO's effects (Talbot *et al.,* 2010) suggesting that RGS7 selectively regulates the action of MOPR agonists depending on the G-protein expressed. This selectivity may be due to a lack of physical interaction between RGS7 and $G\alpha_{i2}$ as increasing concentrations of $G\alpha_{i2}$ effectively disrupted the RGS4/$G\alpha_o$ complex but not the RGS7/$G\alpha_o$ complex (Talbot *et al.,* 2010), suggesting that the inability of RGS7 to regulate MOPR signaling in cells expressing $G\alpha_{i2}$ is due to a failure of RGS7/$G\alpha_{i2}$ complex formation.

RGS9-2 knockout mice have both a baseline decrease in nociceptive responding and an increased antinociceptive response to morphine (Papachatzaki *et al.,* 2011). Similarly, the antinociceptive action of morphine is enhanced in animals with either partial or complete RGS9-2 knockdown in the brain (Sanchez-Blazquez *et al.,* 2003; Garzon *et al.,* 2001; Zachariou *et al.,* 2003). RGS9-2 knockout also reduces thermal hyperalgesia and mechanical allodynia in a spared nerve injury model (Terzi *et al.,* 2014), although the receptor(s) involved in these behaviors were not explored. In mice with endogenous RGS protein levels, nerve injury caused a transient reduction in spinal cord RGS9-2 (as well as reduced expression of the R12 family member RGS10), followed by upregulated RGS9-2 levels in the nucleus accumbens at later points, suggesting possible loci for RGS influence on neuropathic pain (Alqinyah *et al.,* 2017; Terzi *et al.,* 2014).

RGS9-2 knockdown also delays tolerance and enhances antinociception following chronic morphine exposure (Zachariou *et al.,* 2003). When RGS9 was knocked down in HN9.10 cells stably expressing the MOPR, chronic morphine treatment produced less upregulation of adenylyl cyclase activity and no longer shifted the potency of DAMGO, consistent with the reduced tolerance observed in animal models (Xu *et al.,* 2004; Zachariou *et al.,* 2003). Furthermore, morphine treatment enhances the interaction between RGS9-2, MOPR and beta-arrestin, and RGS9-2 overexpression delays morphine induced MOPR internalization (Psifogeorgou *et al.,* 2007). In wild type mice RGS9-2 forms a complex with $G\alpha_{i2}$ and is phosphorylated following

acute morphine treatment (Ibi *et al.,* 2011). However, in mice lacking Nox1, the catalytic subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, both the association of the RGS9-2/$G\alpha_{i2}$ complex and the phosphorylation of RGS9-2 are attenuated (Ibi *et al.,* 2011). This suggests a mechanistic linkage between RGS9-2 and NADPH oxidase, which has previously been associated with the development of morphine tolerance (Doyle *et al.,* 2010). Despite the delayed development of tolerance after loss of RGS9-2, RGS9-2 knockdown animals also somewhat paradoxically displayed increased physical dependence and withdrawal from chronic morphine (Zacharioiu *et al.,* 2003), suggesting that RGS9-2 regulates tolerance and dependence independently.

In animals with endogenous RGS9-2 levels acute peripheral administration of morphine increased RGS9-2 expression throughout the CNS (including in the nucleus accumbens, periaqueductal gray, and spinal cord), while in contrast chronic morphine decreased RGS9-2 levels (Zachariou *et al.,* 2003). Morphine administered i.c.v. produces similar effects, with increased RGS9-2 mRNA observed in the thalamus and elevated RGS7 in the thalamus, however RGS9-2 and RGS11 were reduced in the cortex (Lopez-Fando *et al.,* 2005). By day two of morphine treatment RGS7, RGS9-2, RGS11 and $G\beta_5$ were all increased throughout the brain including the cortex, and these changes persisted over two weeks of continuous morphine delivery (Lopez-Fando *et al.,* 2005). It is unclear why chronic morphine both increased brain RGS9-2 mRNA (Lopez-Fando *et al.,* 2005) and decreased RGS9-2 protein levels (Zachariou *et al.,* 2003) in different studies, however the treatment schedule likely resulted in higher sustained morphine concentrations in the latter study.

RGS9-2 is known to be differentially expressed throughout the pain pathway during different stages of development. RGS9-2 expression is increased in the ventral and dorsal horn, caudate putamen, nucleus accumbens, olfactory tubercle and periaqueductal gray of 1-year old rats compared to 3-week old rats, while expression is decreased in the thalamus and locus coeruleus (Kim *et al.,* 2005). Almost all neuronal cells in the dorsal root ganglion (DRG) are positive for RGS9-2 however expression in this region was not affected by age (Kim *et al.,* 2005).

$G\beta_5$ is unique among Gβ isoforms, with less than 50% homology to other family members and distinct effects on multiple effector pathways including MAPK and adenylyl cyclase (see Simonds and Zhang 2000 for review). Furthermore, evidence suggests that $G\beta_5$ binds directly to

the central G-protein gamma-like (GGL) domain expressed in R7 RGS proteins (Snow *et al.,* 1998; Levay *et al.,* 1999), such that knockdown of $G\beta_5$ may interfere with the activity of these RGS proteins. Knockdown of $G\beta_5$ in the brain by antisense DNA enhanced the potency of morphine and DAMGO to produce antinociception on the tail flick test (Sanchez-Blazquez *et al.,* 2003), which may reflect a reduced ability of R7 RGS proteins (normally in complex with $G\beta_5$) to inhibit MOPR signaling. In addition, morphine failed to produce acute tolerance following $G\beta_5$ knockdown (Sanchez-Blazquez *et al.,* 2003). The fact that the loss of a G-protein subunit enhances the effects of MOPR agonists rather than inhibiting them is on its face paradoxical, however the finding that R7 RGS family members and $G\beta_5$ appear to be co-regulated provides one potential explanation (Chen *et al.,* 2000; Witherow *et al.,* 2000), although R7 protein expression was not assessed following $G\beta_5$ knockdown (Sanchez-Blazquez *et al.,* 2003).

R12 RGS family regulation of MOPR signaling and antinociception

Knockdown of R12 RGS family members RGS12 and RGS14 increased the antinociceptive response to morphine on the tail flick test in the mouse without affecting baseline withdrawal latencies, similar to the effects of RGS16 knockdown (an R4 RGS family member; Garzon *et al.,* 2001). RGS14 knockdown reduced the development of acute tolerance following morphine exposure, and these behavioral changes occurred alongside increased MOPR phosphorylation leading to internalization and recycling of the receptor (Rodriguez-Munoz *et al.,* 2007a). This suggests that in normal circumstances RGS14 limits agonist activity in a way that reduces both MOPR phosphorylation (e.g. by GRKs) and beta-arrestin-mediated endocytosis, leading to more robust receptor desensitization than in systems lacking RGS14 (Rodriguez-Munoz *et al.,* 2007a).

RZ RGS family regulation of MOPR signaling and antinociception

SH-SY5Y cells endogenously express RGS19, as well as MOPR, DOPR and NOPR. Knockdown of RGS19 enhanced MOPR agonist induced MAPK stimulation and adenylyl cyclase inhibition (Wang and Traynor 2013) without affecting DOPR or NOPR agonist effects. RGS19 expression increased after overnight treatment with MOPR agonists, and inhibition of $G\alpha_{i/o}$ protein/receptor interaction with pertussis toxins prevented this upregulation (Wang and Traynor 2013). The upregulation was also prevented by inhibition of either PKC or MEK (Wang and Traynor 2013), suggesting a pathway dependent on MOPR, $G\alpha_{i/o}$ proteins and PKC/MEK leading to RGS19 upregulation.

Knockdown of RGSZ-2 increased the antinociceptive response to morphine and DAMGO, and increased the rate at which tolerance developed to these antinociceptive effects (Garzon *et al.*, 2005). These effects were specific for MOPR agonists as the actions of the DOPR agonists DPDPE and deltorphin II were not affected by RGSZ-2 knockdown (Garzon *et al.*, 2005). Similarly, knockdown of two other RZ RGS family members, RGS19 (aka GAIP) and RGSZ1, enhanced the antinociceptive effects of morphine and DAMGO without affecting the response to DOPR agonists (Garzon *et al.*, 2004). In addition to these effects on antinociception, knockdown of either RGS19 or RGSZ1 increased the rate at which antinociceptive tolerance developed (Garzon *et al.*, 2004). Together these results suggest that all RZ RGS proteins are capable of both inhibiting MOPR agonists induced antinociception and reducing the development of tolerance following agonist exposure, likely through regulation of $G\alpha_z$ and $G\alpha_{i2}$ (Garzon *et al.*, 2004; Garzon *et al.*, 2005; Rodriguez-Munoz *et al.*, 2007b).

Treatment with morphine or DAMGO alters the proteins that $G\alpha_{i2}$ interacts with (detected by co-immunoprecipitation), decreasing associations between MOPR and $G\alpha_{i2}$ but increasing associations between $G\alpha_{i2}$ and RGSZ2 (Rodriguez-Munoz *et al.*, 2007b). This shift is transient, and the time course mimics the duration of antinociceptive tolerance following acute administration of morphine, such that $G\alpha_{i2}$ interactions have returned to normal at time points when acute antinociceptive tolerance has waned (Rodriguez-Munoz *et al.*, 2007b). A similar process occurs with RGSZ2 and $G\alpha_z$, with MOPR agonists increasing association between these proteins while decreasing $G\alpha_z$/MOPR association (Garzon *et al.*, 2005).

RGSZ2 has also been identified as a linker between NMDA receptor signaling and MOPR which may contribute to the ability of the former receptor to attenuate antinociception produced by MOPR activation (Rodriguz-Munoz *et al.*, 2015). This regulation likely involves MOPR induced increases in nitric oxide causing enhanced NMDA receptor signaling in an RGSZ-2 dependent manner (Garzon *et al.*, 2011; Rodriguez-Munoz *et al.*, 2015).

RGS regulation of DOPR signaling and antinociception

Although R7 (RGS6/7/9-1/9-2/11) and RZ (RGSZ1/RGS19) family members are capable of regulating MOPR signaling, knockdown of these proteins does not affect the response to DOPR agonists DPDPE or deltorphin II (Garzon *et al.*, 2003; Garzon *et al.*, 2004). Without agonist treatment RGS19 is segregated from DOPR, with RGS19 found in clathrin-coated membrane

regions whereas DOPR is expressed near $G\alpha_{i3}$ in non-clathrin-coated regions (Elenko *et al.,* 2003). However following DOPR agonist treatment $G\alpha_{i3}$ and RGS19 colocalize in clathrin coated regions and form a complex (Elenko *et al.*, 2003). This is reminiscent of the process described above where MOPR agonist treatment shifts $G\alpha_{i2}$ and $G\alpha_z$ from a complex with MOPR to a complex with RGSZ-2 (Rodriguez-Munoz *et al.*, 2007b). When RGS19 interacts with $G\alpha_{i3}$ in clathrin-coated regions following DOPR agonist treatment, RGS19 promotes the inactivation of $G\alpha_{i3}$ at which point $G\alpha_{i3}$ translocates back to a non-clathrin coated region and reassociates with DOPR (Elenko *et al.*, 2003). This suggests that RGS19 can regulate DOPR signaling despite failing to modulate antinociceptive responses to DOPR agonists (Garzon *et al.,* 2004), or DOPR signaling in SH-SY5Y cells (Wang and Traynor 2013). In fact, RGS19 more effectively attenuated the inhibitory effect of the endogenous DOPR agonist peptide leu-enkephalin in NG-108 cell membranes than did RGS4 (Hepler *et al.*, 1997), suggesting that DOPR regulation by RGS19 may also differ between cell types.

In SH-SY5Y cells a 90% reduction of RGS4 significantly increased the ability of DOPR agonists to inhibit adenylyl cyclase and activate MAPK (Wang *et al.,* 2009) while in HEK293 cells RGS4 overexpression effectively reduced DOPR agonist stimulated signaling and increased the degree of DOPR internalization (Leontiadis *et al.,* 2009). This shows that despite being less effective than RGS19 at regulating DOPR signaling in NG-108 cell membranes (Hepler *et al.*, 1997) RGS4 is still capable of significantly modulating the action of DOPR agonists. Indeed, RGS4 knockout mice had enhanced antinociceptive and antihyperalgesic responses to the DOPR small molecule agonist SNC80 (Dripps *et al.,* 2017). SNC80 also more potently increases striatal MAPK phosphorylation in these animals, suggesting a potential downstream mechanism for the observed antinociceptive effects (Dripps *et al.,* 2017). Interestingly, the peripherally restricted DOPR antagonist N-methylnaltrexone blocked the antinociceptive effects of SNC80 on the acetic acid stretch assay but not its antihyperalgesic effects following nitroglycerin exposure (Dripps *et al.,* 2017) in the RGS4 knockout mice, suggesting that loss of RGS4 enhances DOPR control of both peripheral and centrally-mediated pain perception.

RGS regulation of KOPR and NOPR signaling

In contrast to MOPR and DOPR, very little is known about how RGS proteins affect KOPR and NOPR signaling. The genetic loci for RGS20 and KOPR are separated by only approximately

600 base pairs, suggesting that these proteins may be co-regulated (Sierra *et al.,* 2002). In Xenopus oocytes RGS4 expression inhibits GIRK1 and GIRK2 signaling downstream of KOPR activation, and the presence of RGS4 appears to counteract cellular adaptations to sustained KOPR agonist treatment (Ulens *et al.,* 2000). In PC12 cells stably expressing KOPR, agonist application increased RGS4 mRNA expression in a KOPR antagonist reversible manner, a process that may contribute to desensitization of KOPR agonist responses (Nakagawa *et al.,* 2001).

The genetic loci for NOPR and RGS19 neighbor each other, with RGS19 found only 83 base pairs from the 5' end of the gene encoding NOPR (Ito *et al.,* 2000; Xie *et al.,* 2003). This 83 base pair region functions as a bidirectional promoter for both genes (Ito *et al.,* 2000). Despite this close co-regulation RGS19 and NOPR expression show distinct differences depending on cell type, as RGS19 is found in both undifferentiated and differentiated NT2 cells, while NOPR is expressed only after differentiation (Ito *et al.,* 2000).

Use of RGS Insensitive G-Protein Variants to Study Opioid Signaling

A novel gain-of-function mutation originally identified in yeast Gα protein was found to mimic the effects of RGS knockdown (DiBello *et al.,* 1998). This mutation rendered Gα subunits totally insensitive to the negative regulatory effects of RGS proteins without otherwise affecting G-protein signaling (Lan *et al.,* 1998; Fu *et al.,* 2004), and produces similar effects in all $G\alpha_{i/o}$ proteins as well as $G\alpha_q$ (Kaur *et al.,* 2011). As these mutant RGS insensitive (RGSi) mutations eliminate the effects of all RGS proteins acting at a single Gα subunit there has been considerable interest in using these tools to probe opioid receptor signaling, both in vitro and in knock-in mouse models.

MOPR agonists such as morphine and DAMGO increase intracellular calcium, increase MAPK phosphorylation, and inhibit adenylyl cyclase downstream of $G\alpha_o$ in C6 cells expressing MOPR (Clark *et al.,* 2003). When C6 cells expressing an RGSi variant of $G\alpha_o$ are used only the MAPK and adenylyl cyclase response are enhanced with no change in agonist stimulated calcium levels (Clark *et al.,* 2003) suggesting that endogenous RGS proteins in these cells limit MOPR/$G\alpha_o$ dependent MAPK and adenylyl cyclase activity but not MOPR/$G\alpha_o$ dependent calcium levels.

Morphine's potency is also enhanced in melanophores expressing MOPR and RGSi $G\alpha_{i1}$ compared to cells expressing wild type $G\alpha_{i1}$ (Potenza *et al.,* 1999) and in C6 cells expressing RGSi $G\alpha_{i2}$ or $G\alpha_{i3}$ (Clark *et al.,* 2008). Together these data show that loss of RGS control at any of the four known $G\alpha_{i/o}$ proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_o$) can enhance the acute effects of MOPR agonists in vitro (Potenza *et al.,* 1999; Clark *et al.,* 2003; Clark *et al.,* 2008; Talbot *et al.,* 2010).

Morphine's potency and maximal effect are enhanced to a greater degree than DAMGO's in both C6 and SH-SY5Y cells expressing RGSi $G\alpha_o$ (Clark *et al.,* 2003; Wang *et al.,* 2009). In addition, the efficacy and potency of the MOPR weak partial agonist nalbuphine (Dykstra *et al.,* 1997) was greatly enhanced in cells expressing RGSi $G\alpha_{i2}$ or RGSi $G\alpha_{i3}$, while the higher efficacy agonist DAMGO only increased in potency slightly (Clark *et al.,* 2008). These findings show a consistent pattern where loss of RGS control affects partial agonists to a greater degree than higher efficacy agonists (Clark *et al.,* 2003; Clark *et al.,* 2008; Wang *et al.,* 2009).

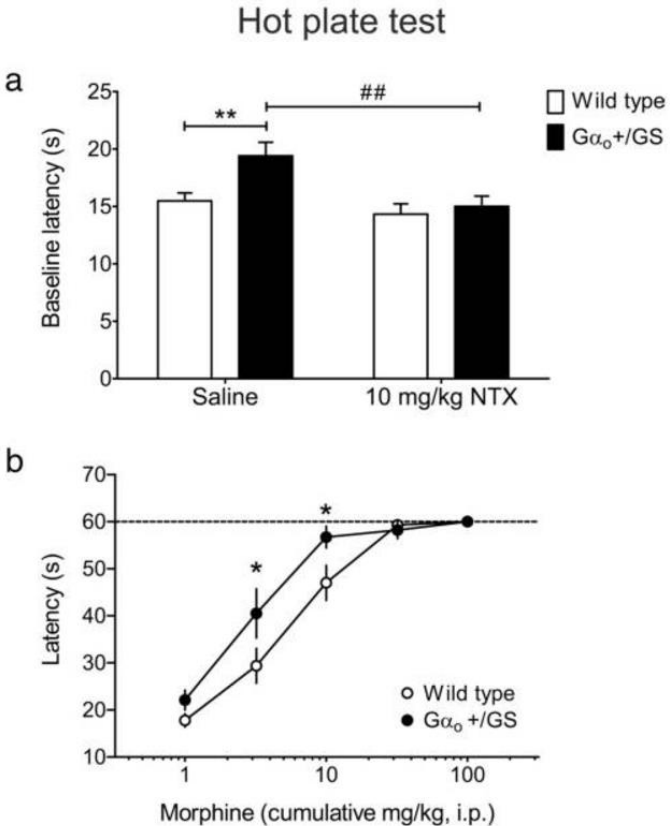

Fig. 3.1: (A) Heterozygous RGSi $G\alpha_o$ knock-in mice ($G\alpha_o$ +/GS) display a naltrexone reversible baseline antinociceptive phenotype on the hot plate test. (B) In heterozygous RGSi $G\alpha_o$ knock-in mice ($G\alpha_o$ +/GS) morphine has increased antinociceptive potency on the hot plate test.

Adapted with permission from Lamberts *et al.,* 2013

In C6 cells with endogenous G-protein expression the withdrawal of MOPR agonists after sustained treatment produces the expected supersensitization of adenylyl cyclase, and this supersensitization is dependent on intact $G\alpha_o$ expression (Clark *et al.,* 2004). In cells expressing RGSi $G\alpha_o$ the magnitude of supersensitization was further enhanced, suggesting that endogenous RGS proteins acting at $G\alpha_o$ normally limit this supersensitizing response (Clark *et al.,* 2004).

Mice expressing RGSi $G\alpha_o$ (Goldenstein *et al.,* 2009) displayed diminished

nociceptive responses on both the hot plate and tail withdrawal tests, a behavioral phenotype that is completely reversible by naltrexone pretreatment (see Figure 3.1A; Lamberts *et al.,* 2013). While the potency of the MOPR agonist morphine is enhanced on the hot plate (see Figure 3.1B), its potency is paradoxically decreased in the tail withdrawal test (Lamberts *et al.,* 2013) suggesting that the loss of RGS control may affect spinal antinociception (e.g. tail withdrawal test; Irwin *et al.,* 1951) differently than supraspinal antinociception (e.g. hot plate test; Heinricher and Morgan 1998). In periaqueductal gray slices GABA administration evokes inhibitory post-synaptic currents, and MOPR agonists inhibit these to a greater degree in mice expressing the RGSi $G\alpha_o$ protein (Lamberts *et al.,* 2013). This effect of MOPR agonists has been suggested to underlie the antinociceptive effects of these drugs (Moreau and Fields 1986; Reichling *et al.,* 1988) suggesting that this signaling change may contribute to the observed behavioral changes. Interestingly these mice also display increased itching behavior dependent both on the expression of KOPR and R7 binding protein (Pandey *et al.,* 2017) suggesting that this mutation may affect behaviors downstream of the other opioid receptors as well.

Conclusion

While an extensive amount of research has been conducted on the interaction between MOPR and certain RGS proteins (e.g. RGS4) for other RGS/opioid receptor pairings, there is still very little information. The effects of RGS proteins on NOPR and KOPR signaling in particular remains understudied, and the investigation of how RGS proteins affect DOPR signaling is just beginning to accelerate. As more small-molecule RGS inhibitors are discovered the rate of progress in this area will likely continue to increase, and the use of RGSi G-protein variants provides an exciting alternative to traditional knockout models.

Together the results summarized above suggest that RGS proteins are attractive targets that may allow more precise control of opioid analgesic effects, and RGS inhibiting molecules may even have stand-alone analgesic efficacy. Despite the considerable challenge of studying a 20+ member protein family with occasionally redundant function, these possibilities warrant further study in how RGS proteins control opioid receptor function.

The next chapter investigates how RGS proteins regulate the interaction between MOPR and NOPR using the RGSi $G\alpha_o$ knock-in mouse model. This will add to the sparse available literature on interactions between NOPR and RGS proteins and will extend previous work which identified a complex opioid-receptor-dependent behavioral phenotype relating to pain perception in mice with genetic alterations in $G\alpha_o$ (Lamberts *et al.*, 2011; Lamberts *et al.*, 2013).

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CHAPTER 4

Loss of RGS Control at $G\alpha_o$ Produces a Nociception/Orphanin FQ Receptor Dependent Hyperalgesia: Implications for a Balance Between Nociceptin Receptor and Mu-Opioid Receptor Systems

Abstract

Regulator of G-protein signaling (RGS) proteins bind to the active GTP-bound Gα subunit of heterotrimeric G-proteins to accelerate hydrolysis of GTP and limit signaling downstream of G-protein-coupled receptors (GPCRs). Studies have shown that mice expressing a modified $G\alpha_o$ protein that is insensitive to modulation by RGS proteins (RGSi $G\alpha_o$) have reduced sensitivity to heat stimuli due to enhanced signaling at the mu-opioid receptor (MOPR). In contrast, preliminary data suggest these mice have a hyperalgesic response to mechanical stimulation. The goal of the present study was to determine the mechanism behind this hypersensitivity to mechanical stimuli using the von Frey test. Behavioral testing was performed following injection of antagonists which we believed would affect von Frey responding, namely antagonists of the opioid receptors and the nociception/Orphanin FQ (N/OFQ) receptor (NOPR). The baseline hyperalgesic phenotype in the RGSi $G\alpha_o$ mice was reversed by pretreatment with systemic or central administration of the NOPR selective antagonist J-113397, while the opioid antagonist naltrexone further intensified the hyperalgesic phenotype. In contrast J-113397 pretreatment did not affect behavior on the 52C hot plate test in either wild type or RGSi $G\alpha_o$ mice. Intraplantar injection of λ-carrageenan produced an inflammatory hyperalgesia in the von Frey test which was also reversed by J-113397. Whole brain homogenates from RGSi $G\alpha_o$ knock-in mice had unchanged levels of NOPR as determined by [^{3}H]N/OFQ saturation binding and no change in the affinity of [^{3}H]N/OFQ for NOPR, while N/OFQ had unaltered potency to

activate G-protein as measured by [^{35}S]GTPγS uptake stimulation. Together, the results are consistent with increased signaling downstream of NOPR leading to a hyperalgesic phenotype in the RGSi $G\alpha_o$ knock-in mice, while increased signaling downstream of MOPR produces a protective effect against NOPR-mediated hyperalgesia. Similar opposing actions of the systems are seen in inflammatory hyperalgesia in wild-type mice, suggesting that in both cases the balance between MOPR and NOPR signaling is disturbed.

Introduction

Regulator of G-protein signaling (RGS) proteins comprise a family of approximately 30 members characterized by a RH domain or RGS box. 20+ members of the RGS family have GTPase accelerating protein (GAP) activity and promote inactivation of the heterotrimeric G-protein complex following G-protein-coupled receptor (GPCR) activation (Watson *et al.*, 1996). They do so by binding to the active GTP-bound Gα subunit and accelerating the conversion to an inactive GDP bound state (De Vries *et al.*, 1995; Berman *et al.*, 1996). This allows the Gα and βγ proteins making up the heterotrimer to reform, thereby negatively regulating signaling (De Vries *et al.*, 2000). Thus, alteration of RGS protein levels can change GPCR signaling *in vitro*. RGS proteins have some degree of selectivity for certain Gα subtypes (Talbot *et al.*, 2010), or even for specific GPCRs (Wang and Traynor 2013). In spite of this, different RGS protein subtypes likely have a considerable amount of functional redundancy. Therefore, attempts to understand RGS function by knocking out individual RGS proteins *in vivo* have often failed to uncover the expected phenotype (Doupnik 2015). However, a single base mutation of a conserved glycine (183 or 184) in Gα to serine prevents the RGS-Gα interaction (see figure 0.2; Tesmer *et al.*, 1997) and renders the Gα protein RGS-insensitive (RGSi). RGSi Gα-proteins are powerful tools to study the impact of RGS proteins *in vivo*, as these mutant proteins allow us to remove all endogenous RGS GAP activity at a particular G-protein and thus to investigate the impact of RGS proteins, even when the cognate RGS protein is not known

The mu opioid receptor (MOPR) is a member of the superfamily of 7-transmembrane domain GPCRs and as such is sensitive to the action of RGS proteins. Signaling downstream of MOPR is enhanced when coupled to RGSi $G\alpha_o$ proteins *in vitro* (Clark *et al.*, 2003, 2004 and 2005). In support of this mice with a RGSi $G\alpha_o$ knock-in have a small baseline antinociceptive response to

thermal stimuli due to enhanced signaling downstream of MOPR (Lamberts *et al.,* 2013). However, in contrast to these data with heat as the nociceptive stimulus, our preliminary studies suggested that RGSi $G\alpha_o$ knock-in mice are more sensitive to mechanical stimuli. We hypothesized this was due to an overactive pronociceptive system, enhanced by the RGS $G\alpha_o$ knock-in, that is working against the antinociceptive opioid system. Many other $G\alpha_o$-coupled receptors can regulate the perception of pain, and therefore may contribute to the observed phenotype in RGSi $G\alpha_o$ mice. Prime amongst these is the 17-amino acid nociceptin peptide (also known as Orphanin FQ or N/OFQ) which has been reported to have either pro or anti-nociceptive effects depending on specific testing conditions. The N/OFQ receptor (NOPR) is a $G\alpha_{i/o}$ coupled GPCR with high expression throughout the brain and spinal cord (Mogil and Pasternak 2001), and there are data suggesting that RGS proteins can affect NOPR signaling *in vitro* (Xie *et al.,* 2005; Wang and Traynor 2013). NOPR is classified as a fourth member of the opioid receptor family due to its close sequence homology with the classical opioid receptors (Mollereau *et al.,* 1994), although it is insensitive to the opioid antagonists naltrexone and naloxone. Administration of NOPR agonists directly to the brain consistently produces hyperalgesic effects in rodent models (Meunier *et al.,* 1995; Citterio *et al.,* 2000; Bytner *et al.,* 2001). Furthermore, NOPR antagonists reverse hyperalgesia induced by various methods including carrageenan injection, spinal nerve ligation, chronic constriction injury and single prolonged stress (Mabuchi *et al.,* 2003; Suyama *et al.,* 2003; Scoto *et al.,* 2009; Zhang *et al.,* 2012). Alongside these behavioral findings, a number of reports have suggested that expression of both NOPR and N/OFQ increase during periods of hyperalgesia (Gabriel *et al.,* 2004; Joseph *et al.,* 2007; Zhang *et al.,* 2012). The extended upregulation of NOPR and its endogenous peptide during periods of hyperalgesia coupled with the ability of NOPR antagonists to relieve these states further suggests this system may be involved in the maintenance of hyperalgesia.

A substantial body of data suggest that the NOPR and MOPR systems interact at a systems level, with NOPR activity in general providing anti-opioid effects (Bertorelli *et al.,* 1999; Khroyan *et al.,* 2009). Therefore, we decided to investigate how the RGSi $G\alpha_o$ knock-in protein affects the activity of NOPR and the interaction between the MOPR and NOPR systems *in vivo*. We hypothesized that these systems would counteract each other, such that increased NOPR activity in the RGSi $G\alpha_o$ knock-in mice would limit the antinociceptive potential of increased MOPR activity.

Materials and Methods

Transgenic mice. Heterozygous RGSi $G\alpha_o$ knock-in mice were generated as described previously (Fu *et al.,* 2004; Fu *et al.,* 2006; Huang *et al.,* 2006; Goldenstein *et al.,* 2009) then backcrossed for 6 generations onto a 129S1/SvImJ background. Animals for experimentation were obtained by breeding a heterozygous RGSi $G\alpha_o$ knock-in mouse with a wild type mouse from this same background. Heterozygous animals (+/GS) and wild types (WT) were obtained at the expected 50:50 Mendelian ratio using this method. All animals used in the described experiments were obtained using this breeding scheme, including experiments using only wild type animals. Animals homozygous for the RGSi $G\alpha_o$ knock-in protein typically die shortly after birth and therefore were not used in any of the experiments described in this report.

Heterozygous $G\alpha_o$ knock-out mice were generated as described previously (Mortensen *et al.,* 1992; Sowell *et al.,* 1997; Duan *et al.,* 2007) and backcrossed for at least 10 generations onto a C57BL/6 background. Animals for experimentation were obtained by breeding heterozygous $G\alpha_o$ knock-out mice with wild type mice from the same background. Heterozygous (+/-) and wild type (WT) animals were obtained at the expected 50:50 Mendelian ratio. Homozygous mice were not used as they are rarely produced and do not survive weaning.

Both male and female mice were used for the described experiments, and animals were typically between 8 and 16 wk of age at time of experiment and typically weighed between 18 and 25 g. Animals were group housed, segregated by sex, with no more than five animals per cage and had access to food and water ad libitum. Animals were maintained on a 12-hour light/dark cycle with lights turning on at 0700 each day. All testing occurred during the 12-hour light phase. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health and all experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Mechanical Sensitivity (von Frey test). Animals were placed in a custom-built apparatus consisting of a series of self-contained clear Plexiglas boxes with open tops and a wire mesh floor (9x18x18 cm). Animals were allowed to acclimate for at least 30 min before the start of testing. Calibrated von Frey filaments (North Coast Medical, Gilroy CA) with 0.16 g, 0.4 g, 0.6 g, 1.0 g, 1.4 g, 2.0 g, 4.0 g, and 6.0 g strengths were used for all experiments. The ascending method was used to determine withdrawal threshold as described by Chaplan *et al.,* 1994.

Briefly, each filament was applied with gradually increasing force perpendicular to the plantar surface of the hind paw until it bent, then was held in place for 2-3 s or until the animal withdrew its paw. Each filament was applied 3 times to each hind paw. Withdrawal threshold was defined as the first filament that caused the animal to withdraw its paw on at least 2 of the 3 applications of a particular filament. The 6.0 g strength filament was considered the maximal cutoff as this filament would lift the animal's paw before bending in most cases. A baseline measurement was obtained for each animal before experimental treatment. Measurements from both hind paws were averaged except in experiments involving intraplantar λ-carrageenan injections in which case each paw was analyzed separately.

Thermal Sensitivity. The hot plate test was used to measure reactivity to a thermal nociceptive stimulus. A hot plate analgesia meter (Columbus Instruments International Corporation, Columbus OH) was maintained at 52.0 C +/- 0.2 C. The mouse was gently placed within an open top clear Plexiglas enclosure on the surface of the hot-plate and the latency to lick forepaws, shake a hind paw or jump off the surface of the hot plate was recorded. Mice were removed from the hot plate immediately after responding. Mice that did not respond within 60 s were immediately removed from the surface of the hot plate to prevent tissue damage. Two baseline measurements separated by 30 min were obtained from each animal before experimental treatment. Experimental results were recorded 30 min after drug or vehicle treatment.

Injections. For experiments involving intraplantar (i.pl.) injections powdered λ-carrageenan was dissolved in a solution containing 10% ethanol, 10% alkamuls, and 80% sterile water to obtain a 2.5% λ-carrageenan solution. 20 μl of this solution was injected into the plantar surface of one hind paw, with administration to left and right paws counterbalanced between animals. A 30 G needle tip was used for injection. Injections were administered slowly over 10-20 s to prevent leakage from the injection site. Animals were returned to their home cage following λ-carrageenan administration and monitored daily for any signs of distress, then sacrificed immediately after the conclusion of the experiment.

Intraperitoneal (i.p.) injections were administered in a 10 ml/kg volume based on animal body weights obtained the same day. A 30 G needle tip was used for injection. Experimental testing typically occurred 30 min following i.p. injection unless otherwise indicated.

Prior to intracerebroventricular (i.c.v.) injections mice were anesthetized in a drop-jar containing 0.2 ml of isoflurane on a gauze pad. Mice were removed from the drop-jar when breathing slowed to approximately one breath per second. Depth of anesthesia was then confirmed by hind paw pinch. A 26 G needle tip was used for injection. i.c.v. injections were administered in a 5 μl volume over a period of two minutes. Needle tip was held in the i.c.v. space for 1 min following injection. Immediately following experiment animals were again anesthetized and a second i.c.v. injection containing Fast-Green dye was administered into the original i.c.v. injection site. Animals were then sacrificed by cervical dislocation followed by decapitation, and the brain was dissected. Animals in which dye was not present throughout the i.c.v. space were excluded from the study.

Membrane preparation. Mice were sacrificed by cervical dislocation followed by decapitation immediately before tissue preparation. The whole brain except for olfactory bulbs was removed and immediately placed in 3 ml of ice cold 50 mM tris buffer pH 7.4 then prepared as described previously (Lester and Traynor 2006). Membrane protein concentration was determined using a BCA assay kit (Thermo Scientific, Rockford, IL).

[^{3}H]N/OFQ saturation binding assay. Whole brain membrane homogenates (100 μg membrane protein per well) were incubated in a 50 mM pH 7.4 Tris buffer with various concentrations of tritiated N/OFQ peptide ([^{3}H]N/OFQ). Total assay volume was 200 μl in each well, and each condition was performed in duplicate. For each concentration of [^{3}H]N/OFQ non-specific binding was determined by addition of the NOPR antagonist J-113397 at 50 μM. Assay was allowed to incubate for 60 min at room temperature (25°C). Assay was then terminated by rapid filtration through a GF/C filter (Whatman) using a MLR-24 harvester (Brandel, Gaithersburg, MD) and bound radioactivity was measured by scintillation counting using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA) as described previously (Lamberts *et al.*, 2013).

N/OFQ stimulated [^{35}S]GTPγS binding. Whole brain membrane homogenates (100 μg membrane protein per well) were added to [^{35}S]GTPγS-binding buffer (50 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA, pH 7.4, with 2 mM dithiothreitol, 30 μM GDP, and 0.4 U/ml adenosine deaminase) with various concentrations of N/OFQ. 0.1 nM [^{35}S]GTPγS was then added to each well followed by a 60 min incubation at 25° C. Assay was then terminated by

rapid filtration through a GF/C filter and bound radioactivity was measured by liquid scintillation counting as described previously (Lamberts *et al.*, 2013). Total assay volume was 200 µl per well and each condition was performed in triplicate.

Statistical Analysis. Experiments involving two independent treatments, and experiments involving both a genetic and a treatment variable were analyzed using 2-way analysis of variance (2-way ANOVA) followed by Tukey's post-hoc test for multiple comparisons. The Ro64-6198 dose/response experiment was analyzed using 1-way analysis of variance (1-way ANOVA) followed by Holm-Sidak's multiple comparisons test. The i.c.v. J-113397 time course experiment was analyzed using 3-way analysis of variance (3-way ANOVA), with time, genotype and treatment factors. If statistics for a main effect, interaction or post-hoc comparison are not detailed in the results these effects did not reach significance. Experiments with only two conditions were analyzed using Student's t-test. $P<0.05$ was the predetermined threshold for significance in all experiments. For *ex vivo* binding experiments Bmax and Kd were determined using a one-site saturation binding curve (Hill slope = 1), while EC_{50} was determined using an agonist versus response curve (Hill slope = 1) as described previously (Lamberts *et al.*, 2013).

Results

Heterozygous RGSi $G\alpha_o$ knock-in mouse hyperalgesic behaviors

Heterozygous RGSi $G\alpha_o$ knock-in mice were used for this study because the full knock-in mice are not viable (Lamberts *et al.*, 2013). The heterozygous RGSi $G\alpha_o$ knock-in mice were shown to be significantly more sensitive to mechanical stimuli than their wild-type littermates as measured using von Frey filaments. (Fig. 4.1A). This baseline hyperalgesic behavior in the RGSi $G\alpha_o$ mice was fully reversed by the NOPR antagonist J-113397 (3.2 mg/kg i.p.) without affecting withdrawal threshold in wild-type littermates (Fig. 4.1B). Two-way ANOVA revealed significant main effects of treatment ($F_{(1,23)}=4.467$, $p=0.0456$), genotype ($F_{(1,23)}=18.23$, $p=0.0003$), and genotype x treatment interaction ($F_{(1,23)}=10.82$, $p=0.0032$). This same dose of J-113397 administered i.p. did not affect latency to respond on the hot plate test in heterozygous RGSi $G\alpha_o$ mice or their wild type littermates (Fig. 4.1C). Two-way ANOVA revealed significant main effects of genotype ($F_{(1,20)}=5.408$, $p=0.0307$), but not treatment ($F_{(1,20)}=0.4999$,

p=0.4877) or genotype x treatment interaction ($F_{(1,20)}$=0.4415, p=0.5140). In contrast, the opioid antagonist naltrexone administered i.p. to heterozygous RGSi $G\alpha_o$ knock-in mice further reduced their baseline hyperalgesic withdrawal threshold while this same treatment did not affect withdrawal threshold of wild-type littermates (Fig. 4.1D). Two-way ANOVA revealed significant main effects of genotype ($F_{(1,25)}$=41.62, p<0.0001), and treatment ($F_{(1,25)}$=6.922, p=0.0144).

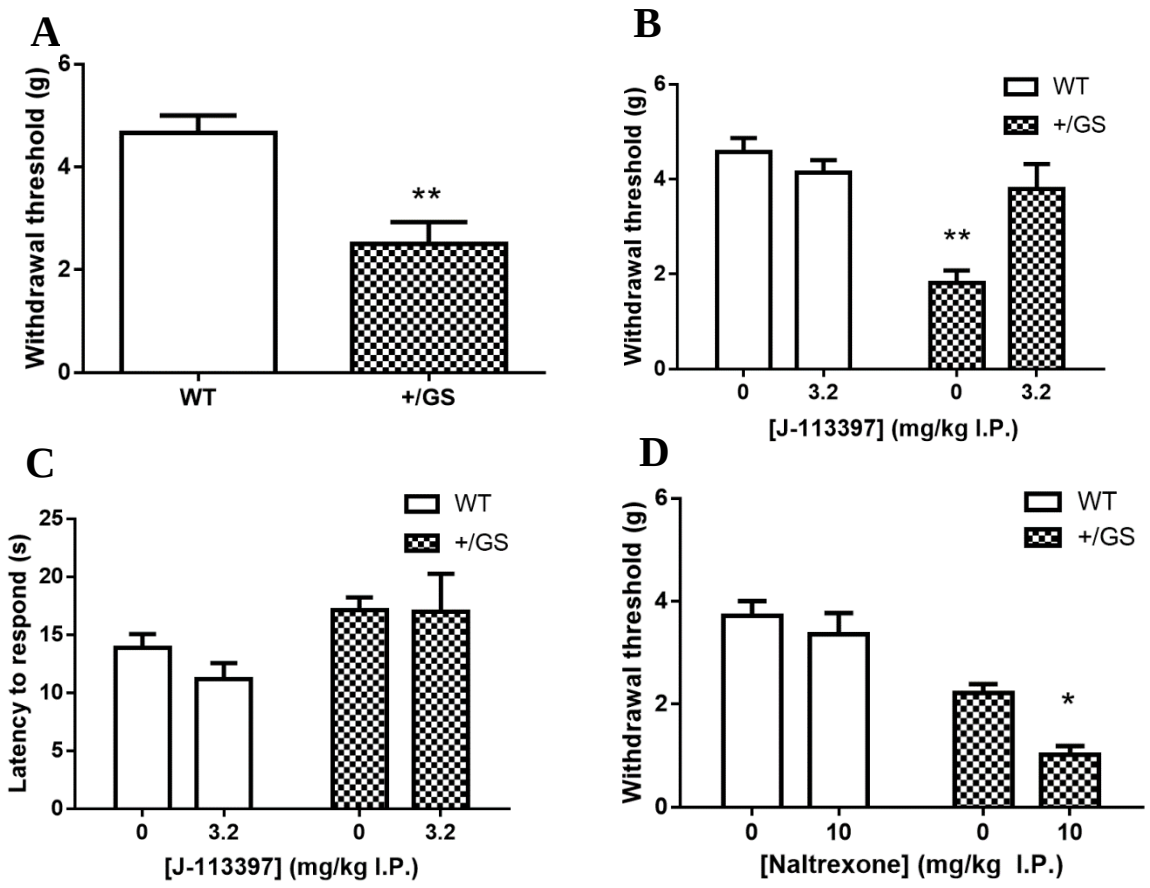

Fig. 4.1: (A) Baseline mechanical nociceptive threshold in heterozygous RGSi $G\alpha_o$ knock-in mice (+/GS) compared to wild type (WT) littermates (von Frey test; **P< .01, compared to wild type littermates; unpaired t-test). **(B)** Effect of NOPR antagonist J-113397 pretreatment on mechanical nociceptive threshold in heterozygous RGSi $G\alpha_o$ knock-in mice and wild type littermates (von Frey test; **P<0.01, compared to RGSi $G\alpha_o$ knock-in mice administered vehicle; significant main effects of genotype, treatment and interaction; 2-way ANOVA followed by Tukey's post-hoc test). **(C)** Effect of NOPR antagonist J-113397 pretreatment on latency to respond to a thermal nociceptive stimulus in heterozygous RGSi $G\alpha_o$ knock-in mice and wild type littermates (hot plate test; significant main effect of genotype; 2-way ANOVA followed by Tukey's post-hoc test). **(D)** Effect of naltrexone pretreatment on mechanical nociceptive threshold in heterozygous RGSi $G\alpha_o$ knock-in mice and wild type littermates (von Frey; *P<0.05, compared to RGSi $G\alpha_o$ knock-in mice administered saline; significant main effects of genotype and treatment; 2-way ANOVA followed by Tukey's post-hot test). Data are expressed as mean ± S.E.M. *n* = 6-8 mice per group.

To confirm a role for NOPR in the hyperalgesic behavior the NOPR partial agonist Ro64-6198 was administered systemically to wild type animals. This produced a bi-phasic pattern of behavior with reduced withdrawal threshold at 1.0 mg/kg but not at 3.2 mg/kg (Fig. 4.2A). One-way ANOVA revealed a significant main effect of treatment ($F_{(5,30)}$=4.867, p=0.0022). A reduction in spontaneous locomotion (or sedative effect) which may explain the biphasic nature of the response was observed following the 3.2 mg/kg dose. Although this locomotor effect was not quantified in our study, locomotor disruption at this dose of Ro64-6198 in mice is consistent with previously published results (Chang *et al.*, 2015). The hyperalgesic behavior caused by 1.0 mg/kg Ro64-6198 was fully reversed by 3.2 mg/kg of the NOPR antagonist J-113397 administered i.p. (Fig. 4.2B). This dose of J-113397 did not affect withdrawal threshold when administered to animals injected with Ro64-6198 vehicle. Two-way ANOVA revealed a significant main effect of J-113397 treatment ($F_{(1,20)}$=8.077, p=0.0101).

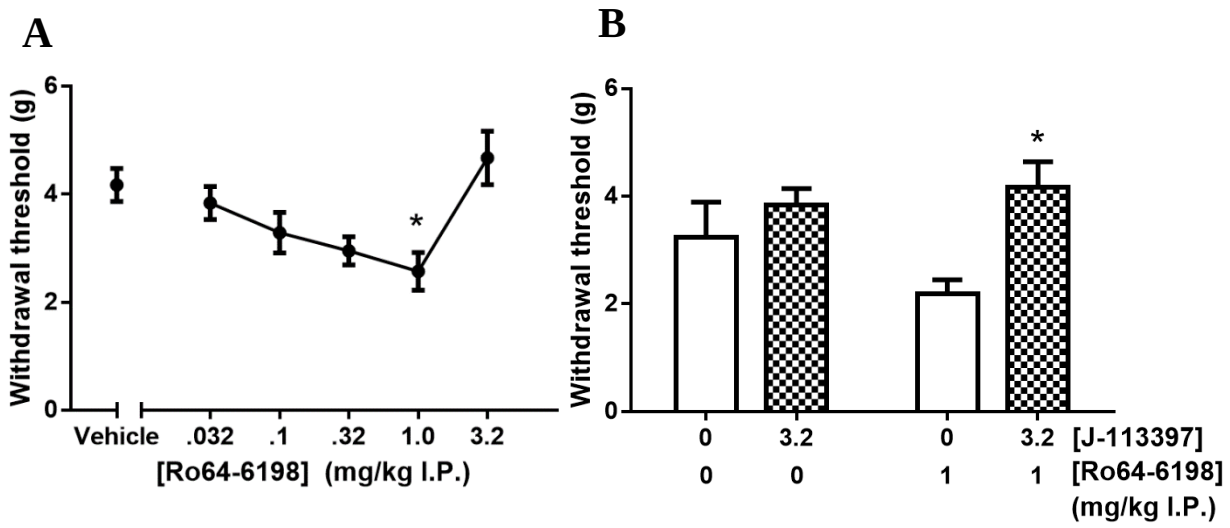

Fig. 4.2: (A) Effect of NOPR agonist Ro64-6198 on mechanical nociceptive threshold in wild type mice (von Frey test). Data are expressed as mean ± S.E.M. *n* = 6 mice for each dose. (*P<0.05, compared to vehicle treated animals; significant main effect of treatment; 1-way ANOVA followed by Holm-Sidak's post-hoc test). **(B)** Effect of NOPR antagonist J-113397 on mechanical hyperalgesia produced by NOPR agonist Ro64-6198 compared to animals administered vehicle. Data are expressed as mean ± S.E.M. *n* = 6 mice per group (*P<0.05, compared to animals administered Ro64-6198 plus vehicle (J-113397); significant main effect of J-113397 pre-treatment; 2-way ANOVA followed by Tukey's post-hoc test).

Finally, to examine if the hyperalgesic effect was dependent on central NOPR activity we administered 10 μg of the NOPR antagonist J-113397 intracerebrovetricularly (i.c.v.). This produced a reversal of the heterozygous RGSi $G\alpha_o$ baseline hyperalgesic phenotype that lasted for at least 60 min without affecting withdrawal threshold in wild type mice (Fig. 4.3). In the time course experiment (Fig. 4.3A) three-way ANOVA revealed significant main effects of time ($F_{(3,3)}$=22.95, p<0.0001), genotype ($F_{(1,3)}$=35.92, p<0.0001), and genotype x treatment interaction ($F_{(1,3)}$=5.141, p=0.0246). At the 30 min time point (Fig. 4.3B) two-way ANOVA revealed a significant main effect of treatment ($F_{(1,46)}$=5.132, p=0.0282).

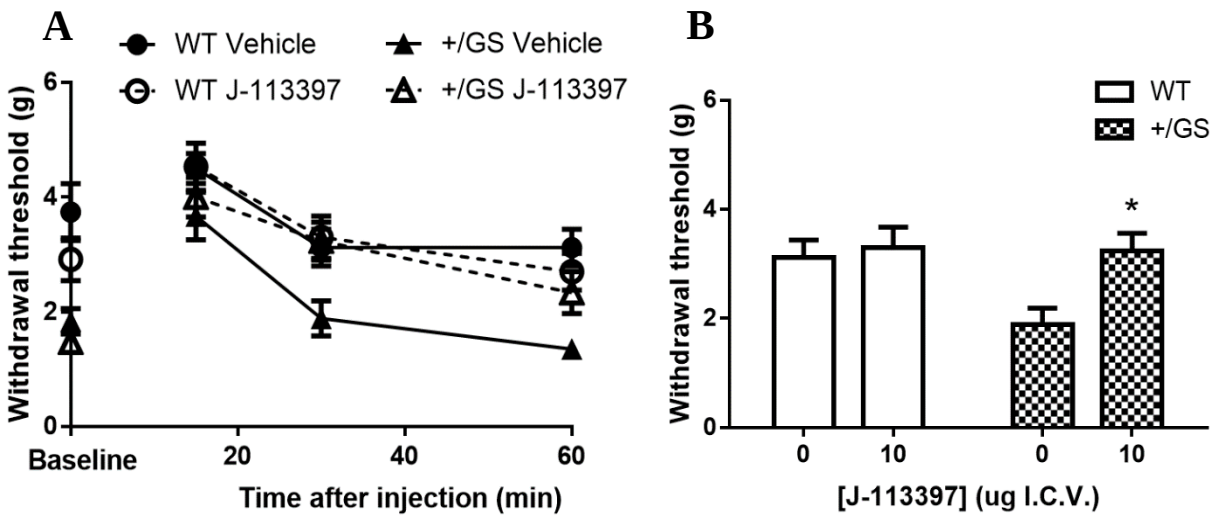

Fig. 4.3: **(A)** Effect of NOPR antagonist J-113397 delivered intracerebroventricularly (i.c.v.) on mechanical nociceptive threshold in heterozygous RGSi $G\alpha_o$ knock-in mice (+/GS) and wild type (WT) littermates (von Frey test; significant main effects of time, genotype, and genotype vs. treatment interaction; 3-way ANOVA). Data are expressed as mean ± S.E.M. *n* = 6-7 mice per group. **(B)** 30-minute timepoint from Figure 4.3A (von Frey test; *P<0.05, compared to +/GS animals administered vehicle; significant main effect of treatment; 2-way ANOVA followed by Tukey's post-hoc test). Data are expressed as mean ± S.E.M. *n* = 6-7 mice per group.

Hyperalgesia caused by intra-plantar λ-carrageenan injection is attenuated by systemic administration of a NOPR antagonist in both wild type and RGSi $G\alpha_o$ mice

λ-Carrageenan (20 μl of 2.5% solution) administered into the plantar surface of one hind paw 72 hr before testing produced a hyperalgesic effect in wild type mice. When these animals were pre-treated with the NOPR antagonist J-113397 at 3.2 mg/kg i.p. 30 min before testing they no longer displayed this hyperalgesic behavior (Fig. 4.4A). J-113397 did not affect withdrawal threshold in the contralateral hind paw of these animals (Fig. 4.4A). Two-way ANOVA revealed

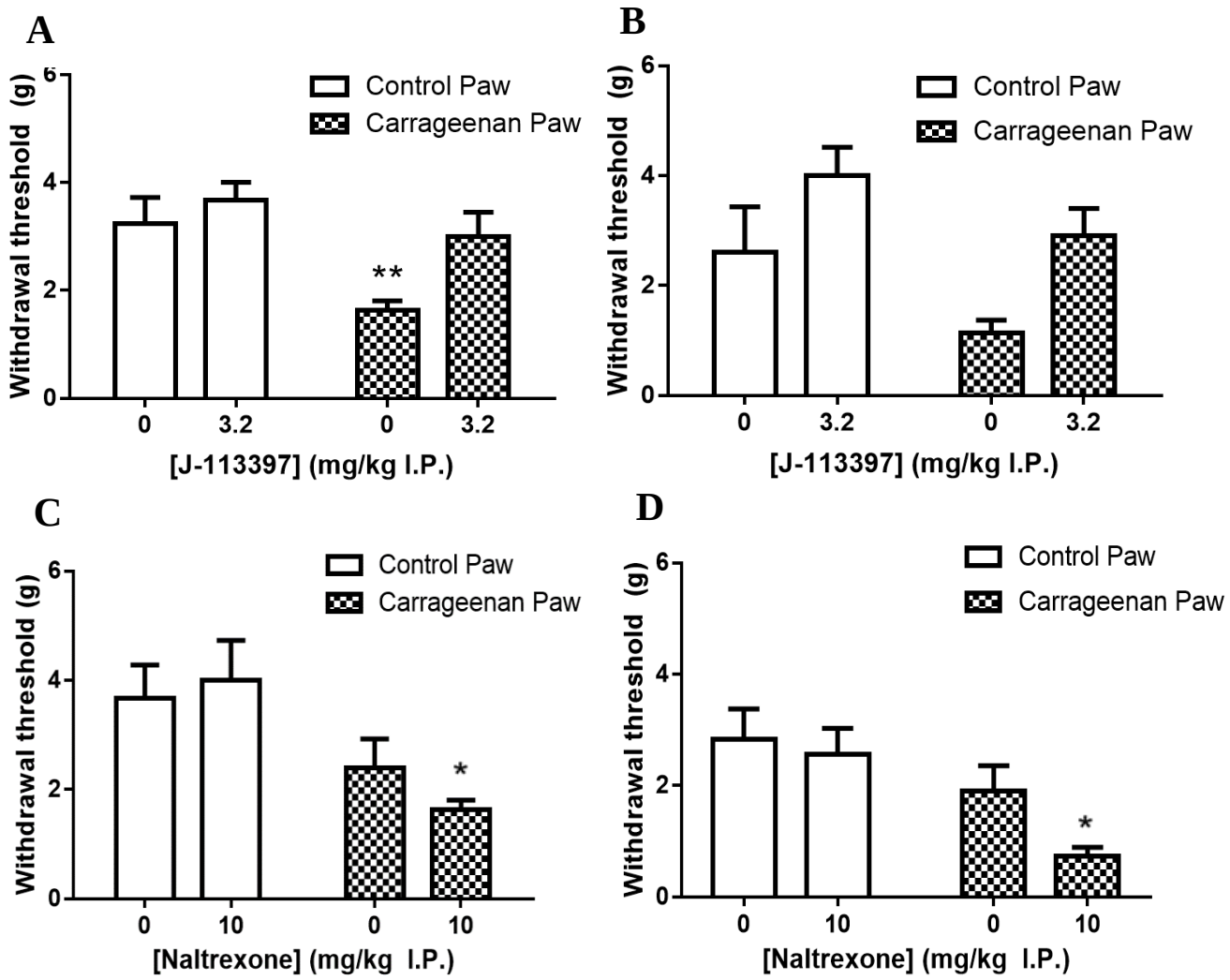

Fig 4.4: (A) Effect of NOPR antagonist J-113397 pretreatment on mechanical hyperalgesia produced by intraplantar administration of a 2.5% λ-carrageenan solution given 72 hours before testing in wild type mice (von Frey test; **P<0.01, compared to the control paw of vehicle treated animals; significant main effects of J-113397 and λ-carrageenan treatments; 2-way ANOVA followed by Tukey's post-hoc test). **(B)** Effect of NOPR antagonist J-113397 pretreatment on mechanical hyperalgesia produced by intraplantar administration of a 2.5% λ-carrageenan solution given 72 hours before testing in in heterozygous RGSi $G\alpha_o$ knock-in mice (von Frey test; significant main effects of J-113397 and λ-carrageenan treatments; 2-way ANOVA followed by Tukey's post-hoc test). **(C)** Effect of naltrexone pretreatment (30 min) on mechanical hyperalgesia produced by intraplantar administration of a 2.5% λ-carrageenan solution given 7 days before testing in wild type mice (von Frey test; *P<0.05, compared to the control paw of naltrexone treated animals; significant main effect of λ-carrageenan treatment; 2-way ANOVA followed by Tukey's post-hoc test). **(D)** Effect of naltrexone pretreatment (30 min) on mechanical hyperalgesia produced by intraplantar administration of a 2.5% λ-carrageenan solution given 7 days before testing in heterozygous RGSi $G\alpha_o$ mice (von Frey test; *P<0.05, compared to the control paw of naltrexone treated animals; significant main effect of λ-carrageenan treatment; 2-way ANOVA followed by Tukey's post-hoc test).

significant main effects of J-113397 treatment ($F_{(1,20)}$=5.561, p=0.0287), and carrageenan injection ($F_{(1,20)}$=8.818, p=0.0076). Heterozygous RGSi $G\alpha_o$ mice treated with λ-carrageenan and administered 3.2 mg/kg J-113397 showed a similar hyperalgesic response and reversal by J-113398, but post-hoc effects did not reach significance (Fig. 4.4B). Two-way ANOVA revealed significant main effects of J-113397 treatment ($F_{(1,20)}$=7.91, p=0.0108), and carrageenan injection ($F_{(1,20)}$=5.196, p=0.0337). Mice with λ-carrageenan injected paws were no longer significantly hyperalgesic 7 days after carrageenan administration. This was true for both wild type (Fig. 4.4C) and heterozygous RGSi $G\alpha_o$ mice (Fig. 4.4D). However, injection of naltrexone (10 mg/kg i.p.) at this time point causes the carrageenan injected paw to show a re-occurrence of increased sensitivity to von Frey filaments in both wild type (Fig. 4.4C) and heterozygous RGSi $G\alpha_o$ mice (Fig. 4.4D) without affecting sensitivity of the non-carrageenan injected paw. In wild type mice injected with naltrexone (Fig. 4.4C) two-way ANOVA revealed a significant main effect of carrageenan injection ($F_{(1,20)}$=6.843, p=0.0165). In RGSi $G\alpha_o$ mice injected with naltrexone (Fig. 4.4D) two-way ANOVA revealed a significant main effect of carrageenan injection ($F_{(1,20)}$=10.28, p=0.0044).

NOPR expression level and potency of N/OFQ to stimulate G-protein is unchanged in RGSi $G\alpha_o$ mice

MOPR receptor numbers and G-protein activation by MOPR agonist is not changed in brain homogenates from the RGSi $G\alpha_o$ mice compared to their wild-type littermates (Lamberts *et al.*, 2013). To determine if the integrity of the NOPR system was similarly maintained between the genotypes, we evaluated [^{3}H]N/OFQ binding and stimulation of [^{35}S]GTPγS binding in whole brain membrane homogenates prepared from wild type mice and heterozygous RGSi $G\alpha_o$ littermates (Fig. 4.5). There was no change in the Bmax (110 +/- 14 vs. 120 +/- 18 fmol/mG-protein) or affinity (0.58 +/- 0.19 v 0.77 +/- 0.27 nM) of N/OFQ or in the EC50 (2.8 +/- 1.6 vs. 1.6 +/- 0.69 nM) or maximal effect of N/OFQ to stimulate [^{35}S]GTPγS binding (Table 4.1).

Effect of the NOPR agonist Ro64-6198 in $G\alpha_o$ knockout animals

To confirm a role for $G\alpha_o$ in the actions of NOPR activation, we tested the activity of the NOPR agonist Ro64-6198 in mice with a 50% reduction in $G\alpha_o$ expression. Note that as stated previously the complete $G\alpha_o$ knockout animals are not viable (Lamberts *et al.*, 2013). These animals showed a similar hyperalgesic response to Ro64-6198 as their wild-type littermates (Fig.

4.6). Two-way ANOVA revealed a significant main effect of treatment ($F_{(1,20)}$=11.711, p=0.0027).

	WT	**+/GS**
[^{35}S]GTPγS stimulation		
EC50 (nM)	2.9 ± 1.6	1.6 ± 0.69
R^2	0.85	0.86
Saturation Binding		
Bmax (fmol/mg)	110 ± 14	120 ± 18
Kd (nM)	0.58 ± 0.19	0.77 ± 0.28
R^2	0.79	0.86

Table 4.1: [^{3}H]N/OFQ saturation binding and potency of [^{35}S]GTPγS uptake stimulation by N/OFQ**.**

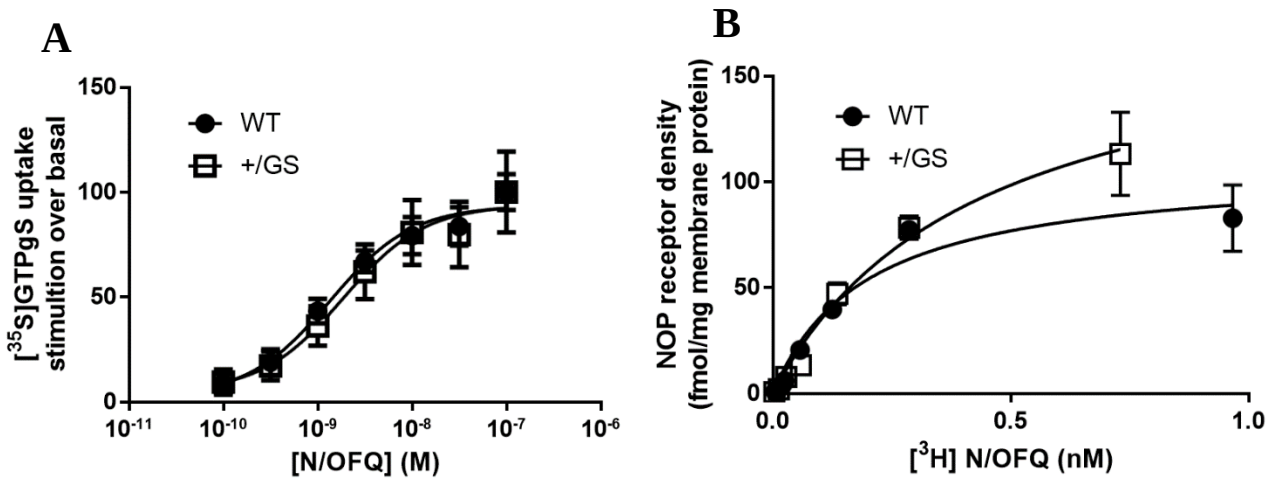

Fig 4.5: Stimulation of [^{35}S]GTPγS binding by N/OFQ in whole brain membrane homogenates from heterozygous RGSi $G\alpha_o$ knock-in mice (+/GS) and wild type (WT) littermates **(A)** and comparison of [^{3}H]-N/OFQ saturation binding in whole brain membrane homogenates from wild type (WT) mice and heterozygous RGSi $G\alpha_o$ knock-in (+/GS) littermates **(B)**. Non-linear fit of [N/OFQ] vs. [^{35}S]GTPγS uptake was used to determine the EC_{50} of N/OFQ potency (see Table 4.1). Non-linear fit of [N/OFQ] vs. specific N/OFQ binding was used to determine Bmax and Kd (see Table 4.1). Unpaired t-tests used to compare EC50, Kd and Bmax between RGSi $G\alpha_o$ knock-in and wild type animal's whole brain membranes, no significant differences. Data presented are representative experiments selected from *n* = 3 independent experiments performed in duplicate over the entire listed concentration range. Data are expressed as mean ± S.E.M for each concentration tested. Each experiment used membrane tissue prepared from different animals.

Discussion

The results described demonstrate that in a mouse model loss of RGS control at the heterotrimeric Gα protein $G\alpha_o$ produces a hyperalgesic behavioral phenotype in response to a mechanical stimulus. This contrasts with the MOPR-mediated antinociceptive behavioral phenotype in these mice as measured by the hot plate and tail withdrawal tests (Lamberts *et al*, 2013). The increased responding to mechanical nociceptors in the mutant mice was fully reversed by the NOPR antagonist J-113397 given systemically or i.c.v. suggesting that overactive signaling downstream of NOPR within the central nervous system is responsible for the observed behavior. Opioid receptor blockade by naloxone worsened the hyperalgesia. Carrageenan-induced mechanical hyperalgesia was also reversed by J-113397. These findings indicate that in wild type mice there is a fine balance between the pronociceptive activity of NOPR and the antinociceptive activity of the opioid systems that is altered by increasing $G\alpha_o$ signaling, via loss of RGS control, or by inflammation.

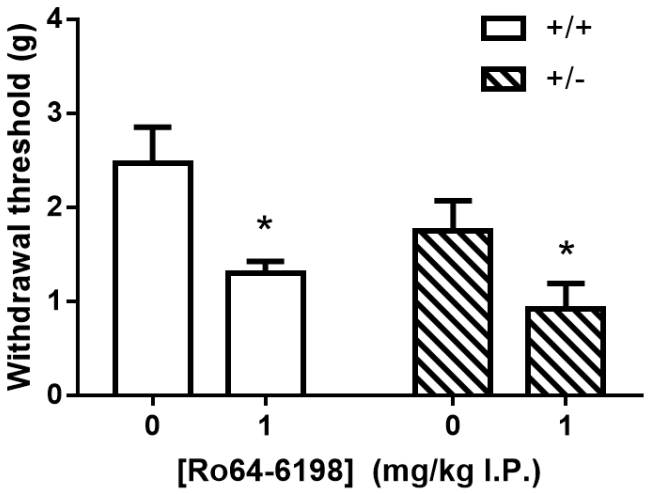

Fig 4.6: Effect of NOPR agonist Ro64-6198 on mechanical nociceptive threshold in heterozygous $G\alpha_o$ knock-out (+/-) mice and wild type (+/+) littermates (von Frey test; *P<0.05, compared to vehicle treated wild type animals. Significant main effect of Ro64; 2-way ANOVA followed by Tukey's post-hoc test). Data are expressed as mean ± S.E.M. *n* = 6 mice for each condition.

The finding that the NOPR antagonist J-113397 given i.c.v. reverses the behavioral phenotype indicates a central location as the source of the hyperalgesia in the RGSi $G\alpha_o$ knock-in mice. This is consistent with many published findings that supraspinal administration of a NOPR agonist produces pronociceptive effects (reviewed in Lambert, 2008; Schroder *et al.*, 2014; Kiguchi *et al.*, 2016) and that N/OFQ itself administered i.c.v. induces hyperalgesia (Zhu *et al.*, 1997). Moreover, i.c.v. administration of a NOPR antagonist peptide derivative (UFP-101) provides an antinociceptive action in the mouse formalin test (Rizzi *et al.*, 2006) and UFP-101 injected directly into the periaqueductal gray (PAG) reverses carrageenan-induced mechanical allodynia and chronic constriction tactile allodynia in rats (Scoto *et al.*, 2009). These data indicate that, as in the RGSi $G\alpha_o$ knock-in mouse model, the NOPR system is constitutively

active (Kiguchi *et al.,* 2016) and that the PAG is a potential locus of this activity. The PAG has high levels of NOPR (Neal *et al.,* 1999; Mollereau and Mouledous 2000) and N/OFQ (Houtani *et al.,* 1996). Moreover, in the PAG N/OFQ inhibits presynaptic GABA release onto a subset of PAG neurons but increases an inwardly rectifying K+ conductance (GIRK) in all neurons, thereby inhibiting descending PAG output (Vaughan *et al.,* 1997). Thus, the antinociceptive action of morphine given in the PAG, measured by the tail flick assay, is reversed by intra-PAG injection of N/OFQ (Morgan *et al.,* 1997).

In support of a role for increased NOPR signaling in the RGSi $G\alpha_o$ hyperalgesic phenotype we observed that peripheral administration of the brain-penetrant, NOPR selective agonist Ro64-6198 produced a hyperalgesic response in wild-type mice that was fully reversed by the NOPR antagonist J-113397. Similarly, hyperalgesia produced by i.pl. injection of λ-carrageenan was fully reversed by J-113397 in wild type mice. This NOPR antagonist significantly attenuated the more severe hyperalgesic response in RGSi $G\alpha_o$ knock-in mice given carrageenan, while the opioid antagonist naloxone tended to make the hyperalgesia more severe. These data are consistent with observations of increased circulating N/OFQ in various rodent models of hyperalgesia, suggesting that increased NOPR activity may be a general feature during hyperalgesic states (Joseph *et al.,* 2007; Zhang *et al.,* 2012).

On the other hand, these findings contrast with reports that peptidic NOPR agonists administered directly to peripheral sites can produce antinociception (Sakurada *et al.,* 2005) and that systemic non-peptide NOPR agonists attenuate models of inflammatory bowel disease (Sobczak *et al.,* 2014). Moreover, mice lacking NOPR or N/OFQ exhibit increased inflammatory hyperalgesia (Depner *et al.,* 2003). A considerable amount of data also show that spinal administration of NOPR agonists results in antinociception in acute pain models and antihyperalgesic and antiallodynic actions during chronic pain (for review see Kiguchi *et al.,* 2016). Data such as these have been used to suggest that NOPR agonists may have therapeutic potential for the treatment of inflammatory pain. However, our current findings and others support the notion that NOPR's effects on pain are highly variable (Lambert 2008; Schroder *et al.,* 2014; Kiguchi *et al.,* 2016) and depend on the pain modality, source of pain, whether there are compensatory changes in the NOPR system induced by the noxious insult, and if so where they occur.

The fact that loss of RGS regulation of $G\alpha_o$ enhances the signaling downstream of NOPR is evidence that $G\alpha_o$ plays a major role in NOPR and well as MOPR signaling. This is not surprising given the abundance of this Gα protein in the brain and its importance for transduction of signaling via many $G\alpha_o$-coupled GPCRs (Birnbaumer *et al.,* 2001). The behavioral phenotype we observed in the RGSi-$G\alpha_o$ mice occurred without changes in brain NOPR expression or the ability of N/OFQ to stimulate G-protein. Our previous results show that there is no significant difference in protein expression of $G\alpha_z$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\beta_{1\text{-}4}$, or $G\gamma_2$ in either the brain or spinal cord of RGSi $G\alpha_o$ knock-in mice compared to wild type controls, suggesting that the behavioral changes are due to increased signaling downstream of G-protein activation rather than compensatory changes in other G-proteins (Lamberts *et al.,* 2013). The mice do however have a slight reduction in $G\alpha_o$ protein expression in both the brain and the spinal cord (~25% reduction compared to wild type littermates), likely as a compensatory response to the increased signaling activity of the knock-in protein (Lamberts *et al.,* 2013). This may lead to an underestimate of the level of signaling enhancement caused by loss of RGS control, although the reduction of $G\alpha_o$ expression was not sufficient to reduce the maximal stimulation of G-protein activity by N/OFQ in brain tissue, suggesting that there is more than sufficient $G\alpha_o$ coupled to NOPR even in the heterozygotes. In support of this we found the NOPR agonist Ro64-6198 produced the same degree of hyperalgesia in mice with a 50% reduction in $G\alpha_o$ expression, showing that there is more than sufficient $G\alpha_o$ available for efficient NOPR signaling and that the minor reduction of $G\alpha_o$ in the RGSi $G\alpha_o$ mice is likely insignificant to the behavioral phenotype.

Our previous results indicating that removal of RGS control at $G\alpha_o$ may reduce sensitivity to painful stimuli in a MOPR dependent manner led us to speculate that RGS proteins might provide a target for analgesic drug development (Lamberts *et al,* 2013). Other evidence supports this, for example, intrathecal administration of an RGS4 inhibitor produces an antinociceptive effect and enhances the action of the MOPR ligand DAMGO in the formalin test in the mouse (Yoon *et al.,* 2015). However, this must now be tempered with the knowledge that in some situations the pronociceptive effects of increased NOPR signaling may predominate. Nonetheless, rather than a pan inhibition of all RGS protein activity as seen in the RGSi $G\alpha_o$ genetic model, our present and previous findings suggest it is feasible that selectively targeting specific members of the RGS protein family will produce useful analgesic effects. There are over 20 RGS proteins with GTPase accelerating (GAP) activity (De Vries *et al.,* 2000) and

several target $G\alpha_o$; however, there are reports that RGS proteins show selectivity for particular receptors (Ghavami *et al.*, 2003; Wang *et al.*, 2011; Xie and Palmer 2007). Most RGS proteins have not been thoroughly investigated for their ability to differentially regulate MOPR and NOPR signaling, although RGS19 has been reported to act at MOPR but not NOPR in SH-SY5Y cells (Wang and Traynor 2013) suggesting that RGS19 may be a suitable target. On the other hand, overexpression studies indicate RGS19 can act as a GAP for NOPR (Xie *et al.*, 2005). Due to these contradictory findings, more work is needed to determine suitable RGS proteins which regulate MOPR, but not NOPR signaling.

In conclusion, the finding that the RGSi $G\alpha_o$ mice show hyperalgesic behavior towards a mechanical stimulus is in stark contrast to the opioid receptor dependent antinociceptive phenotype seen in the hot-plate and tail withdrawal tests (Lamberts *et al.*, 2013). We saw no effect of the NOPR antagonist J-113397 on the baseline increased latency in the hot-plate test. However, we did observe that the hyperalgesia in the RGSi $G\alpha_o$ mice was prevented by J-113397 and made worse by the addition of the opioid antagonist naloxone, with similar observations during λ-carrageenan-mediated inflammation. These data show that the MOPR and NOPR systems are working in opposition, but that the pronociceptive action of NOPR signaling predominates in hyperalgesic states. This serves to highlight a fine homeostatic balance between the two systems that in some situations have opposing actions on pain perception.

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CHAPTER 5

Overall Discussion

The work described in this thesis investigates the role of the regulator of G-protein signaling (RGS) proteins in pain and depression, using RGS insensitive (RGSi) Gα proteins. Chapters 1 and 2 cover the role of RGS proteins in depression and depressive-like behaviors with a focus on the serotonin 1A receptor (5-HT1AR), while chapters 3 and 4 describe the involvement of RGS proteins in pain and antinociceptive effects with a focus on the interaction between the mu opioid receptor (MOPR) and nociceptin receptor (NOPR). Using mice expressing either RGSi $G\alpha_{i2}$ or RGSi $G\alpha_{o}$ this work highlights substantial differences between the intracellular signaling processes that affect antidepressant-like and hyperalgesic responses downstream of these receptors. Therefore, I have broken this discussion chapter into two sections, one summarizing the findings involving 5-HT1AR-mediated antidepressant-like effect, and the other on the interplay between MOPR and NOPR in hyperalgesic responses.

5-HT1AR-Mediated Antidepressant-Like Behaviors

Work described here shows that RGSi $G\alpha_{i2}$ knock-in mice have spontaneous antidepressant-like behavior that is reversible not only by systemic injection of a 5-HT1AR antagonist (WAY100-635; Talbot *et al.,* 2010) but also by intra-hippocampal injection of the same antagonist (Fig. 2.1). This 5-HT1AR dependent phenotype is not due to an upregulation of 5-HT1AR expression (Fig. 2.5A) nor changes in hippocampal G-protein expression (Fig. 2.5B). The 5-HT1AR dependent phenotype of these animals (i.e. increased antidepressant-like but not hypothermic effects of 5-HT1AR agonists; Talbot *et al.,* 2010) is mimicked by intra-hippocampal administration of a 5-HT1AR agonist (8-OH-DPAT), which produces antidepressant-like effects in a 5-HT1AR antagonist reversible manner (Fig. 2.2A), but not hypothermic effects (Fig. 2.2B).

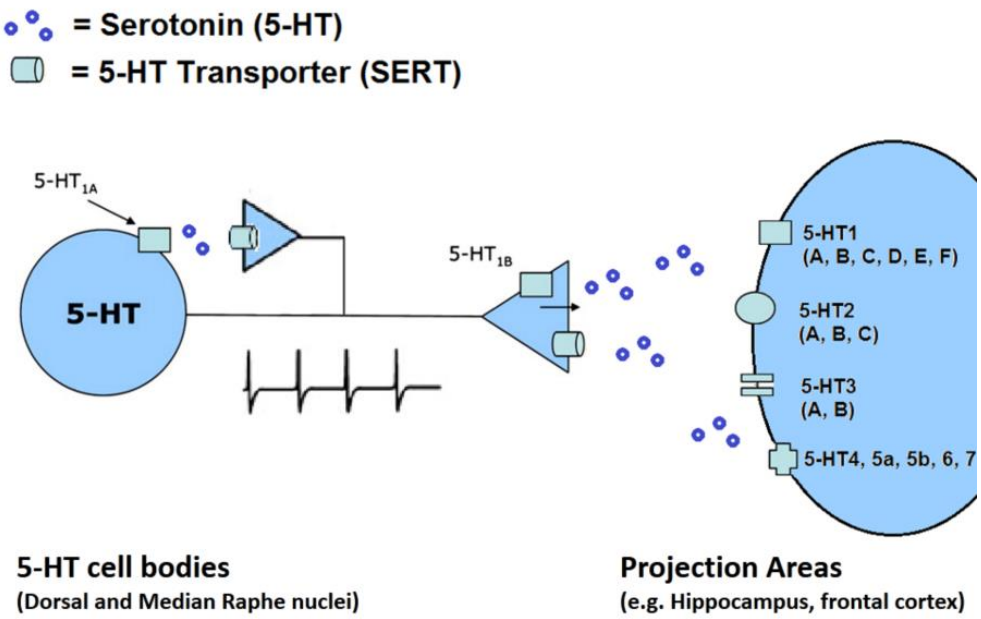

Fig. 5.1: Diagram of brain 5-HT network. Serotonergic cells originating in the dorsal and median raphe nuclei exhibit a slow constant firing rate that is inhibited by activation of the 5-HT autoreceptors (e.g. 5-HT1AR). Serotonergic axons project into areas such as the hippocampus where any of the 14 5-HT receptors may be activated by 5-HT released from these cells. Extracellular 5-HT is cleared primarily by the action of the 5-HT transporter (SERT).

Together these data show that hippocampal 5-HT1AR activity is necessary for the expression of antidepressant-like behavior in the RGSi $G\alpha_{i2}$ knock-in mouse in the tail suspension test, and that activating this same receptor population in wild type mice is sufficient to mimic this behavior.

The lack of hypothermic effects following intra-hippocampal 5-HT1AR agonist was unsurprising as 5-HT1AR-mediated hypothermia is known to depend on 5-HT1AR expressed on serotonergic neurons in the raphe nucleus (Goodwin *et al.*, 1987; Higgens *et al.*, 1988; Hillegaart 1991). This supports the hypothesis that the RGSi $G\alpha_{i2}$ knock-in mouse has enhanced activity downstream of post-synaptic 5-HT1AR (e.g. in the hippocampus) but not pre-synaptic 5-HT1AR in the raphe nuclei (Fig. 5.1) which would produce hypothermia as well as inhibit antidepressant-like effects due to reduced serotonin release. Based on these results we predict that other pre-synaptic 5-HT1AR-mediated effects (e.g. reduced extracellular 5-HT concentrations throughout the brain) would similarly be unaltered by the RGSi $G\alpha_{i2}$ mutation, although this has not yet been tested. It's possible that 5-HT1A autoreceptor responses have a relatively high receptor reserve and thus loss of RGS control is unable to further increase these responses, however the fact that i.p. 8-

OH-DPAT produced a dose dependent hypothermia in wild type mice (Fig 2.2B) and heterozygous littermates (Talbot *et al.*, 2010) suggests that this response can be enhanced further by increased receptor activation, but loss of RGS control at $G\alpha_{i2}$ fails to do so.

We also show that the 5-HT1AR agonist 8-OH-DPAT has a greater hyperpolarizing effect on CA1 hippocampal neurons from heterozygous RGSi $G\alpha_{i2}$ knock-in mice compared to wild type littermates (Fig. 2.3A-D). This involves a reduction in spontaneous action potentials and resting membrane potential, as well as an increase in the minimal current injection required to produce an action potential (rheobase) in neurons from heterozygous RGSi $G\alpha_{i2}$ knock-in mice after 8-OH-DPAT application (Fig. 2.3F). There was no significant change in these measures in wild type mice, although we expect higher doses of 8-OH-DPAT would be effective (Fig. 2.3E). The increased ability for 8-OH-DPAT to reduce resting membrane potential and spontaneous neuronal firing (as well as increase rheobase) in neurons from heterozygous RGSi $G\alpha_{i2}$ knock-in mice supports the hypothesis that hippocampal neurons from these mice are more sensitive to the effects of 5-HT1AR agonists. Going forward we propose a next step would be to record from raphe nuclei neurons to test the hypothesis that the RGSi $G\alpha_{i2}$ mutation preferentially affects post-synaptic 5-HT1AR compared to pre-synaptic receptors in the raphe.

After identifying the hippocampus as a relevant locus of action for 5-HT1AR-mediated antidepressant-like effects, we next attempted to determine relevant RGS proteins that could be inhibited in this region to produce antidepressant-like effects. We set out to inhibit RGS19 due to its known regulation of hippocampal 5-HT1AR in both *in vitro* and *ex vivo* models (Wang *et al.*, 2014), as well as relatively high hippocampal expression compared to any other brain region (Grafstein-Dunn *et al.*, 2001). As no highly selective RGS19 inhibitors are available we used the RGS4/19 inhibitor CCG-203769, which inhibits RGS4/19 with over 100x increased potency compared to other RGS proteins examined (Blazer *et al.*, 2015). Although acute intra-hippocampal injection of CCG-203769 did not produce antidepressant-like effects, repeated administration surprisingly produced antidepressant-like effects only in female animals (Fig 2.4A). This sex difference was not explained by compensatory changes in RGS19 protein expression (Fig 2.4C) or sensitivity to the antidepressant-like effects of 5-HT1AR agonist treatment (Fig 2.4B). A number of hypotheses for this sex difference have yet to be tested,

including that this inhibitor has more potent actions in female animals, or that different RGS proteins are involved in the antidepressant-like response in male mice.

While the tail suspension test experiments with CCG-203769 produced promising results suggesting that an RGS inhibitor could produce antidepressant-like effects through a hippocampal action, these studies did not conclusively identify the mechanism of action by which CCG-203769 produced these effects. Therefore, in the future we hope to characterize the effects of intra-hippocampal CCG-203769 in mice lacking either RGS4 or RGS19. To accomplish this RGS4 knockout mice can be used as the homozygous knockout animals are viable and overall healthy (Dripps *et al.,* 2017). Although RGS19 knockout mice have now been generated by Crispr Cas9 technology and are commercially available (https://www.jax.org/strain/030071), these animals have not been characterized and the homozygous knockouts may not be appropriate for behavioral experiments, either due to health issues or behavioral abnormalities (e.g. locomotor dysfunction) that would complicate interpretation of CCG-203769's effects. Considering that RGS4 knockout mice do not have a baseline antidepressant-like phenotype (Dripps *et al.*, 2017), and that RGS19 is known to regulate the effect of 5-HT1AR receptor agonists in hippocampal neurons (Wang *et al.*, 2014), we predict that CCG-203769 will be equally effective in RGS4 knockout mice as in wild type controls, while CCG-203769 will not produce antidepressant-like effects in RGS19 knockout mice (assuming these animals are appropriate for behavioral testing). It is possible that RGS19 knockout mice will display spontaneous antidepressant-like behavior without drug treatment, in which case we would not expect CCG-203769 treatment to produce further antidepressant-like effects.

If the RGS19 knockout mice have a very robust antidepressant-like phenotype at baseline, then it may not be possible to produce further antidepressant-like effects in these animals. In this case the basis of their antidepressant-like phenotype can instead be probed. We would expect an RGS19 knockout mouse antidepressant-like phenotype to be dependent on hippocampal 5-HT1AR receptor activity, such that either systemic or intra-hippocampal 5-HT1AR antagonist administration should reverse their antidepressant-like behavior, similar to the results obtained with RGSi $G\alpha_{i2}$ knock-in animals. Furthermore, we would predict that similar signaling changes would occur in the hippocampus of RGSi $G\alpha_{i2}$ knock-in mice and RGS19 (but not RGS4)

knockout mice, including increased phosphorylation of GSK3β (Talbot *et al.,* 2010) and an increased hyperpolarizing effect of 5-HT1AR agonists on hippocampal neurons.

In addition to working with the knockout mice, RGS4 and RGS19 can be knocked down in the hippocampus using intra-hippocampal delivery of lentivirus expressing shRNA against either RGS protein. While this strategy will produce a much more localized reduction of RGS protein this will provide valuable information on how these RGS proteins affect antidepressant-like behavior. We expect that hippocampal knockdown of RGS19, but not RGS4, would produce antidepressant-like behaviors. Furthermore, RGS4 knockdown would not affect the antidepressant-like behavior produced by intra-hippocampal CCG-203769. As lentiviral protein knockdown in brain tissues does not always remove 100% of the target protein, sufficient RGS19 protein may remain available for CCG-203769 to inhibit and produce additional antidepressant-like effects. In either case, we expect the effects of hippocampal RGS19 knockdown to depend on 5-HT1AR receptor activity, and to produce similar changes in signaling downstream of hippocampal 5-HT1AR receptors as observed in the RGSi $G\alpha_{i2}$ knock-in mice.

It is possible that neither knockdown of RGS4 or RGS19 will produce antidepressant-like effects. In this case other RGS proteins that CCG-203769 targets less potently (RGS7, RGS8; Blazer *et al.,* 2015) and are expressed at high levels in the hippocampus (Gold *et al.,* 1997; Grafstein-Dunn *et al.,* 2001) can be knocked down as well. If these RGS proteins all inhibit 5-HT1AR signaling in the hippocampus to some degree then only knocking down these proteins together would mimic the effects of CCG-203769 and the RGSi $G\alpha_{i2}$ phenotype, and would suggest a considerable redundancy exists between these RGS proteins for this response. In this case lentivirus delivering shRNA against all four of these RGS proteins can be used.

While we were surprised to observe a sex difference in the antidepressant-like effects of CCG-203769, and this difference was not explainable by differential RGS19 expression or sensitivity to 5-HT1AR agonists, we hope to explore the basis of this sex difference further. One possibility is that female mice are more sensitive to the antidepressant-like effects of CCG-203769 than male mice, and that higher doses would produce antidepressant-like effects in male mice as well. Unfortunately, intra-hippocampal administration of CCG-203769 above 3 μg produced catatonia in both male and female animals, precluding accurate behavioral testing. It is possible that this

catatonia occurs due to off target effects, and that other RGS inhibitors would not produce such side effects. In this case other RGS inhibitors tested at a wider dose range could identify potency differences between male and female animals. The use of newer RGS inhibitors would also help to validate the results obtained with CCG-203769, and may aid in narrowing down the relevant mechanism of action. For example, if intra-hippocampal delivery of any RGS inhibitor with activity at RGS19 produces antidepressant-like effects in female animals, while inhibitors lacking RGS19 activity did not, then this would bolster the evidence that hippocampal RGS19 plays a critical role in this behavior.

Rather than a drug specific sensitivity difference, female mice may be more sensitive to the effects of hippocampal RGS protein knockdown in general. In this case intra-hippocampal lentiviral delivery of shRNA against the RGS proteins both expressed in the hippocampus and targeted by CCG-203769 (RGS4, RGS7, RGS8, RGS19) may also produce differential effects between male and female animals. This would suggest that there is enhanced downstream signaling effects (e.g. increased MAPK or GSK3β phosphorylation) in the hippocampus of female animals compared to male animals following RGS inhibition. A general increased sensitivity to RGS inhibition in female animals would be surprising however, as no sex differences were reported in the phenotype of RGSi $G\alpha_{i2}$ knock-in mice (Talbot *et al.*, 2010), although the possibility remains that CCG-203769 is affecting behavior through signaling pathways that do not depend on $G\alpha_{i2}$. It is also possible that the estrous cycle plays a role in the observed sex difference, and if so sensitivity to the antidepressant-like effects of CCG-203769 may be altered in animals at different stages of the cycle.

Finally, we compared the antidepressant-like phenotype of the RGSi $G\alpha_{i2}$ knock-in mice to the behavior of RGSi $G\alpha_{o}$ knock-in mice. Heterozygous RGSi $G\alpha_{o}$ knock-in mice also display antidepressant-like behavior, however in contrast to RGSi $G\alpha_{i2}$ knock-in mice this behavior is not reversed by 5-HT1AR antagonist pretreatment (Fig. 2.6). This suggests that while loss of RGS control at either G-protein produces antidepressant-like effects, it does so through distinct mechanisms. Although the receptors involved in the RGSi $G\alpha_{o}$ antidepressant-like phenotype have not been thoroughly investigated at this time, these mice are known to have an enhanced antidepressant-like response to the DOPR agonist SNC80 on the tail suspension test (personal communication with Jutkiewicz lab), and more generally DOPR activation is known to produce

antidepressant-like effects in wild type animals (Jutkiewicz *et al.,* 2005; Dripps *et al.,* 2017). Therefore, we hypothesize that increased DOPR activity in RGSi $G\alpha_o$ knock-in mice produces an antidepressant-like phenotype such that DOPR antagonist pretreatment should reverse this behavior.

Opioid Receptor-Mediated Hyperalgesic Behaviors

Based on a previous report showing that heterozygous RGSi $G\alpha_o$ knock-in mice have a complex phenotype related to pain perception, including increased sensitivity to opioid analgesics on the hot plate test and decreased sensitivity on the tail withdrawal test (Lamberts *et al.,* 2013), we set out to determine the behavior of these animals in response to a mechanical nociceptive stimulus as measured using von Frey filaments. Based on their baseline antinociceptive behavior in both the hot plate and tail withdrawal tests we hypothesized that these animals would have a baseline antinociceptive response on the von Frey test as well. Heterozygous RGSi $G\alpha_o$ knock-in mice surprisingly had a baseline hyperalgesic response on the von Frey test (Fig. 4.1A) that was reversed by pretreatment with the NOPR antagonist J-113397 (Fig. 4.1B). As all previous results suggest that these animals have increased activity of opioid receptor systems, we next set out to test the effects of the opioid antagonist naltrexone on the observed hyperalgesic behavior. Naltrexone increased the hyperalgesic behavior in the RGSi $G\alpha_o$ knock-in mice even further without affecting wild type behavior (Fig 4.1D). We also show that the blood brain barrier permeable NOPR partial agonist Ro64-6198 (Dautzenberg *et al.,* 2001) administered systemically produces a hyperalgesic response in wild type mice on the von Frey test (Fig. 4.2A-B). This suggests that increased activity downstream of NOPR in RGSi $G\alpha_o$ knock-in mice counteracts the antinociceptive effects of opioid receptor activity in these animals. This behavioral phenotype was not due to increased NOPR expression in the brain, nor an increased ability of the endogenous peptide ligand for NOPR (N/OFQ) to stimulate G-protein (Fig. 4.5A-B) suggesting that signaling changes downstream of $G\alpha_o$ due to the loss of RGS control drive this behavior.

In addition, we show that i.c.v. administration of the NOPR antagonist J-113397 also reverses the RGSi $G\alpha_o$ knock-in mouse hyperalgesia (Fig 4.3A and B), suggesting a central locus for this phenotype. This is consistent with a series of studies showing that NOPR agonists have pro-

nociceptive and anti-opioid activity when delivered i.c.v. (Mogil *et al.*, 1996a; Mogil *et al.*, 1996b) or directly into the periaqueductal gray (PAG) (Morgan *et al.*, 1997). We therefore hypothesize that increased sensitivity to endogenous nociceptin peptide in the PAG of RGSi $G\alpha_o$ mice produces a pro-nociceptive response that should be reversible by specific delivery of a NOPR antagonist to the PAG. This should be examined in future studies.

As the RGSi $G\alpha_o$ knock-in mice had an opioid antagonist reversible antinociceptive phenotype on the hot plate test (Lamberts *et al.*, 2013), and opioid receptor and NOPR activity appear to produce opposing effects on the von Frey test, we set out to determine whether a NOPR antagonist would have a similar pro-nociceptive effect on the hot plate test in these animals. The NOPR antagonist J-113397 did not affect hot plate responding in either wild type or RGSi $G\alpha_o$ knock-in mice (Fig 4.1C), consistent with published results showing this antagonist does not affect behavior on the hot plate test in wild type mice (Ozaki *et al.*, 2000). This suggests that while the opioid receptors and NOPR have a push/pull effect on pain threshold in response to a mechanical stimulus (von Frey test) leading to an overall hyperalgesic phenotype in RGSi $G\alpha_o$ knock-in mice, in response to a thermal stimulus (hot plate test) only the antinociceptive action of opioid receptor activity appears to contribute to the phenotype. We expect that NOPR antagonist treatment will similarly fail to affect RGSi $G\alpha_o$ knock-in mouse behavior on the tail withdrawal test as well, and preliminary results support this hypothesis (Fig. 5.2).

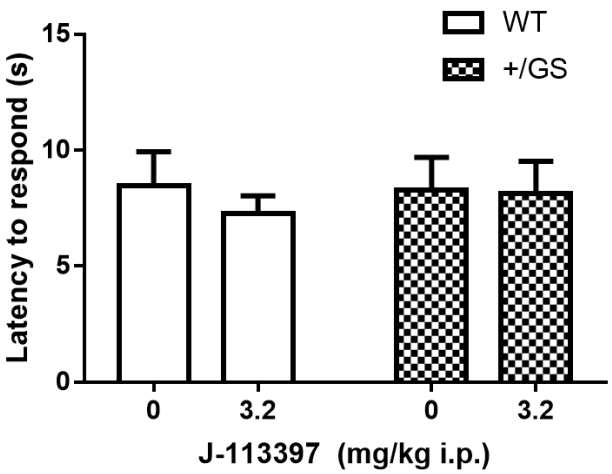

Fig. 5.2: Pilot 50C warm water tail withdrawal test results in wild type (WT) and heterozygous RGSi $G\alpha_o$ knock-in mice (+/GS) treated with NOPR antagonist J-113397 (n = 3-4).

We next sought to determine whether this push/pull phenomenon between the NOPR and opioid systems observed in the RGSi $G\alpha_o$ knock-in mouse model also occurs during a model of inflammatory hyperalgesia. Intraplantar λ-carrageenan injection produced a hyperalgesic response on the von Frey test that was alleviated by NOPR antagonist pretreatment 72 hr after carrageenan injection (Fig. 4.4A and B). In a pilot experiment naltrexone administered 72 hr after carrageenan administration did not make

hyperalgesia significantly worse (data not shown). Seven days following carrageenan administration mice were no longer significantly hyperalgesic, however naltrexone administration caused the carrageenan injected paw to again become hyperalgesic (Fig. 4.4C and D). Combined with the lack of effect of these antagonists on healthy wild type mice shown earlier (Fig. 4.1B and D) this suggests that both NOPR and opioid receptor activity increases during inflammatory states, either due to constitutive activity (Corder *et al.,* 2013), or increased opioid peptide release, and that this increased (non-NOPR) opioid receptor activity contributes to the apparent recovery of hyperalgesia at later time points.

Naltrexone and naloxone have shown the ability to reverse recovery from hyperalgesic states in other models as well, including human post-operative hyperalgesia (Koppert *et al.,* 2003), human electrically induced hyperalgesia (Koppert *et al.,* 2005), hyperalgesia after plantar incision in mice (Campillo *et al.,* 2011) and mouse complete Freund's adjuvant (CFA) induced inflammatory hyperalgesia (Corder *et al.,* 2013). The finding that MOPR expression increases in the dorsal root ganglia (DRG) during inflammatory injury (Ji *et al.,* 1995; Endres-Becker *et al.,* 2007) may provide an explanation for the ability of opioid antagonists to reverse recovery from hyperalgesia, as opioid antagonist application would counteract the antihyperalgesic effects of MOPR upregulation.

Prepronociceptin (the precursor peptide to the endogenous peptide ligand for NOPR) mRNA also increases in a subpopulation of neurons in the DRG following intraplantar carrageenan administration (Andoh *et al.,* 1997; Itoh *et al.,* 2001). Interestingly the increase in prepronociceptin occurred as soon as 30 minutes after carrageenan injury (Andoh *et al.,* 1997) while MOPR expression changes did not reach a maximal level until 3 days after injury (Ji *et al.,* 1995). These results suggest that endogenous ligands acting at MOPR and NOPR in the DRG may be driving the changes in nociceptive threshold observed following carrageenan injury. It is possible that increased activity downstream of MOPR and NOPR in the DRG also occurs in the RGSi $G\alpha_o$ knock-in mice, contributing to a phenotype that resembles carrageenan injury on the von Frey test. Although our results showing that i.c.v. administration of a NOPR antagonist reverses hyperalgesia in RGSi $G\alpha_o$ knock-in mice (Fig. 4.3A) suggest that NOPR activity in the brain is required for the expression of this behavior, it is possible that a pathway involving both spinal and brain NOPR contributes to this behavior, such that antagonism of NOPR at either site

could alleviate their hyperalgesia. We hope to test this hypothesis by administering NOPR and MOPR antagonists i.t. and comparing their effects to systemic administration of the same antagonists, both in inflammatory hyperalgesia models and in the RGSi $G\alpha_o$ knock-in mice.

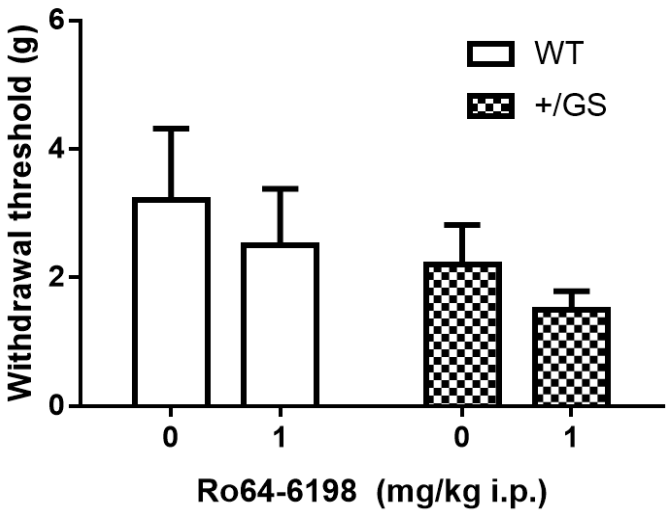

Fig. 5.3: Pilot von Frey test results in wild type (WT) and heterozygous RGSi $G\alpha_{i2}$ knock-in mice (+/GS) treated with NOPR agonist Ro64-6198 (n=2).

We also wanted to determine a role for other Gα proteins in the hyperalgesic activity. RGSi $G\alpha_{i2}$ knock-in mice were not significantly hyperalgesic compared to wild type littermates in a pilot experiment (Fig. 5.3) although there was a slight trend towards hyperalgesia. This suggests that removing RGS control downstream of $G\alpha_o$ promotes hyperalgesia to a greater degree than removing RGS control downstream of $G\alpha_{i2}$, and that $G\alpha_o$ is the primary G-protein involved in this NOPR dependent behavior. This hypothesis should be tested more fully in the future. If a full study shows that RGSi $G\alpha_{i2}$ knock-in mice are significantly hyperalgesic antagonist studies will provide information on the receptor systems involved in this behavior.

While behavioral results on the von Frey test suggest that RGSi $G\alpha_o$ knock-in mice have enhanced activity of the NOPR system, this enhancement has not been conclusively demonstrated at a signaling level. In a pilot experiment the NOPR agonist Ro64-6198 was administered i.p. to wild type and heterozygous RGSi $G\alpha_o$ knock-in mice followed 15 minutes later by brain dissection and processing of whole brain tissue for Western blot analysis. Whole brain tissue was analyzed for preliminary studies as relevant brain regions have not yet been determined for this behavior. The phosphorylation state of MAPK was analyzed as NOPR expressing cells exhibit increased MAPK phosphorylation following NOPR agonist treatment *in vitro* (Zhang 2012), and stimulation of MAPK activity by $G\alpha_{i/o}$ proteins is well characterized (see Belcheva and Coscia 2002 for review). Unexpectedly, vehicle treated RGSi $G\alpha_o$ knock-in mice appeared to have reduced MAPK activity, and Ro64-6198 further reduced activity in RGSi

$G\alpha_o$ knock-in mice but not wild type littermates (Fig. 5.4), although the Ro-64-6198 effect did not reach significance. Two-way ANOVA revealed a significant main effect of genotype ($F_{(1,8)}$=6.845, p=0.0308). In the future, it should be determined whether the decreased MAPK activity in the brains of heterozygous RGSi $G\alpha_o$ knock-in mice is consistent enough to reach significance with repeated experimentation, and if so whether that decrease is reversible by NOPR antagonist pretreatment. This would suggest that the observed decrease in MAPK activity is in fact due to increased baseline NOPR activity in these animals, while a failure to reverse this difference with a NOPR antagonist would indicate that one of the many other GPCRs signaling through $G\alpha_o$ may be reducing MAPK activity instead.

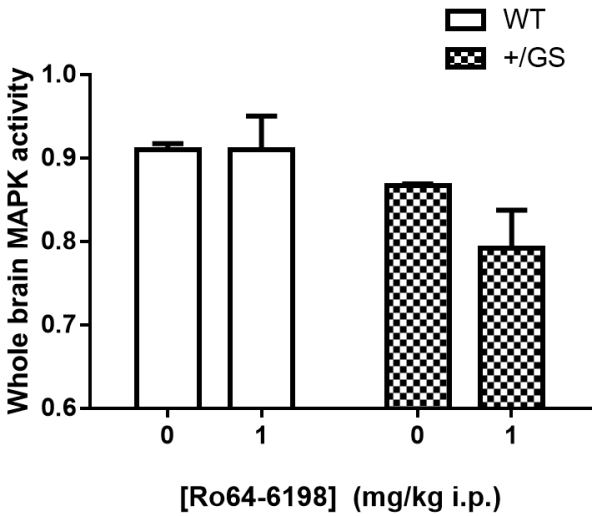

Fig. 5.4: MAPK activity (quantified as ratio of pERK/total ERK) in whole brain homogenates from heterozygous RGSi $G\alpha_o$ knock-in mice (+/GS) and wild type (WT) littermates administered Ro64-6198 or vehicle i.p. (Significant main effect of genotype, no significant post-hoc effects; 2-way ANOVA followed by Tukey's post-hoc test; n=3).

While the decrease in MAPK activity was unexpected, this may reflect a network effect whereby increased NOPR action on some neurons does produce increased MAPK activity in those cells (as predicted by *in vitro* models), but due to downstream effects on other neurons the net effect is a reduction in MAPK activity. A similar process has been observed in the hippocampus of animals treated systemically with the 5-HT1AR agonist 8-OH-DPAT, where a decrease in MAPK activity is observed instead of the expected increase due to network effects downstream of the direct site of action (Chen *et al.*, 2002). If a similar process is occurring in the brains of RGSi $G\alpha_o$ knock-in mice following NOPR agonist treatment then we might expect to see regional differences in MAPK activity following agonist administration, similar to the differential results across brain regions with 5-HT1AR agonists (Chen *et al.*, 2002). Direct versus indirect effects of NOPR agonists on central MAPK activity across brain regions could then be distinguished by comparing the effects of *ex vivo* NOPR agonist application to primary neurons or brain slices from various regions with high NOPR expression and relevance to antinociceptive action such as the periaqueductal gray (PAG) or

striatum (Calo' *et al.*, 2000). A more detailed level of signaling information could prove useful for determining the brain regions involved in the RGSi $G\alpha_o$ behavioral phenotype.

Once relevant brain regions are identified NOPR/opioid antagonist microinjections can be performed in those regions to determine how more specific regional receptor blockade affects the behavior of these animals. Furthermore, the relevant RGS proteins expressed in each region can be identified, with the aim of inhibiting/knocking down RGS proteins that would normally inhibit $G\alpha_o$. This would allow testing of the hypothesis that specific RGS protein knockdown/inhibition in relevant brain regions can mimic the RGSi $G\alpha_o$ behavioral phenotype, providing novel information on how RGS proteins control pain perception.

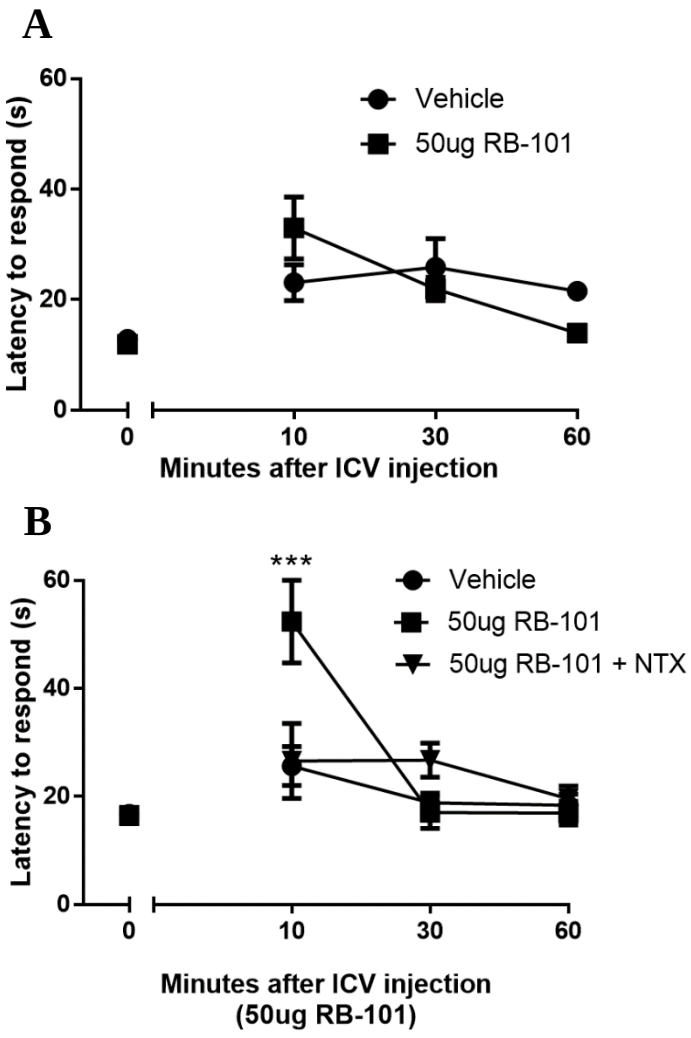

Fig. 5.5: Effects of 50 µg enkephalinase inhibitor RB-101 administered i.c.v. **(A)** in wild type and **(B)** heterozygous RGSi $G\alpha_o$ knock-in mice (*** indicates 10-minute vehicle treated animals are significantly different from both 10-minute 50ug RB-101 and 10-minute 50ug RB-101 + NTX treated animals; 2-way ANOVA followed by Tukey's post-hoc test; n = 6).

Current work which suggests that RGSi $G\alpha_o$ knock-in mice have an enhanced antinociceptive response to endogenous opioid peptides on thermal tests should also be extended. The initial behavioral screening of these animals revealed a naloxone reversible antinociceptive phenotype on both the hot plate and tail withdrawal tests (Lamberts *et al.*, 2013). I have extended these results to show that RGSi $G\alpha_o$ knock-in mice have an enhanced response to the enkephalinase inhibitor RB101 (Fig. 5.5A and B) supporting the hypothesis that endogenous opioids produce increased antinociception in these animals. In wild type mice (Fig. 5.5A) two-way ANOVA

revealed a significant main effects of time ($F_{(3,32)}$=9.781, p<0.0001). In RGSi $G\alpha_o$ knock-in mice (Fig. 5.5B) two-way ANOVA revealed significant main effects of time ($F_{(3,52)}$=14.44, p<0.0001), and treatment x time interaction ($F_{(6,52)}$=4.98, p=0.0004). Furthermore, preliminary data suggest that these animals also exhibit increased swim stress induced antinociception (Fig. 5.6A and B) although these results did not reach significance. The development of opioid antinociceptive tolerance should be determined in these animals compared to wild types (Fig. 5.7) in order to determine whether the RGSi $G\alpha_o$ mutation enhances opioid antinociception selectively, or whether the development of tolerance is increased as well.

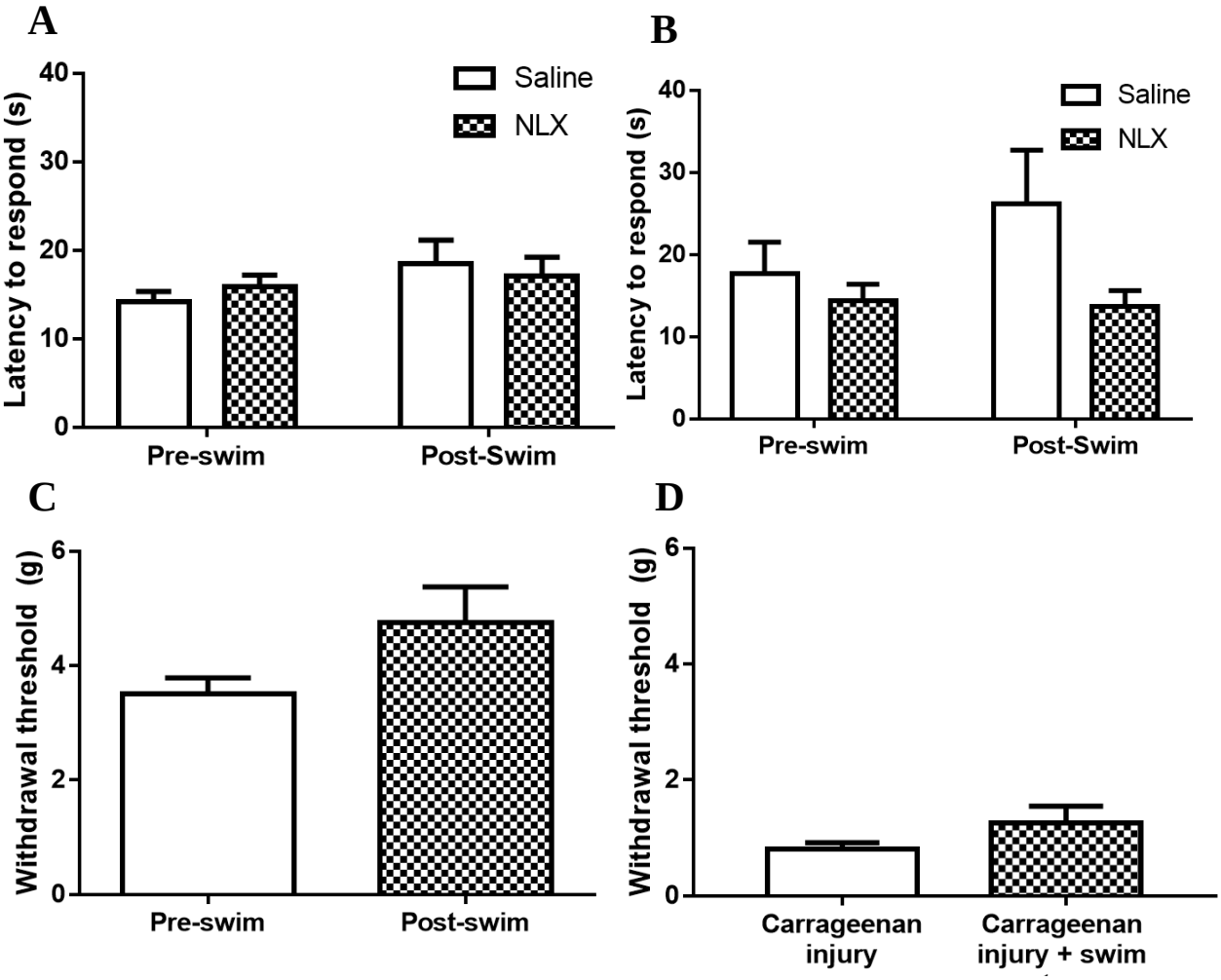

Fig. 5.6: Effects of a 15-minute swim stress in room temperature water on 52C hot plate test responding before and after 10 mg.kg i.p. naloxone **(A)** in wild type and **(B)** heterozygous RGSi $G\alpha_o$ knock-in mice (n = 5-9). **(C)** Effect of 15-minute swim stress in room temperature water on von Frey responding in wild type mice (n = 4). **(D)** Effect of 15-minute swim stress in room temperature water on von Frey responding in wild type mice 3 hours after intraplantar carrageenan injection (20ul, 2.5%; n = 4)

Swim stress induced antinociception is a naloxone reversible antinociceptive response thought to occur due to the release of endogenous opioid peptides following a brief forced swim in room temperature water (Mogil *et al.*, 1996c), and so we expected this response to increase in RGSi $G\alpha_o$ knock-in mice. When initially piloting this experiment, we were able to produce a robust antinociceptive response in wild type mice on a C57/Bl6 background following a 15-minute swim stress (data not shown), however wild type mice on a 129 background did not have a significant antinociceptive response to this same procedure on either the hot plate test (Fig. 5.6A) or von Frey test (Fig. 5.6C). In order to eliminate the possibility that a ceiling effect was preventing the presentation of swim stress induced antinociception on the von Frey test we next tested the effects of a 15 min swim stress 3 hr after intra-plantar λ-carrageenan injection in wild type animals (Fig. 5.6D) and still did not detect a swim stress induced antinociception. Nonetheless, compared to the total lack of response in wild type animals heterozygous RGSi $G\alpha_o$ knock-in mice appeared to have a slight swim stress induced antinociception on the hot plate that was reversed by NLX pretreatment (Fig. 5.6C), but these effects did not reach significance.

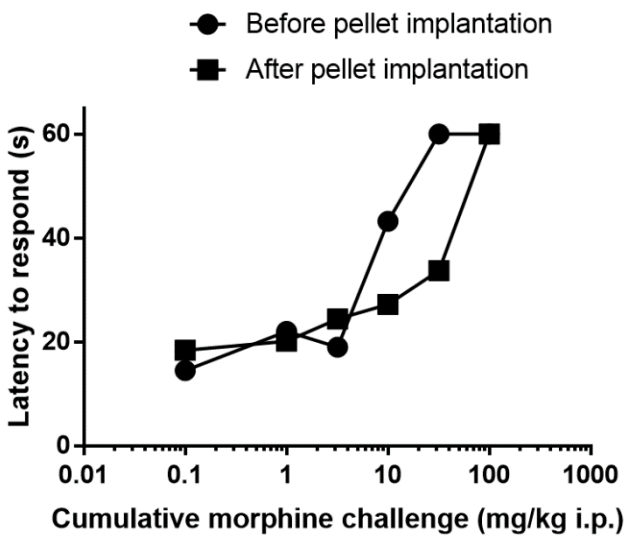

Fig. 5.7: Pilot study of tolerance to morphine challenge one week after morphine pellet implantation in the flank of a wild type mouse on the 52C hot plate test.

The lack of a swim stress induced antinociception in wild type mice on a 129 background may be due to a tonically active stress response during behavioral testing which precludes further stress induced responses in this strain of mice. These animals are especially prone to freezing while being handled compared to other strains (personal observation), a known response to fear in rodent models (Curzon *et al.*, 2009). In addition, in the elevated plus maze test for anxiety wild type 129 mice have a low baseline time spent in open arms (~4.5% of total test time vs. ~10% for C57/Bl6 mice; Fig. 5.8; Komada *et al.*, 2008) suggesting that wild type 129 strain mice may be anxiogenic at baseline. A thorough study of 16 different mouse strains found marked differences in behavior between strains, with BALB/cJ mice spending the most time in open

arms and A/J mice spending the least (Trullas and Skolnick 1993). Although 129 strain mice were not one of the 16 strains compared, our results (~4.5% of time spent in open arms; Fig. 5.8) would make 129 mice one of the most anxiogenic strain out of the 16 tested (Trullas and Skolnick 1993). As the RGSi $G\alpha_o$ knock-in mice are also available on a C57/Bl6 background this experiment should be repeated in the future using C57/Bl6 background RGSi $G\alpha_o$ knock-in mice instead of 129 background mice to produce a more robust swim stress induced antinociception. On this background strain we expect to see a clear naloxone reversible swim stress induced antinociception in wild type animals on both the hot plate and von Frey tests (either with or without carrageenan), with even greater swim stress induced antinociception observed in C57/Bl6 background RGSi $G\alpha_o$ knock-in mice. This would support the hypothesis that RGSi $G\alpha_o$ knock-in mice are not only more sensitive to the antinociceptive effects of exogenous opioid agonists, but to endogenous opioid peptide release as well.

The elevated plus maze experiment was initially run to determine whether other NOPR dependent behaviors unrelated to pain response are altered in RGSi $G\alpha_o$ knock-in mice. Centrally acting NOPR agonists are known to produce anxiolytic effects on the elevated plus maze (Gavioli *et al.*, 2008), and so we predicted that RGSi $G\alpha_o$ knock-in mice would similarly have an anxiolytic phenotype on this test. RGSi $G\alpha_o$ knock-in mouse have no apparent behavioral differences compared to wild type littermates on the elevated plus maze (Fig. 5.8), although the time spent in open arms for wild type 129 mice is already so low (~4.5% of test duration) that if the RGSi $G\alpha_o$ knock-in mouse instead have an anxiogenic phenotype we may not be able to detect it using this test. This low baseline should allow us to study the anxiolytic effects of NOPR agonists however, and this response has already been well characterized on the elevated plus maze in wild type animals treated systemically with Ro64-6198 (Dautzenberg *et al.*, 2001; Jenck *et al.*, 2000; Wichman *et*

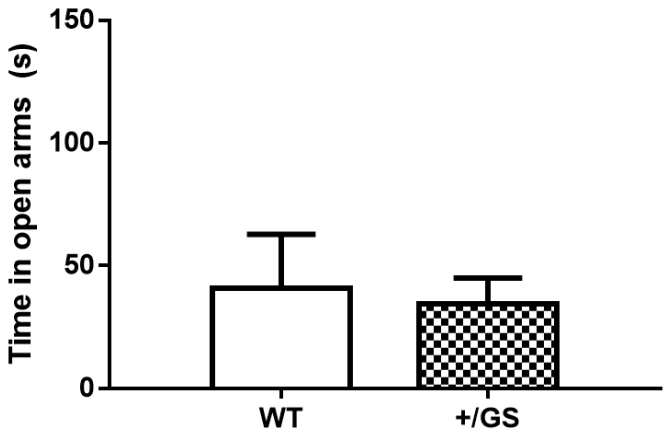

Fig. 5.8: 15-minute elevated plus maze results with wild type (WT) and heterozygous RGSi $G\alpha_o$ knock-in mice (+/GS) following i.p. saline administration(n = 5). Average time for a C57/Bl6 mouse on this test with these conditions often exceeds 150 s (personal communication with Jutkiewicz lab).

al., 2000). We predict that Ro64-6198 will promote anxiolytic behavior more potently in RGSi $G\alpha_o$ knock-in mice compared to wild type controls. Alternatively, RGSi $G\alpha_o$ may differentially affect different NOPR-mediated behaviors, such that its pronociceptive action is enhanced while anxiolytic activity is unaffected. If this is the case then Ro64-6198 should produce anxiolytic effects with similar potency in both the mutant mice and wild type littermates. This line of experimentation would allow us to determine whether NOPR dependent hyperalgesic and anxiolytic effects can be separated by promoting activity downstream of one G-protein compared to others, and if so whether potential therapeutics targeting NOPR can separate these effects.

Conclusions

The results with RGSi $G\alpha_{i2}$ knock-in mice could have important implications for the treatment of depression, as they appear to achieve a long-standing goal within this field of research: to promote signaling downstream of postsynaptic 5-HT1AR receptors without stimulating presynaptic 5-HT1AR receptor activity. This could lead to antidepressant drugs acting at new targets (such as RGS19) with improved effects compared to the current generation of antidepressant drugs.

The RGSi $G\alpha_o$ knock-in mouse results provide novel insight into the interplay between opioid and NOPR systems, but the observation of hyperalgesic behavior in these animals must temper our enthusiasm for any novel treatment strategy that will increase signaling downstream of the NOPR/$G\alpha_o$ complex. At the same time, the results suggesting that these animals have an antidepressant-like phenotype through a yet-to-be determined mechanism should be explored further, as it remains possible that the antidepressant-like and hyperalgesic effects could be separated.

Together these results demonstrate the value of using RGSi G-protein knock-in mouse models to study complex behaviors. These models can reveal differences between closely related G-proteins, aid in identifying RGS proteins relevant to therapeutic responses, and increase our knowledge of how various receptor systems interact. This information has the potential to uncover new therapeutic targets, and has already improved our understanding of antidepressant and analgesic drugs acting through these pathways.

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