Inhibitory G-protein Modulation of CNS Excitability

by

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DEDICATION

For Bud, Gagi, and Judy

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LIST OF ABREVIATIONS

129S: 129S1/SvImJ mouse strain

5-HT: Serotonin

8-OH-DPAT: 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol

AC: Adenylyl cyclase

ANOVA: Analysis of variance

B6: C57BL/6J mouse strain

CA3: Cornu Ammonis region 3

Chr: Chromosome

CNS: Central nervous system

DAMGO: [D-Ala2, N-MePhe4, Gly-ol]-enkephalin an opiod agonist

ES: embryonic stem

F1: 129 x B6 first generation

G184S: Glycine to serine mutation preventing RGS proteins from GAPing mutant G-protein (See RGSi)

GABA: *gamma*-Aminobutyric acid

GAP: GTPase accelerating protein

GDP: Guanosine diphosphate

GIRK: G-protein inwardly rectifying potassium channel

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinase

GTP: Guanosine triphosphate

HVA: homo-vanilic acid

IED: interictal epileptiform discharge

KA: Kainic acid

Mb: Millions of base pairs

Mogs1: Modifier of g-protein induced seizure 1

N[x]: Backcrossed x times onto the following genetic background

NE: Norepinephrine

PAM: Positive allosteric modulator

PTX: Pertussis toxin

PTXi: Pertussis toxin insensitive

PTZ: Pentylenetetrazol, a non-competitive GABAA antagonist

RGS: Regulator of G-protein signaling

RGSi: Regulator of G-protein signaling insensitive (See G184S)

RH: RGS homology domain

SCG: Superior cervical ganglion

SCN1A: Sodium channel, voltage-gated, type I, alpha subunit

SMEI: Severe myoclonic epilepsy of infancy

SNP: Single nucleotide polymorphism

SSRI: Selective-serotonin-reuptake inhibitor

SUDEP: Sudden unexpected death in epilepsy

tm2Rneu: Allele of G_o that is insensitive to RGS action

ABSTRACT

Epilepsy represents a major health burden upon society. Approximately 30% of patients still remain symptomatic despite therapy. Complicating development of more efficacious therapies is the fact that patients with similar underlying genetic causes display a range of phenotypes and those with similar phenotypes may display a range of genetic mutations. While not a current medicinal target for epilepsy, the family of inhibitory G-proteins appears to play an important role in this disease, as blocking their function in animal models increases both acute seizure susceptibility and the rate of spontaneous seizure development in kindling, a research model with parallels to epilepsy.

The most abundant inhibitory G-protein in the brain is G_o, composing roughly 2% of membrane bound protein. To further clarify the role of G_o in epilepsy a *Gnao1* gain-of-function mouse line (RGSi/G184S) was employed. Hippocampal slices from *Gnao1* $^{+/RGSi}$ mice show enhanced epinephrine-mediated suppression of epileptiform burst firing of neurons demonstrating that *Gnao1* $^{+/RGSi}$ mice have enhanced G_o signaling.

The aforementioned work lead me to hypothesize that C57BL/6J *Gnao1* $^{+/RGSi}$ mice would be protected from kindling. In fact, the mice experience premature death, enhanced kindling susceptibility and over a ten-fold increase in frequency of electrical disturbances within their brain. C57BL/6J *Gnao1* $^{+/RGSi}$ mice also display an unexpected

loss of inhibitory signaling within specific brain regions. This loss may be due to changes in brain development as spine density on pyramidal cells within the CA1 is reduced as early as four weeks of age.

Interestingly, the mutation is only lethal on the C57BL/6J background. The progenitor 129S1/SvImJ strain demonstrates no change in seizure susceptibility or viability. A genome-wide SNP analysis identified a region on chromosome 17 between 41-70 megabases that affords protection from spontaneous lethality. Further, this region also reduces the rate at which mice develop seizures in response to a chemically induced model of epilepsy (kindling). This region was further refined to a subregion from 41-51 megabases which was found sufficient to afford protection to kindling. Consequently, I have identified two genomic loci, *Gnao1* and the Chr17 modifier which I term *Mogs1* (modifier of G-protein seizures), that should be examined as candidates in human epilepsy. Additionally, future use of this model should prove informative in assessing the utility of novel therapeutics to prevent the progression of epilepsy.

Chapter 1

Introduction

Statement of purpose

The goal of this thesis is to improve our understanding of the role of G-protein coupled receptors (GPCRs), a major target of current medications for other diseases, in convulsive epilepsy. My thesis will focus on a highly abundant protein within the brain, $G_{o,}$ that is downstream of many GPCRs, and dissect its role in controlling neuronal excitability.

This introduction will start with a historical overview of epilepsy and background on the traditional focus of ion channels in disease pathology. The limitations upon current therapies will then be highlighted followed by support for the potential of novel therapeutics targeting GPCR signaling. Details of GPCR signaling will then be discussed. This comprehensive background will then be leveraged to outline the approach I employed to achieve the goal of my thesis.

History and health burden of epilepsy

Epilepsy is one of the most common neurological disorders and is associated with a large societal burden. Epilepsy affects approximately 0.7% of the population (Hirtz *et al.* 2007) with current treatments leaving roughly one-third of patients without relief (Organization 2012). Epilepsy is defined as recurrent spontaneously occurring seizures.

Seizures may involve involuntary body movements of all or part of the body and consciousness can be lost during these periods. Because of the potential to interfere with daily activities, epilepsy dramatically reduces the quality of life of those afflicted. Life expectancy is reduced by 18 years and patients are at greater risk of developing depression or a panic disorder (de Boer *et al.* 2008).

The refractory nature of this disease may be, in part, due to two major factors. The first factor is the common focus of current therapies on modulating ion channels. The second factor is that many different rare genetic mutations likely play an important role in epilepsy (Michelucci *et al.* 2012). For about 30% of patients (Michelucci *et al.* 2012) the specific genes affected are not known. Further, even in patients with known genetic causes, the severity of their disease and the response to treatment are modulated by interactions with their genetic background (Loscher *et al.* 2009).

How the brain communicates

To understand how large networks of neurons fire in unison to create a seizure, we must first understand the basic principles that modulate firing of an individual neuron. Excitatory and inhibitory signals converge on the axon initial segment (AIS) of a neuron. This region acts as a gate between the cell body and the axon (Mantegazza & Catterall 2012). A variety of ion channels in the region integrate the signals received from the soma leading to either the initiation of an action potential or termination of any depolarization further along the axon. If initiated, an action potential propagates down the myelinated axon via sodium channel-mediated depolarization at nodes of Ranvier (Mantegazza & Catterall 2012). Eventual depolarization at axon terminals promotes release of neurotransmitters at the synapse. These neurotransmitters then act as ligands to

activate various receptors on target cells, some of which are ionotropic ligand-gated ion channels which allow ions to flow in or out of the cell to depolarize or hyperpolarize the target. Neurotransmitters may also bind metabotropic G-protein coupled receptors (GPCRs) to induce longer term changes to cellular processes. Metabotropic receptors are transmembrane receptors that generate intracellular signals through generation of second messengers which influence a wide range of cellular processes. The major neurotransmitters in the brain are glutamate for excitation, gamma-amino-butyric acid (GABA) for inhibition, as well as acetylcholine (ACh), norepinephrine, dopamine, serotonin, and histamine for more specific functions within the brain. The following sections will discuss many of the steps in this process while highlighting literature relevant to epilepsy.

Sodium Channels

Voltage-gated sodium channels are large multi-subunit channels that play a critical role in the initiation and propagation of action potentials. They consist of a large pore forming α subunit with voltage sensing properties in the S4 segments (Mantegazza & Catterall 2012). During membrane depolarization the S4 segments rotate causing the pore to open (Mantegazza & Catterall 2012). After sustained opening, an inactivation gate blocks the pore, limiting the time the pore can remain open (Mantegazza & Catterall 2012). One or two β subunits also compose a mature sodium channel where, among other roles, they regulate channel levels at the plasma membrane and participate in cell-cell adhesion(Brackenbury *et al.* 2008).

Sodium channels play a role in the pathophysiology of many types of epilepsy. mRNA levels of many α subunits are altered in animal models of epilepsy (Bartolomei *et*

al. 1997). Changes are also observed in human tissue taken from epileptic brains (Lombardo *et al.* 1996). Although mRNA does not always directly correlate to protein levels, these changes do result in functional consequences. Temporal lobe neurons taken from an animal model of epilepsy involving repeated electrical stimulation of the amygdala have larger persistent sodium channel current (I_{NaP}) than control (Blumenfeld *et al.* 2009). I_{NaP} is the result of incomplete inactivation of sodium channels after an action potential. This phenomenon leads to a persistent current lasting on the order of 10s of seconds. Typically I_{NaP} is the result of sustained opening of less than 1% of previously activated sodium channels, but it plays key roles in modulating repetitive firing patterns and with small changes can have a large impact on rhythmicity of neuronal activity, a key disturbance in seizures (Mantegazza & Catterall 2012). Similar changes in I_{NaP} are also found in resected brain tissue from human patients with temporal lobe epilepsy (Vreugdenhil *et al.* 2004). Thus increased I_{NaP} may contribute to human epilepsy in some cases.

While the data above focused on gain-of-function changes, loss-of-function within inhibitory cells may also result in epilepsy. This is due to less inhibitory neuron firing leading to disinhibition of excitatory neurons for a net gain in excitation. For example, Dravet Syndrome can result from mutations in *SCN1A*, encoding the Nav1.1 channel, which reduce sodium channel function within inhibitory neurons (Catterall *et al.* 2010). This simplified view has been recently challenged by others in the field. Testing patient derived neurons demonstrates that *SCN1A* mutations initially thought to reduce sodium currents only within inhibitory neurons may, in fact, increase sodium currents

within both inhibitory and excitatory neurons through an unknown mechanism (Liu *et al.* 2013).

Potassium channels

Among the diverse roles that potassium channels play in modulating neuronal excitability I will highlight their critical role in modulating the likelihood of an action potential leading to neurotransmitter release. The voltage-dependent potassium channel family is large, but all members are selective for potassium. Due to the high relative concentration of intracellular potassium, family members typically lead to repolarization of the cell and reduce neuronal excitability. Despite these similarities there are many differences. Each family member serves a distinct role in modulating cellular excitability resulting from their specific cellular localization and activity tuned to a specific membrane potential. This section will only discuss Kv1.1, encoded by *KCNA1*, in its role in modulating transmitter release, and mutations in *KCNA1* involved in epilepsy.

Mutations in *KCNA1* result in epilepsy through loss of function mutations. A T226R mutation in KCNA1 was initially discovered in patients with episodic ataxia (Browne *et al.* 1994). A subset of episodic ataxia patients with the T226R mutation experience epilepsy. This indicates the potential for environmental or genetic modifiers to play a role in modifying outcome (Eunson *et al.* 2000). When studied *in vitro*, the mutation reduces surface expression of the protein (Rea *et al.* 2002) implicating loss-of-function as the likely mechanism.

A causal link between loss of Kv1.1 function and epilepsy is further supported by *Kcna1* $^{-/-}$ mice. These mice have spontaneous seizures accompanied by a decreased rate

of axonal repolarization (Smart *et al.* 1998). This is in concordance with data that Kv1.1 channels predominately localize to axons and presynaptic terminals (Wang *et al.* 1994). Isolated neurons expressing T226R also exhibit enhanced release probabilities without enhanced intrinsic excitability (Heeroma *et al.* 2009).

Calcium channels

Voltage-dependent calcium channels are responsible for the calcium influx in response to cellular depolarization. The family is quite large, with various family members performing distinct roles. For an exhaustive review see (Catterall 2011). This section shall focus on N, P/Q-type calcium channels (Cav2), the activity of which is inhibited by GPCR signaling (Tedford & Zamponi 2006). Activation of Cav2 channels promotes release of neurotransmitters. Similar to sodium channels, calcium channels require membrane depolarization, only activate for a brief period before being inactivated, and require repolarization of the membrane before reopening. Despite Cav2 channels being particularly important in regulating neurotransmitter release, few mutations have been reported in connection with convulsive human epilepsy.

GABA

$GABA_A$ receptors play a critical role in the response to GABA, the major inhibitory neurotransmitter in adult mammals. They are ligand-gated ion channels generally composed of two α, two β and one γ or δ subunits. In response to GABA binding, they allow influx of chloride leading to membrane hyperpolarization and a decreased probability of an action potential. Over 15 different mutations across all the subunits have been linked to a various idiopathic epilepsy syndromes (Macdonald *et al.* 2012).

To more directly assess the correlation of the mutations to epilepsy and to determine the mechanisms of action, transfected cell systems have been employed. Though the mechanisms vary, many mutations appear relevant as they have been found to reduce chloride conductance. Mechanisms include reduced mRNA stability, trafficking defects, loss of proper oligermization or altered inactivation kinetics (Ding *et al.* 2010; Frugier *et al.* 2007).

Another approach to determine the impact mutations have on a disease is to cluster the type of mutation with the associated phenotype. If the mutation is causative similar mutations should result in similar syndromes. This hypothesis has been borne out in studies of $GABA_A$ mutations in epilepsy. Distal N terminal missense mutations are associated with the less severe epilepsies while proximal N terminal missense mutations are associated with the more severe epilepsy (Macdonald *et al.* 2012).

Current therapies

Current therapies predominantly act through modulation of ion channels. In fact, progress over the last several decades in treatment of epilepsy has focused on improving the side-effect profiles of drugs acting through the same mechanism of action (Loscher 2002, 2011). While improving the quality of life for those whose disease is well-treated by current drug classes, it has continued to leave seizures in a third of patients poorly controlled. There are several obstacles to the successful development of new therapies for epilepsy which are highlighted below.

Complications limiting current therapies

Genetics

Genetics play a significant role in epilepsy with many genetic modifiers of epilepsy still to be discovered. Mutations in over 20 genes are known to lead to inherited epilepsies, examples of which were highlighted in prior sections. Risk is increased two to four-fold in relatives of those who suffer from idiopathic epilepsy (unknown cause) (Ottman *et al.* 1996). There is also a higher rate of idiopathic epilepsy among genetically identical twins compared to fraternal twins (Berkovic *et al.* 1998). Mendelian inheritance of epilepsy is however quite uncommon since many patients have no afflicted relatives (Ottman *et al.* 1996).

There are several explanations for the genetic complexity of epilepsy. One model proposes epilepsy as multifactorial disease requiring multiple "hits". In this model many detrimental alleles have a modest effect on their own (low penetrance). However when inherited together they act in an additive or synergistic fashion to promote epilepsy. In this model relatives would statistically be unlikely to have inherited a sufficient number of low penetrance alleles. A second model highlights the role of *de novo* mutations, the significance of which is already well-recognized in Dravet-Syndrome where almost 70% of patients have *de novo* mutations in *SCN1A* (Chakravarti 2001). *De novo* mutations are often associated with severe juvenile epilepsies whereby the severity of the phenotype generally prevents transmission of the mutation to subsequent generations.

A major limiting factor in elucidating the genetics of epilepsy stems from the fact that there is not a one-to-one correspondence between phenotype and genotype. Many

syndromes can be caused by either complex inheritance or high penetrance genetic mechanisms which limits correlation of phenotype to genotype. Even in clearly inherited epilepsies, mutant alleles in a variety of genes or different mutant alleles of the same gene can lead to the same syndrome (Ottman & Risch 2012).

Complex genetic and environmental modifiers further complicate the picture as even the same genetic mutation does not always result in the same phenotype. This is exemplified by a family with an inherited mutation in *SCN1A* whereby various family members were classified with epilepsy syndromes from mild febrile seizures to severe temporal lobe epilepsy despite all having the same *SCN1A* mutation (Ottman & Risch 2012).

Remodeling

In addition to the difficulty complex genetics raises, there are also complications due to the progressive nature of the disease. In many ways epilepsy becomes a positive feed-forward loop, making intervention more difficult, and the best course of action likely dependent upon changes that have already occurred in a patient's brain. This is highlighted by three basic observations. Following seizures, the ion-channel composition of many neurons changes which promotes excitation, and potentially alters the effectiveness of current therapies (Houser *et al.* 2012). However, even further complicating matters is that the network of neurons itself changes. Death of inhibitory neurons leads to less inhibition of excitatory neurons (Andre *et al.* 2001; Dudek & Shao 2003; Fritsch *et al.* 2009). A positive feed-forward loop can also develop as excitatory neurons sending new processes out onto other excitatory neurons in a process called mossy fiber sprouting (Buckmaster 2012). Lastly, new neurons can even be

inappropriately integrated into the network, further promoting seizures (Parent & Kron 2012).

GPCRs: a classic target underutilized in the treatment of epilepsy

In the treatment of other diseases, the family of G-Protein Coupled Receptors (GPCRs) is a common target. Approximately 40% of prescription drugs act either directly or indirectly on GPCRs (Whalen *et al.* 2011). GPCRs reside on the plasma membrane where they function to convert signals from the extracellular space into intracellular changes. They have been a predominant focus of drug discovery efforts due to their distinct tissue distributions, each family member's involvement in a limited subset of physiological processes, and well defined pockets for easy drugability. As of yet, however, they are not widely targeted in the treatment of epilepsy.

Both pharmacological and genetic literature support the role of GPCRs in modulation of seizures and the development of epilepsy in rodent models. Seizures are modulated by signaling through many GPCRs including: adenosine A1 (Chen *et al.* 2012), galanin R2 (Lu *et al.* 2010), α2 adrenergic (Shouse *et al.* 2007), serotonin 5HT-1(Bagdy *et al.* 2007; Pericic *et al.* 2005), and μ-opioid receptors (Grecksch *et al.* 2004).

Most interestingly, in several of these models GPCR signaling appears to impact the remodeling process. An experimental paradigm referred to as kindling has many parallels to the remodeling process in human epileptogenesis (Golarai *et al.* 1992; Morimoto *et al.* 2004). μ-opioid receptor null mice are more susceptible to kindling which supports a protective role for their action in epileptogenesis (Grecksch *et al.* 2004). Genetic models, however, can involve complex development changes. Further buttressing

a protective role for acute GPCR signaling, adenosine A1 activation during kindling suppresses the development of seizures (Li *et al.* 2007), indicating that activity during embryonic and neonatal development is not required for GPCR signaling to impact remodeling.

My thesis focuses on understanding the role of GPCR signaling in epilepsy. Instead of focusing on a specific receptor, I will explore common downstream pathways of GPCRs with the goal of facilitating drug development targeting these pathways. Targeting downstream pathways as opposed to the GPCR itself should avoid several potential pitfalls that are highlighted below.

Limitations of drugs targeting a specific GPCR

One drawback to the use of drugs that activate GPCRs (agonists) on a chronic basis is the development of tolerance (Whalen *et al.* 2011), whereby an equivalent dose of a drug becomes less effective. This is a consequence of receptor phosphorylation by G-protein Receptor Kinases (GRKs) in response to agonist binding and subsequent receptor inactivation. β-arrestins may also be recruited to the receptor leading to receptor internalization (Whalen *et al.* 2011).

A second challenge of developing drugs targeting specific GPCRs is the need to differentiate among structurally similar receptors that respond to a single endogenous ligand. For example the five muscarinic acetylcholine receptors are involved in a variety of cellular processes. They have a very similar binding pocket for endogenous ligand recognition. This has made development of subtype-specific agonists extremely challenging. However, without subtype-specific agonists, therapeutic utility is reduced,

since other family members have opposing actions or create adverse side effects (Conn *et al.* 2009). Developing therapies that act not on the receptor itself, but downstream to potentiate desired signaling, may eliminate these potential pitfalls. It does, however, raise concerns about greater side-effects due to lower specificity.

The $GABA_B$ GPCR, a practical example of GPCR drug development limitations

GABA, the major inhibitory neurotransmitter of the CNS, not only acts through the ionotropic $GABA_A$ receptor, as highlighted previously, but also acts through a single GPCR, the metabotropic $GABA_B$ receptor. $GABA_B$ receptors can be found both in presynaptic and postsynaptic locations. The major effects of $GABA_B$ activation include: autoreceptor inhibition of GABA release, inhibition of glutamate release, and hyperpolarization of cells through increased potassium conductance (Bettler *et al.* 2004).

The literature supports $GABA_B$ receptors as a therapeutic target for many diseases. One currently used drug used is baclofen, a $GABA_B$ agonist, which serves as a muscle relaxant (Bettler *et al.* 2004). Baclofen administration can also be of assistance in reducing cravings in many paradigms of addiction (Bowery *et al.* 2002). Rats that receive low doses of baclofen do not self-administer heroin as often (Di Ciano & Everitt 2003; Xi & Stein 1999), nor do alcohol-preferring strains of mice consume as much alcohol (Besheer *et al.* 2004). Additionally, baclofen has pain relieving effects (Bowery *et al.* 2002). Blockade of $GABA_B$ is also an area of interest. Antagonists are efficacious in the treatment of absence seizures (Vergnes *et al.* 1997) and improve cognitive deficits in animal models (Helm *et al.* 2005; Mondadori *et al.* 1996).

Further development of drugs modulating $GABA_B$ signaling is limited due to adverse side effects and the development of tolerance. Therapeutic actions occur at lower doses of baclofen than those required for general sedative effects (Bettler *et al.* 2004; Bowery *et al.* 2002). Unfortunately the difference in the dose of baclofen required for the beneficial effect and the sedative effect is not sufficiently large to be separable in a less controlled clinical setting. Tolerance also develops rapidly to baclofen's pain-relieving properties through an unknown mechanism (Bettler *et al.* 2004). Development of $GABA_B$ antagonists for cognitive improvements (Froestl *et al.* 2004) is also likely to face significant difficulty due side-effects, as antagonists are likely to phenocopy $GABA_{B(1)}$ knock-out mice which display a host of negative effects including increased pain sensitivity (Schuler *et al.* 2001).

$GABA_B$ signaling occurs through only one receptor subtype, and thus selective agonists/antagonists with limited side-effect profiles probably cannot be developed. This is demonstrated by the fact that a knock out of the $GABA_B$ subunit known to bind GABA, $GABA_{B(1)}$, prevented the classical effects of baclofen administration and ablated radio-ligand binding of a $GABA_B$ antagonist (Schuler *et al.* 2001). Alternatively, if one could potentiate signaling of $GABA_B$ receptors to only reduce glutamate release without influencing other functions, this may be a novel method for the control of seizures. To understand how this might be possible we must understand GPCR signaling and its regulation in more depth.

GPCR signaling

GPCRs function in part through the activation of heterotrimeric G-proteins. There are several families of heterotrimeric G-proteins, each activates or inhibits a specific set

of effectors. Effectors may include enzymes or ion channels in the cell. As previously mentioned, activation of a number of GPCRs appears protective from seizures. The one commonality of these receptors is that they activate the $G_{i/o}$ family of heterotrimeric G-proteins. The relevance of this family is further highlighted by the fact that *in vivo* administration of pertussis toxin (PTX), which inactivates the $G_{i/o}$ family, lowers the amount of excitatory stimulus required to induce seizures in rats (Ormandy & Jope 1991).

The $G_{i/o}$ family consists of several members which generally act to reduce neuronal excitability. In recombinant systems, all members of the $G_{i/o}$ family are capable of reducing neurotransmitter release by suppressing the prerequisite activity of N-type calcium channels Family members can also activate G protein-coupled inwardly-rectifying potassium (GIRK) channels which hyperpolarize neurons. Lastly, the family blocks the production of cyclic AMP, a common signaling molecule within cells that influences a variety of cellular events (Albert & Robillard 2002). When studied in more physiologically relevant systems, however, distinct roles for particular family members begin to emerge. In these cases, each $G_{i/o}$ family member is thought to preferentially signal through only a subset of potential effectors, some of which are unique to the family member (Duan *et al.* 2007).

In an attempt to understand G-protein signaling, G-protein null mice have been generated. Mice lacking a single inhibitory G-protein display surprisingly mild phenotypes. This is likely due to compensation by other family members. For example, despite data showing that inhibition of N-type calcium currents by $GABA_B$ is G_o-dependent under physiological conditions (Campbell *et al.* 1993), G_o knock-out mice have altered kinetics, but not diminished signaling (Greif *et al.* 2000). Results such as

these clearly limit the utility of using knock-out mice to study the G-protein component in physiological GPCR signaling. This background raises three questions.

1) How can one understand the role of individual family members?
2) Further, even if one were able to enhance their inhibitory action would it have a beneficial effect?
3) Lastly, how would one pharmacologically target the function of a single family member?

To answer these questions we need a better appreciation for the details of the system.

The G protein cycle

In their inactive state, heterotrimeric G-proteins exist as trimers, consisting of an α, β and γ subunit with the α subunit bound to a βγ complex and guanosine diphosphate (GDP) (Figure 1.1 step 1). Agonist binding to a GPCR causes a conformational change within the GPCR which induces the G-protein to exchange GDP for guanosine triphosphate (GTP) (Figure 1.1 step 2). The α subunit then dissociates from the βγ complex and each part of the trimer may then interact with downstream effectors (Figure 1.1 step 3). The α subunit has intrinsic GTP hydrolysis activity allowing for cleavage of the GTP terminal phosphate, regenerating α bound to GDP. Inactive trimer is then able to reform (Birnbaumer 2007a). To understand how to leverage this system in the study of individual G-proteins requires one additional component which is introduced below.

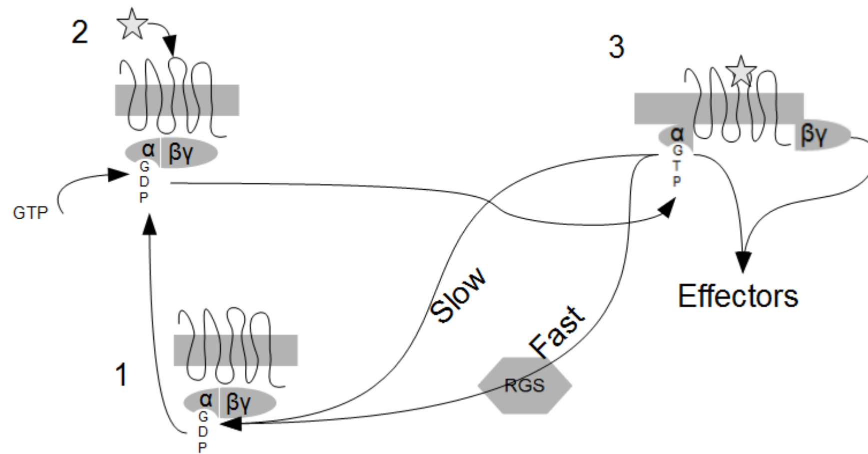


Figure 1.1GPCR cycle
GPCRs form complexes with heterotrimeric G-proteins in the basal state (Step 1). Upon agonist binding (star) GPCRs induce heterotrimeric G-proteins to exchange GDP for GTP (Step 2). The α and βγ subunits of the G-protein dissociate and interact with effectors (Step 3). Activation of some G-proteins is then terminated by a family of proteins called Regulators of G-protein Signaling (RGS). If RGS proteins are unable to act on the α subunit only a substantially slower self-catalyzed pathway is available to terminate signaling.

Discovery of Regulators of G-protein Signaling (RGS) proteins

After the isolation of G-proteins in 1981 (Sternweis *et al.* 1981), a clear understanding emerged about the role of GTP hydrolysis in the termination of GPCR signals (Ross & Gilman 1980). However, it became obvious by the late 1980s and early 1990's that there was a discrepancy between the biochemical GTPase activity of Gα subunits and the turn-off rate of physiological signals. This was clearly documented in the visual system where the measured GTPase activity of transducin (Gαt) was about 10 times slower than the turn-off of physiological responses to light (Chabre & Deterre 1989). This was attributed in part to effects of the phosphodiestrase (Arshavsky & Bownds 1992) but other studies suggested the involvement of another protein (Dratz *et al.* 1987). This was also about the time when GTPase accelerating proteins (GAPs) for the oncogene ras were discovered (Adari *et al.* 1988) leading to the suggestion that there

might be similar GAPs for heterotrimeric G-proteins. The solution to this conundrum ultimately came from studies of yeast and worms.

Pheromone signaling in the mating of yeast (*Saccharomyces cerevisiae*) utilizes GPCRs and its study has revealed a number of key insights into G-protein signaling mechanisms including the role of Gβγ subunits as active signaling elements and the identification of RGS proteins. Haploid yeast secrete pheromones which act on GPCRs in yeast of the opposite mating type. This induces growth arrest and events that that promote fusion of the two cells for mating. Chan and Otte discovered a mutant yeast strain (sst2) that was supersensitive to the α factor pheromone (Chan & Otte 1982). The mutant strain also had a prolonged pheromone response; in continuous presence of α factor, the budding returned to baseline after about 4 hrs for the wild type cells but in sst2 yeast the effect of α factor lasted more than 6 hours after just 1 hr of exposure. This effect of the Sst2 protein was through direct actions on the G protein α subunit (Dietzel & Kurjan 1987; Dohlman *et al.* 1995) and Sst2p is now known to represent the first member of the family of RGS proteins.

Studies about the same time in the model organism *Caenorhabditis elegans* identified the gene EGL-10 which suppresses serotonin signaling through the Goa protein (Koelle & Horvitz 1996). This study and several others (Druey *et al.* 1996) defined the large mammalian RGS family, members of which have sequence homology to the G-protein regulators from the model organisms. Soon thereafter, the mechanism of action was shown to be GAP activity on the α subunits of $G\alpha_i$ and $G\alpha_q$ G-protein family members (Berman *et al.* 1996).

One primary principle of RGS action on Gα subunits relates to specificity of different RGS proteins for the Gα subunits. Surprisingly, this specificity is relatively limited when examined in biochemical studies with purified RGS and Gα proteins. The $G_{i/o}$ and G_q family are the primary targets and to date there have not been convincing demonstrations of an RGS protein acting as a GTPase Accelerating Protein (GAP) at a G_s protein. Figure 1.2 summarizes extensive literature on this point.

Beyond this simple analysis of purified proteins – generally in solution – there is clear specificity at the level of expression in different cell types and brain regions. Also substantial emerging literature addresses specificity driven by complex formation between RGS proteins and other signaling molecules in cells – including receptors and several scaffold proteins such as spinophilin, R7 binding protein, and RGS-GAIP-interacting protein.

Combined these data indicate the potential role for RGS inhibitors as possible therapeutics in the modulation of GPCR signaling. It remains to be determined which RGS would be most useful to target. Each family member is distributed in a subset of tissues indicating a potential role in modulating specific disease states (Blazer & Neubig 2009). In an attempt to identify the function of individual family members in specific disease states, RGS null mice were generated for many different RGS family members.

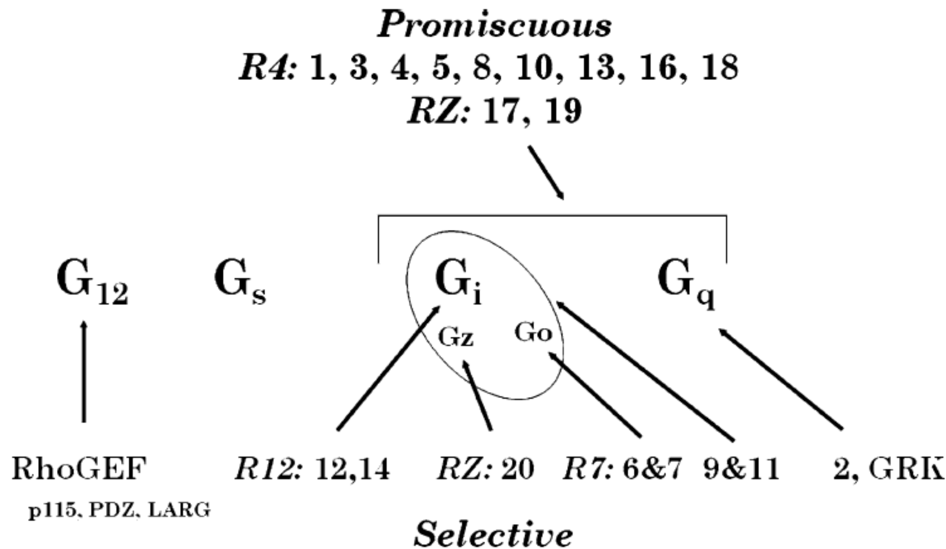


Figure 1.2 Selectivity of RGS proteins for heterotrimeric G-proteins

Drawback of individual RGS null models

Since RGS proteins reduce GPCR signaling, Genetic deletion of an RGS should potentiate signaling by the G-protein upon which the RGS acts physiologically. Several RGS proteins for which there is quite specific tissue expression show prominent effects in knockouts that relate to their tissue locus (RGS1 in lymphocytes, RGS4 in SA node, RGS9 in eye and striatum, and RGS13 in mast cells). Unfortunately, the phenotypes of most RGS null mice have been surprisingly modest; many RGS null mice appearing normal unless careful testing is done to elicit a specific phenotype. This is probably due at least in part to the functional redundancy among the ~20 RGS protein family members (Zhong & Neubig 2001). For example, in atrial myocytes there are 7 RGS proteins with abundant RNA expression (Doupnik *et al.* 2001) and 5 of them (RGS3, 4, 10, 17, and 19) have broad specificity for G_i and G_q proteins.

These findings create some concern about the utility of RGS inhibitors as potential therapeutics. However this concern is mollified through several lines of

evidence. One potential reason for the limited phenotypes is that RGS proteins may play a more prominent physiological role under stress or in pathological situations, thus requiring analysis of these animals in specific disease models. This is supported by the fact that RGS mRNA levels are modulated by seizures (Gold *et al.* 2002). Also, while RGS4 null mice do not display striking baseline phenotypes, in a mouse model of fragile X syndrome with spontaneous seizures, elimination of RGS4 function is able to ameliorate many of the pathophysiological phenotypes (Pacey *et al.* 2009). This suggests that RGS inhibitors should have minimal side-effects in normal individuals, but hopefully provide benefit in disease processes.

Given their likely functional redundancy and the large number of different RGS proteins, it will be virtually impossible to undertake combinatorial knockouts of all RGS combinations to understand the full contribution of RGS proteins to physiological processes. Despite the large family size, and lack of specificity of many RGS proteins for specific Gα subunits how can the goal of my thesis, to identify the function of G_o *in vivo* be addressed? Genetics studies in yeast will provide a solution in the form of a mutation that prevents the action of all RGS proteins on a particular Gα subunit. This eliminates the need for genetic deletion of RGS proteins and provided the ability to dissect the role of specific $G_{i/o}$ family members due to the significant role RGS proteins play in negatively relating $G_{i/o}$ signaling.

Development of a novel model to study GPCR signaling

In 1998, Dohlman and colleagues undertook a genome-wide mutagenesis study in yeast to discover mutations that could phenocopy the enhanced pheromone sensitivity of the sst2 RGS knockout allele. The only mutation identified was a glycine to serine

mutation at residue 302 (RGSi) in the Gα subunit Gpa1 (DiBello *et al.* 1998). The mutation had no effect on nucleotide binding to or release from Gpa1. However, the RGS protein GAIP (a.k.a. RGS19) failed to increase GTP hydrolysis by the G302S mutant Gpa1.

Subsequent biochemical studies showed that this mutation had general effects across multiple Gα and RGS proteins. In the Neubig lab, Lan et al showed that the analogous G184S mutations in $G\alpha_o$ and $G\alpha_{i1}$ blocked GAP activity and binding of the mutant Gα subunits to RGS4 and 7 (Lan *et al.* 1998). There was a >100-1000-fold reduction in the affinity of AlF_4^- activated Gα subunits for the RGS proteins and GAP activity was undetectable on those mutants. Due to nature of the mutation it will be referred to throughout this thesis either by the amino acid substitution G184S or as RGSi for RGS insensitive. This mutation is in the critical switch II glycine of Gα subunits which occurs at the contact interface between RGS4 and $G\alpha_{i1}$ (PDB: 1agr) (Tesmer *et al.* 1997) as well as in other RGS proteins whose co-crystal structures with a Gα subunit have been solved.

A different situation exists for RGS homology (RH) domain-containing proteins that don't function primarily as GAPs for a Gα subunit (including the RH-domain-containing RhoGEFs such as LARG, PDZRhoGEF, and p115rhoGEF as well as the G protein coupled receptor kinase or GRK RH domains). Their interaction mode with Gα is quite different. Distinct surfaces on those RH domains bind to the Gα subunit and they contact different surfaces on the Gα subunit as well. In the case of these RH domains, the G184S mutation in switch II of the Gα subunit does not prevent Gα/RH binding or function (Kaur *et al.* 2011).

For all examples to date in the “classical” RGS proteins (families R4, R7, R12, and RZ) the RGSi mutation appears to abolish RGS binding to Gα and GAP function. Also, there has been no demonstrated effect on other functions of the Gα subunit including: binding to Gβγ, activation by receptor, or coupling to effector (Fu *et al.* 2004). Consequently, the switch II G184S mutation provides a clean molecular phenotype for analysis of the role of individual Gα subunits and RGS binding and/or GAP activity in biological function.

Utilizing this mutation to study inhibitory G protein signaling provides three advantages compared to RGS null mice. First, this approach eliminates the action of all RGS proteins at the expressed mutant Gα subunit, overcoming potential functional redundancy. Second, the results differ from an RGS deletion in that only effects mediated through the RGS domain/Gα interaction would be affected. The role of other functional domains of the RGS protein (e.g. GoLoco or RhoGEF) would not be altered in these mutants but would be lost through genomic RGS deletion. In that sense, the Gα G184S mutants give better insights into the actions of potential drugs that would block RGS binding to Gα subunits (Neubig & Siderovski 2002); (Jin *et al.* 2004) (Roman *et al.* 2007) than a null mouse might. Third, this approach allows one to study the role of specific $G_{i/o}$ family members because signaling will only be potentiated through pathways which depend upon the mutant Gα subunit. This therefore provides a key method to accomplish one of my thesis goals, dissecting the role of G_o within the CNS. To support these ideas prior *in vitro* work is presented.

Cellular studies

In the original paper defining the yeast G184S mutation, DiBello *et al*. also showed a functional effect of the analogous mutation in mammalian $G\alpha_q$ (DiBello *et al.* 1998). In CHO cells, transfected with the 5-HT_{2c} receptor, serotonin increases calcium mobilization through G_q activation. RGS7 co-expression along with wildtype G_q reduced this calcium mobilization but the effect of RGS7 was abolished when a G188S mutant $G\alpha_q$ subunit was co-transfected with the RGS.

Similarly strong effects of the G184S mutation have been defined on $G_{i/o}$ functions in cellular systems. One commonly used tool to study inhibitory G-proteins (i.e. the $G_{i/o}$ family) is pertussis toxin (PTX) which modifies the Gα to prevent activation by GPCRs. Protecting the Gα through an amino acid mutation can protect it from modification, making it insensitive to PTX (i.e. PTXi). Treating cells with PTX and then transfecting in PTXi G-proteins allows one to study their unique contribution without any endogenous activity due to PTX treatment.

Clark et al used this approach to determine the effect of the G184S mutation in $G\alpha_o$ on opioid-induced inhibition of adenylyl cyclase (AC) in C6-mu cells – a rat glioma cell line stably expressing mu opioid receptors (Clark *et al.* 2003). PTX abolished the opioid-dependent AC inhibition which was restored by stable expression of the PTXi-$G\alpha_o$. Use of a PTXi/RGSi double mutant showed a strikingly greater inhibition of AC with morphine being converted from a weak partial agonist to a full agonist. Also, the full agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) showed a nearly 50-fold left shift of the dose response curve. These data were interpreted to indicate that endogenous RGS proteins were strongly suppressing the opioid inhibition of AC through $G\alpha_o$ and that

elimination of the RGS GAP on $G\alpha_o$ activity enhances inhibition. Subsequent studies extended this finding to other $G_{i/o}$ family members and substantially larger effects were seen on the maximum AC inhibition for partial agonists while full agonists generally showed an increase in potency (decrease in EC_{50}) when RGSi $G\alpha$ subunits were expressed (Clark *et al.* 2008).

The RGSi mutant $G\alpha$ subunits also profoundly change ion channel regulation in primary neuron cultures. Jeong and Ikeda (Jeong & Ikeda 2000) showed, using this approach, that norepinephrine (NE) induced inhibition of N-type calcium currents in rat superior cervical ganglion (SCG) is subject to regulation by RGS proteins. SCG neurons were transfected by intranuclear injections with PTXi $G\alpha_o$ or PTXi/RGSi $G\alpha_o$. The kinetics of calcium current inhibition and recovery with a 60 sec pulse of NE were similar for neurons with PTXi $G\alpha_o$ compared to control neurons. PTXi/RGSi $G\alpha_o$ mutants showed a similar maximum NE-induced inhibition of the calcium current but the rate of recovery from the inhibition was greatly slowed in PTXi/RGSi mutants as compared to that seen in PTXi-transfected or normal cells (from 10-30 seconds in controls to >1-3 minutes with RGSi $G\alpha_o$). In addition to the change in channel kinetics, the PTXi/RGSi mutants also resulted in an 8-fold leftward shift in the dose response curve for NE induced current inhibition. This was one of the first demonstrations that elimination of endogenous RGS function could strongly potentiate agonist function in a mammalian system. These actions of RGS function on calcium channels also have important implications for synaptic function. Chen and Lambert (Chen & Lambert 2000) showed that adenosine-mediated presynaptic inhibition in primary cultures of rat hippocampal neurons was restored to PTX-treated cells by viral transduction with PTXi

G-proteins ($G\alpha_o$ or $G\alpha_{i1}$). Furthermore, the recovery from adenosine-induced presynaptic inhibition was much slower in neurons expressing PTXi/RGSi Gα subunits as compared to those with only PTXi G protein. The time constant for recovery in the RGSi/PTXi $G\alpha_o$ mutant was increased to 40 sec as compared to 3 sec in the PTXi mutant $G\alpha_o$.

Based upon this evidence, RGS protein function, as detected by use of the RGSi Gα subunits is profound – especially in neural systems. Furthermore, these data support the premise that Gα function can be elucidated using an RGSi approach as RGS proteins are significant negative regulators of $G_{i/o}$ signaling and selective blockade of their action on a single $G_{i/o}$ family member *in vivo* is likely to yield profound molecular changes.

In vivo studies

Given the pronounced effects of the RGSi Gα subunit mutations in the cellular studies described above, the Neubig lab embarked on an effort to apply this system to *in vivo* studies in whole animals. One key consideration was how to maintain normal patterns and levels of expression of the mutant Gα. To address this, a knock-in strategy was employed where the mutant gene replaces the wild-type gene at its normal genomic locus. The details of how this was accomplished are outlined in previous studies (Fu *et al.* 2004). In brief, the targeting construct contains the mutant codon (G184S) in exon 5 of *Gnao1* or *Gnai2* as well as a diagnostic restriction site (PvuI) that is compatible with the coding sequence in the mutant protein. The *neo* selection marker for isolating targeted embryonic stem (ES) cells was placed in the intron between exons 5 and 6. Preliminary studies (Fu *et al.* 2004) showed that leaving the entire *neo* marker intact lead to markedly reduced expression of the mutant $G\alpha_o$ so loxP sites flanking the *neo* marker were introduced to permit its removal after the mice were generated. Introduction of cre

recombinase by transfection in ES cells or by breeding mutant strains with cre-expressing mice left only the small single loxP site in the intron which permitted normal levels of Gα subunit expression (Fu *et al.* 2004).

Gene dosage effects

The Gα G184S mutation, unlike RGS null mutants, has a dominant gain-of-function phenotype. To understand this, it is worth considering a scenario in which there is one G protein and one RGS protein in a system and the RGS protein suppresses the action of the G protein by 99% due to acceleration of turn off. In Table 1.1, the predicted effects of a heterozygous mutation of *Gnai2* $^{+/G184S}$ (or generically $G\alpha^{+/G184S}$) compared to a RGS $^{+/-}$ is illustrated using a simple model based on rates of RGS-mediated Gα subunit deactivation.

	Signal Strength		
	$RGS^{+/+}$	$RGS^{+/-}$	$RGS^{-/-}$
$G\alpha^{+/+}$	1	***2***	91
$G\alpha^{+/RGSi}$	***46***	46.5	91
$G\alpha^{RGSi/RGSi}$	91	91	91

Table 1.1 Predicted effects due to mutations in Gα subunit and RGS protein
This table illustrates predictions of a simple model of G protein activation and deactivation and compares results for loss-of-function mutations in the RGS vs RGSi mutations in the Gα subunit. A simple equilibrium is assumed between an inactive G protein (G) and an active G protein G*. The total amount of G protein is 100. The rate of activation is constant for all situations (1 sec^{-1}). The rate of deactivation is equal to 0.1 sec^{-1} for G protein with no RGS present and is 100 sec^{-1} with the full amount of RGS present (1000x stimulation). A heterozygote $RGS^{+/-}$ is presumed to have half as much RGS so would have half the rate of deactivation. A heterozygous $G_{\alpha}^{+/RGSi}$ is presumed to have half of its G protein behave like the $RGS^{+/+}$ situation and half like the $RGS^{-/-}$ situation. The signal strength is calculated to be equal to the amount of G* with 100 being full activation.

It is striking that, with these parameters, a $RGS^{+/-}$ shows only a 2-fold increase in signaling while a heterozygous G_{α} mutant ($G\alpha^{+/RGSi}$) shows a 46-fold increase. Homozygotes of both sorts show a strong 91-fold increase since both produce a complete loss of RGS function. Different parameters for the rates of activation and basal and stimulated deactivation would alter the magnitude of the effects but the qualitative result that the heterozygous RGSi G_{α} mutants give a much larger effect than the heterozygous $RGS^{+/-}$ would always be the case. Also, if there was more than one RGS acting on the system, a complete knock-out of a single RGS might behave more like a heterozygote $RGS^{+/-}$ in this model in which only one RGS is present. Consequently, the G_{α} G184S mutants are expected to have much stronger phenotypes, with $G_{\alpha}^{+/RGSi}$ heterozygotes showing clear effects perhaps approaching those of a homozygous RGSi mouse.

RGS-regulated, Gα subtype-selective signaling in the central nervous system

The Cornu Ammonis region 3 (CA3) of the hippocampus has one of the lowest seizure thresholds in the CNS due to recurrent collaterals that can mediate synchronous

burst firing behavior in neurons, a feature found among large populations of neurons during many seizures (Goldenstein *et al.* 2009). Synchronous firing within this region can be brought on experimentally by bicuculline-mediated blockade of the inhibitory $GABA_A$ receptors. The synchronous firing is suppressed by α_2 adrenergic agonists such as epinephrine and UK14304, an effect mediated by α_{2a} adrenergic receptors (Goldenstein *et al.* 2009). *Gnao1*$^{+/G184S}$ RGSi mice show an 8-fold increase in epinephrine potency compared to wild-type mice, whereas *Gnai2*$^{+/1G84S}$ RGSi mice are comparable to control mice. The selective potentiation of the α_{2a} adrenergic receptor effect by the RGSi $G\alpha_o$ mutant suggests that this mechanism is primarily mediated by $G\alpha_o$ (at least compared to $G\alpha_{i2}$). While it is possible that $G\alpha_{i2}$ is involved but is not regulated by RGS proteins, the contrast of this result to those in serotonin signaling (see below) suggests otherwise.

Another exciting phenotype that further supports the role of differential $G_{i/o}$ subtype function in the CNS relates to the actions of serotonin in murine models of antidepressant action. In tail suspension tests, antidepressants reduce the immobility time of mice. The *Gnai2*$^{G184S/G184S}$ mice show a spontaneous and nearly maximal reduction of immobility time that is reversed by a serotonin $5HT_{1A}$ antagonist suggesting that endogenous serotonin signals sufficiently strongly in the mutants to produce an antidepressant-like effect. The *Gnai2* $^{+/G184S}$ mice produce an intermediate reduction of immobility times and, in those mice, the dose-response for the $5HT_{1A}$ agonist 8-OH-DPAT is “left-shifted” 14-fold while that for the selective-serotonin-reuptake inhibitor (SSRI) fluvoxamine is left-shifted 6-fold (Talbot *et al.* 2010). Consequently, an RGS protein action at $G\alpha_{i2}$ seems to be strongly suppressing the antidepressant-like actions of serotonin. Intriguing evidence of the specificity of this effect is seen in two observations.

First, norepinephrine-dependent antidepressants (e.g. amitriptyline) are not potentiated in the *Gnai2* $^{+/G184S}$ mutant mice. Second, there is no effect of the mutation on the hypothermic effect of 8-OH-DPAT. The strong effect that the RGSi $G\alpha_{i2}$ mutation has on 5HT-dependent antidepressant-like effects with no effect on hypothermia raises the possibility that modulating RGS actions could greatly enhance the specificity of drug action – enhancing beneficial effects while not potentiating side effects.

Overview of findings

My thesis will accomplish the goals set forth at the outset of this chapter through the use of the unique model that the G184S mutation provides. I will outline a significant role for G_o in modulating kindling susceptibility within a murine model. In contrast to prior work that only highlighted a protective role for G_o in the development of seizures, my thesis will highlight the detrimental effects enhanced activity has in a murine model of epilepsy. Both pharmacological and physiological changes will demonstrate that enhanced G_o activity induces epileptogenesis. Genetics will also be employed to identify, for the first time, a putative modifier region of epilepsies of GPCR signaling origin.

In Chapter 2, I determine that mice heterozygous for the *Gnao1* G184S gain-of-function allele display phenotypes indicative of enhanced presynaptic dopamine autoreceptor inhibition of transmitter release. I also demonstrate that mutants experience spontaneous adult lethality, abnormal spontaneous electrical activity within the brain, and marked sensitization to an experimental model of epilepsy.

In Chapter 3, I define a novel epilepsy modifier locus, *Mogs1*, on Chr 17 (41-70Mb) which mitigates both premature death and sensitization to the experimental model

of epilepsy in mutant mice on the B6 background. Development of recombinant mice allow for the identification of the 41-51Mb subregion of *Mogs1* as sufficient to afford protection.

In Chapter 4, I demonstrate that the *Gnao1* G184S mutation reduces GIRK action among three GPCRs known to modulate seizure and/or kindling susceptibility. Further, I confirm that these findings are not due to a loss of G_o function or disruption of transcriptional control of GIRK channels.

Chapter 2

Initial characterization of *Gnao1* $^{+/RGSi}$ mice reveals a pro-seizure phenotype

Summary

Many physiological processes, including those involved in epilepsy, are modulated by the $G_{i/o}$ family; however the specific family member involved is controversial in many cases. Here, I examine the role of one family member, G_o, the most abundant inhibitory G-protein in the brain. A gain-of-function knock-in mutation that prevents termination of G_o-mediated signaling by Regulators of G protein signaling (RGS) proteins results in enhanced G_o signaling. Homozygous *Gnao1*$^{G184S/G184S}$ mutations are perinatal lethal. Heterozygotes are viable, but experience strain-dependent death between 15-40 weeks of age and increased frequency of interictal epileptiform discharges. *Gnao1*$^{+/G184S}$ mice also have an increased rate of sensitization to pentylenetetrazol (PTZ) kindling, a model with parallels to human epileptogenesis. One potential mechanism for these findings would be enhanced suppression of GABA release. While suppression of GABA release could not be directly measured, autoreceptor function in another neurotransmitter system, dopamine, was assessed. A reduction in novelty-induced locomotion and levels of a common dopamine metabolite are indicative of less dopamine release, possibly through enhanced autoreceptor function.

This work was the effort of many people. Kadee Luderman in the Gnegy lab was instrumental in planning and execution of dopamine studies. However, I physically performed all the dopamine experiments. The Parent lab performed all video EEG work. Undergraduates who contributed meaningfully to this work by doing genotyping and assisting with kindling experiments include Kristen Gilbert, Hans Dalton and Kevin Kohut.

Introduction

Epilepsy

Despite epilepsy being one of the most common serious neurological disorders, current treatments leave roughly one third of patients without relief (Engel 1996). Many current therapies target ion channels. This common mechanism of action may contribute to the high percentage of patients that are refractory to current medication. New therapeutics targeting metabolic pathways might be an effective adjuvant for a portion of the refractory population.

One potential new target to treat epilepsy is the family of G-Protein Coupled Receptors (GPCRs). Seizures can be suppressed by increased signaling through many $G_{i/o}$ coupled receptors such as adenosine A_1 (Chen *et al.* 2012), GAL_2 (Lu *et al.* 2010), α_2 (Shouse *et al.* 2007), $5HT_{1A}$ (Bagdy *et al.* 2007; Pericic *et al.* 2005), μ-opioid (Grecksch *et al.* 2004), and metabotropic glutamate (Alexander & Godwin 2006) receptors. Also, pretreatment of rats with pertussis toxin, which inhibits $G_{i/o}$ family function, greatly potentiates seizure induction through pilocarpine or kainic acid administration (Ormandy

& Jope 1991). Further, genetic ablation or chemical inhibition of RGS4, a negative regulator of $G_{i/o}$ and G_q signaling, reduces seizures (Chen *et al.* 2012; Pacey *et al.* 2009).

The picture, however, is more complicated than simple protection by $G_{i/o}$ family member activation. While increased activation or expression of the $G_{i/o}$ coupled $GABA_B$ receptor can suppress generalized tonic-clonic seizures (Pacey *et al.* 2009), it can also lead to absence seizures (Hosford *et al.* 1992). This complexity may be partially explained by the fact that $GABA_B$ receptors activate multiple downstream effectors, likely including multiple $G_{i/o}$ family members. Thus it is possible that specific effectors are preferentially involved in specific outcomes. The specific effector involved in $GABA_B$-mediated protection against convulsive seizures has yet to be identified.

Another potential pitfall in the current development of epilepsy medications is the use of acute seizure models in initial screening for efficacy. Acute seizure models (Loscher 2011) do not assess epileptogenesis (ie the maladaptive changes that occur after excitatory stimuli that lead to subsequent enhancement of seizure susceptibility) which is a process thought to occur in human epilepsy (Morimoto *et al.* 2004). Electrical or chemical kindling models (Bialer & White 2010) attempt to assess this with repeated sub-threshold excitatory stimuli that eventually result in generalized seizures. Pentylenetetrazol (PTZ) is a non-competitive $GABA_A$ antagonist and a single large dose induces seizures by engaging the brainstem (Peterson & Albertson 1998). However, lower doses of PTZ, in a repeated-dose kindling protocol, preferentially activate the deep prepiriform cortex (Peterson & Albertson 1998). This causes remodeling that parallels changes in human epileptic brains such as mossy fiber sprouting within the dentate gyrus

(Golarai *et al.* 1992; Lee *et al.* 2012). Therefore to better assess the role of inhibitory G-proteins in epilepsy I employed a PTZ kindling model.

I hypothesized that G_o ***is the major inhibitory G protein responsible for suppressing the development of seizures.***

The rationale includes the following observations:

1) *Gnao1* $^{+/G184S}$ mice show enhanced epinephrine-mediated suppression of epileptiform bursting in hippocampal slices (Goldenstein *et al.* 2009)

2) G_o is the most highly expressed $G_{i/o}$ family member within the brain, composing 2% of membrane bound protein (Birnbaumer 2007b)

3) G_o suppresses neurotransmitter release paralleling the actions of many epilepsy medications (Chen & Lambert 2000)

4) G_o is activated by the adenosine A_1 receptor (Fu *et al.* 2006), the activation of which is protective in several seizure and epilepsy models (Masino *et al.* 2011); (Chen *et al.* 2012; Li *et al.* 2007)

Contrary to my hypothesis, enhanced G_o signaling resulted in spontaneous death by 15-40 weeks of age, frequent interictal epileptiform discharges (IEDs), and accelerated sensitization in a PTZ kindling model. Possible explanations for these unexpected findings represent a focus of my thesis.

Enhanced presynaptic suppression of GABA release is one potential mechanism to explain the aforementioned data. While I was not able to directly assess this

hypothesis, the dopamine system was used as a model to study the effect of the *Gnao1* G184S mutation on neurotransmitter release.

Dopamine D_2 receptors, a model of presynaptic inhibition by G_o signaling

Existing literature supports the hypothesis that G_o is one of the major heterotrimeric G proteins to be activated by dopamine D_2 autoreceptors. *Gnao1* $^{-/-}$ mice lack the high affinity D_2 binding conformation which indicates that the majority of D_2 receptors are likely to associate with G_o. Further, mice lacking both $G_{i1/2}$, or $G_{i1/i3}$ did not display a similar loss in high affinity binding (Jiang *et al.* 2001). *In vitro* data also provides functional evidence of D_2 coupling to G_o. In a pituitary cell culture model using GH4ZR7 cells, the D_2 receptor inhibits calcium channel activity primarily through G_o (Banihashemi & Albert 2002).

Results

Gnao1 $^{+/G184S}$ mutant mice die perinatally

Gnao1 $^{G184S/G184S}$ mice die perinatally. Of mice surviving to weaning from N4 heterozygote x heterozygote crosses on the 129S1/SvImJ background only 5% (2 of 41, 1 male & 1 female) were homozygous mutants, with heterozygotes also underrepresented (Figure 2.1a Chi-squared χ^2 df(2)=16.02 $p < 0.001$). On the N4 B6;129S background, no homozygotes survived to weaning out of fifty-five offspring from heterozygote x heterozygote crosses and heterozygotes were underrepresented (Figure 2.1b Chi-squared χ^2 df(2)=45 $p < 0.0001$). Sex does not influence genotype distributions on either background (data not shown). To understand the loss of viability of mice carrying the G184S allele, timed pregnancies were performed for N7 B6 heterozygote x heterozygote crosses and embryos were collected. At E18.5, genotypes did not differ from the expected

Mendelian ratio (Figure 2.1c) indicating that the loss of viability occurred after E18.5 but before two weeks of age. Pregnant females were then monitored starting at E19 for birth of pups every 4-5 hours. There were very few viable homozygous offspring and heterozygotes were also under-represented (Figure 2.1d, Chi-squared χ^2 df(2)=8.76 $p < 0.02$). Many homozygotes and some heterozygotes were found dead but there were no obvious anatomical abnormalities. This does not appear to be due to reduced G_o expression as $G\alpha_o$ protein expression in the brain of heterozygotes at 8-12 weeks of age was found to be normal (Figure 2.2).

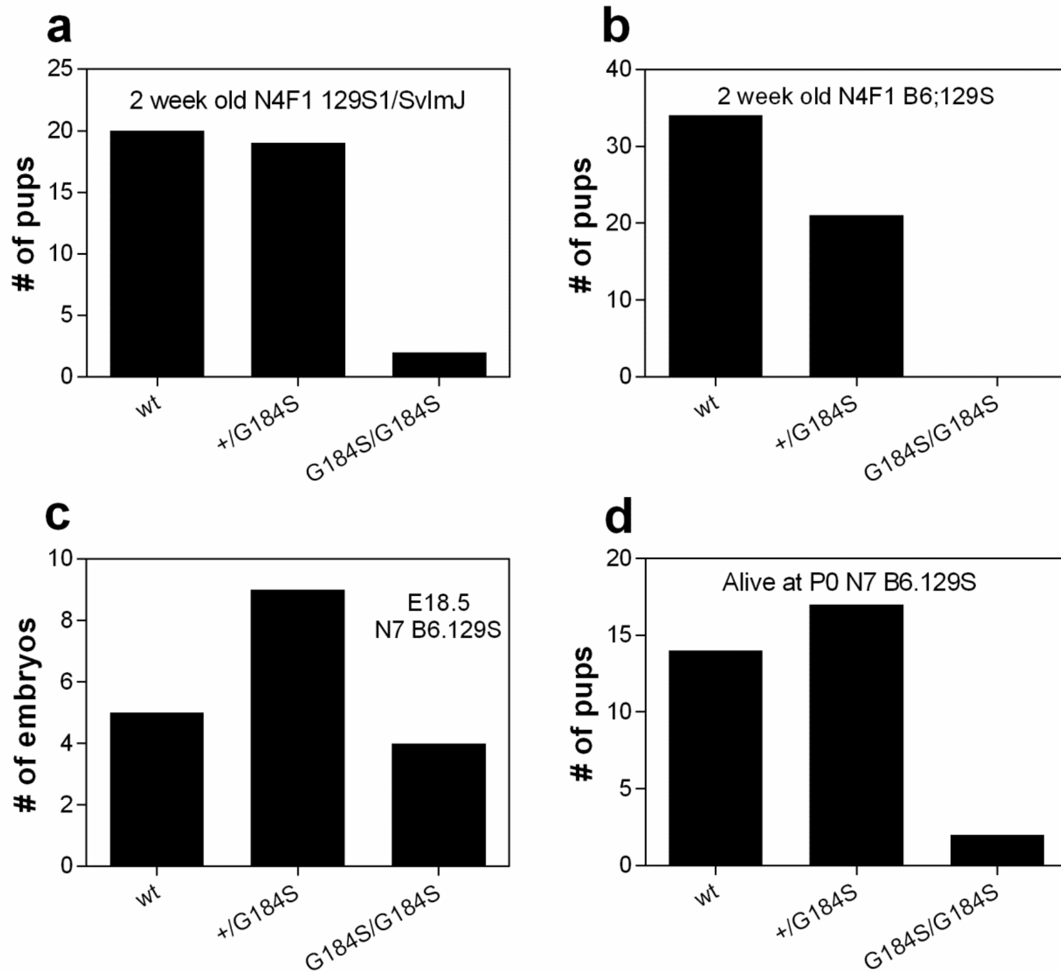


Figure 2.1 ***Gnao1*** $^{+/G184S}$ **mutant mice experience decreased perinatal viability**

Heterozygotex heterozygote crosses were performed on either the 129S1/SvImJ or C57BL/6J background as indicated. Viability was determined at various ages from E18.5 to weaning. a) N4 129S *Gnao1* G184S mutant mice are under-represented at weaning (Chi-squared χ^2 df(2)=16.02 $p < 0.001$). b) No N4 B6;129S *Gnao1* $^{G184S/G184S}$ mice were observed by 2 weeks of age and heterozygotes are under-represented (Chi-squared χ^2 df(2)=45 $p < 0.0001$). c) N7 B6 *Gnao1* $^{G184S/G184S}$ and *Gnao1* $^{+/G184S}$ mice are present at expected numbers at E18.5. d) Viable N7 B6 G_o mutant mice are underrepresented within a few hours of birth (Chi-squared χ^2 df(2)=8.76 $p < 0.02$).

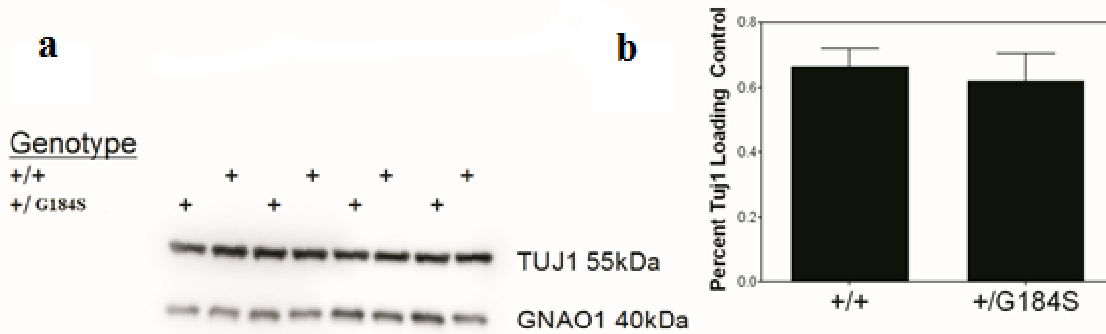


Figure 2.2 ***Gnao1*** **$^{+/G184S}$ mice have normal Gα_o protein levels**
a) Gα_o expression in whole brain lysates from drug-naïve, B6129F1-*Gnao1* $^{+/G184S}$ female mice at 8-12 weeks of age was assessed by Western blot. b) The ratio of Gα_o immunoreactivity to that of the Tuj1 loading control was quantified and found to be similar regardless of mutation status.

Epilepsy

Gnao1$^{+/G184S}$ mice experience spontaneous death in adulthood

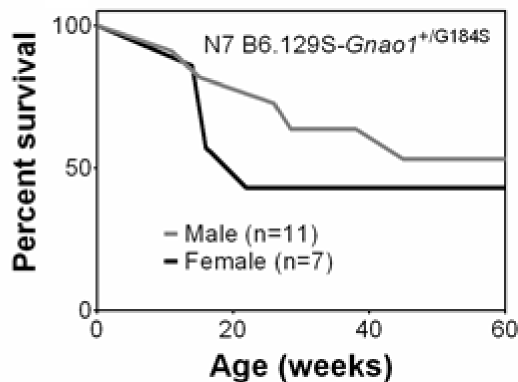


Figure 2.3 ***Gnao1*** **$^{+/G184S}$ mice experience spontaneous death in adulthood**
N7 B6-*Gnao1* $^{+/G184S}$ mice die prematurely regardless of gender

Both male and female N7 B6 heterozygotes, sired from a single N6 B6 heterozygote male, experience lethality starting at ~80 days (11-12 weeks) with ~50% dead by 25 weeks of age (Figure 2.3 Gehan-Breslow-Wilcoxon df(1)=12.8 p<0.001 B6 mutant vs wildtype control). Interestingly, there appeared to be heterogeneity in survival as the ~50% that survived to 25 weeks showed little further premature lethality.

Gnao1$^{+/G184S}$ mice have EEG abnormalities

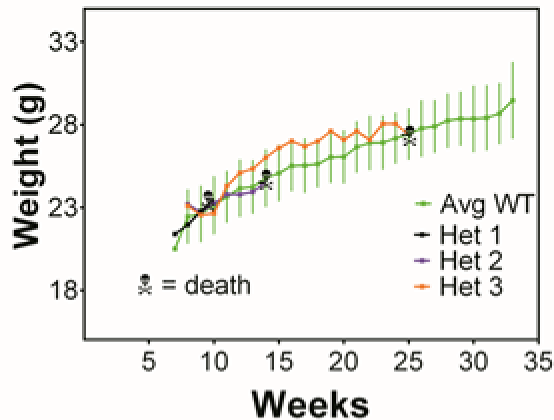
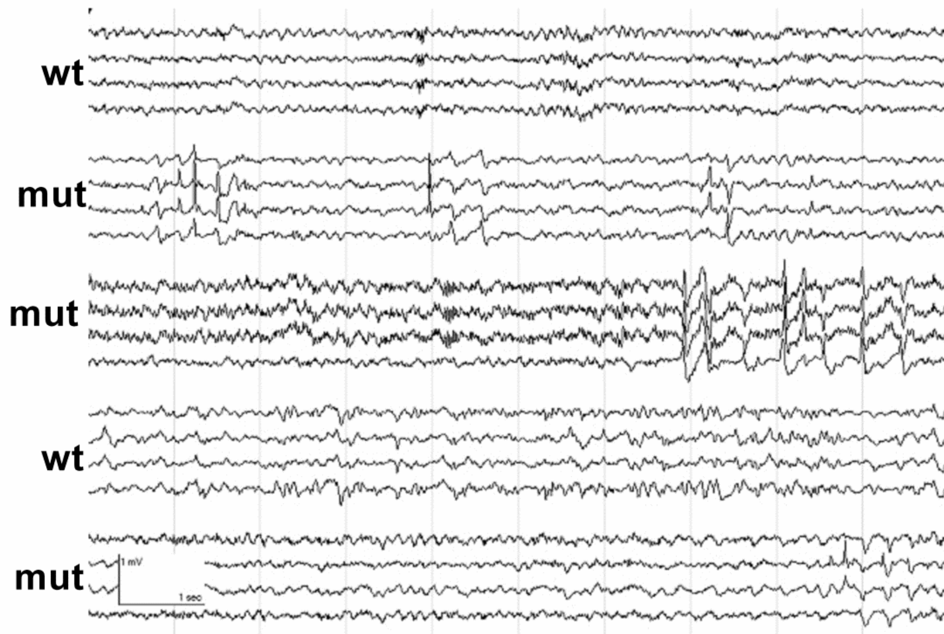


Figure 2.4 No changes are observed in mouse behavior or weight prior to sudden death
Male N7 B6 *Gnao1* $^{+/+}$ (average shown with SEM) and B6 *Gnao1* $^{+/G184S}$ mice were observed three times per week. Behavior, weight, and deaths were recorded 3 times per week. No reproducible trend was observed in mutant mice prior to death. Three deaths were observed as indicated by skull and crossbones.

To try to identify a cause of death, N7 B6-*Gnao1* $^{+/G184S}$ mice (n=11) were observed three times per week for 30 weeks for changes in weight, grooming behavior, activity level and evidence of seizures. Three out of four male mice died during the observation period. No seizures were observed, nor were changes in weight or behavior in the days prior to death (Figure 2.4). However, subsequent IR video surveillance conducted around the clock (8 mice for 16 weeks) revealed a spontaneous seizure in one mouse just prior to death.

To further support seizures as a cause of death we employed video EEG on B6 females. A representative EEG trace of all mice at one point in time is presented in Figure 2.5a. Mutant mice had a substantially elevated frequency of interictal epileptiform discharges (IEDs) (Figure 2.5b, unpaired t-test $t=7.37$ $df=2.2$, $p < 0.02$). The IEDs seemed to occur more frequently during electrographic sleep than during wakefulness. Frequently, they occurred in runs of 1-3 Hz, but they did not meet criteria of electrographic seizures due to their short duration. Tapes of mice were also observed during the lights-on period for behavioral signs of seizure activity, as it is possible that cortical electrodes would not detect localized seizure activity. During this time multiple potential seizures (with modified racine score above 1 & lasting > 10 seconds) were observed in the mutant mice, however they could not be confirmed via EEG data.



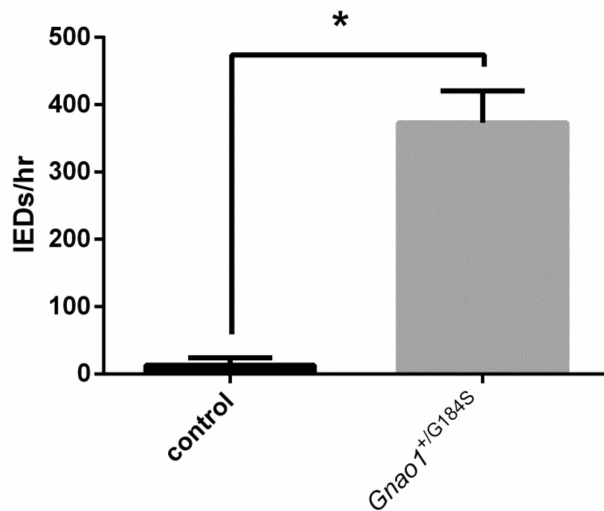


Figure 2.5 B6 *Gnao1* $^{+/G184S}$ mice have a greater frequency of IEDs

a) Simultaneous EEG of heterozygote (n=3) and wildtype (n=2) mice is presented at a representative time point. Vertical bars demark the passage of 1 second. B) Mutant B6 females experience greater than 10 fold increase in IEDs compared to control (unpaired t-test t=7.37 df=2.2, $p < 0.02$).

Gnao1 $^{+/G184S}$ mice are sensitized to PTZ kindling

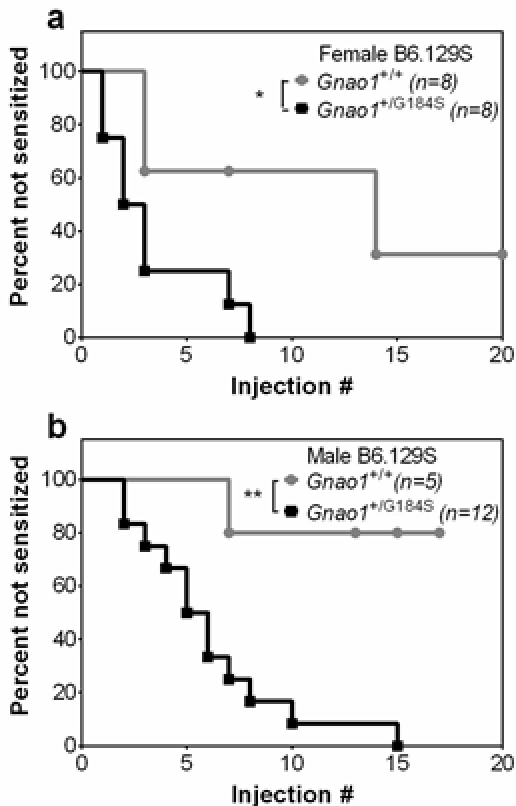


Figure 2.6 *Gnao1* $^{+/G184S}$ mice have a strain-dependent reduction in time to sensitize to repeated low dose PTZ The time to undergo sensitization to PTZ kindling (see Methods for definition) was recorded. a) B6-*Gnao1* $^{+/G184S}$ females sensitize more rapidly than littermate controls (Gehan-Breslow-Wilcoxon df(1)=5.74 $p<0.05$). b) B6-*Gnao1* $^{+/G184S}$ males also sensitize more rapidly than littermate controls (Gehan-Breslow-Wilcoxon df(1)=6.88 $p<0.01$).

To determine the physiological significance of the elevated IEDs we assessed susceptibility to epileptogenesis in mutant mice. Female B6 *Gnao1*$^{+/G184S}$ mutants were more sensitive to PTZ kindling than control (Figure 2.6a Gehan-Breslow-Wilcoxon df(1)=5.74 $p<0.02$). The increased PTZ kindling susceptibility on the C57BL/6J background was independent of gender as males also showed sensitization (Figure 2.6b, Gehan-Breslow-Wilcoxon df(1)=6.88 $p<0.01$) just as was the case for spontaneous death.

Dopamine

Gnao1 $^{+/G184S}$ mice have reduced novelty-induced locomotion

Mice were exposed to a new environment, which activates reward pathways leading to an increased exploratory behavior. More dopamine release is associated with more novelty-induced locomotion. Both male and female *Gnao1* $^{+/G184S}$ mice have reduced novelty induced locomotion (Figure 2.7). One potential explanation for this finding is that mutant mice release less dopamine due to enhancement of G_o action. However, the fact that males display differences in baseline locomotion complicates interpretation.

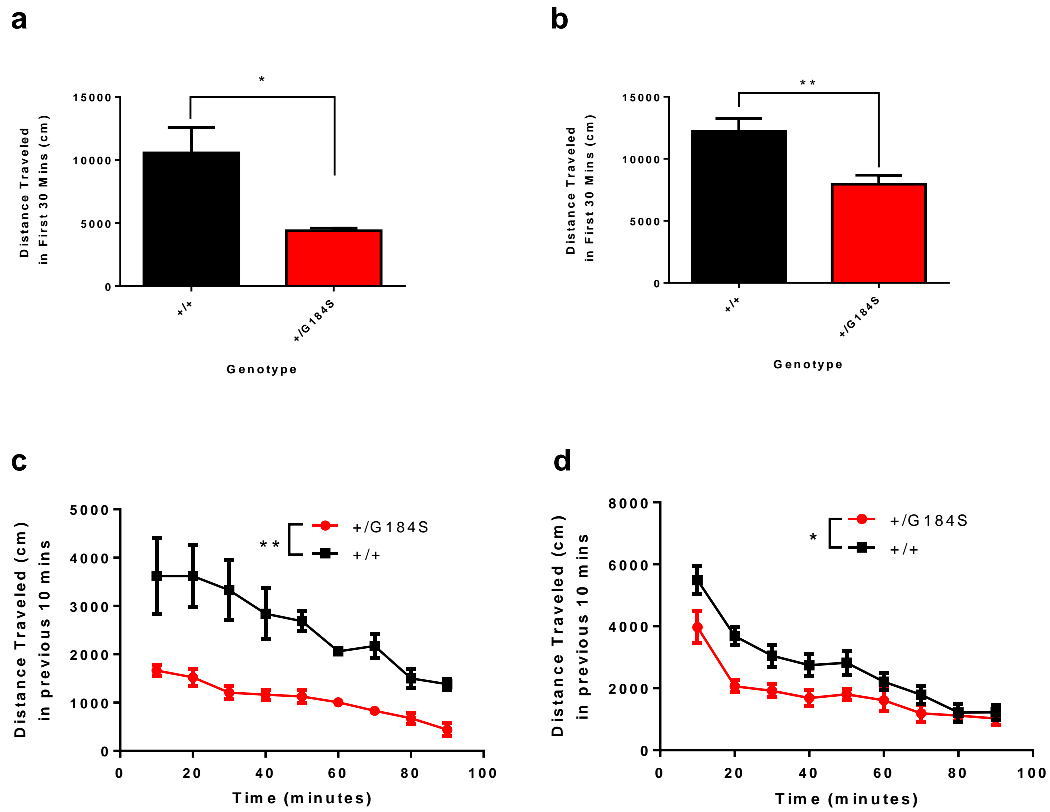


Figure 2.7 *Gnao1* $^{+/G184S}$ mice experience less novelty-induced locomotion
a) Male mutant mice travel less distance within the first 30 minutes of a novel environment. [n=3, $p < 0.02$, unpaired t-test t=3.636 df=5] b) Female mutants also experience a reduction in distance traveled within the first 30 minutes. [n=10, $p < 0.01$, unpaired t-test t=3.438 df=19] c) Male wildtype mice do not reach an equivalent baseline level of locomotion after acclimation complicating interpretation d) Female wildtype mice do reach an equivalent baseline.

Total dopamine is unaffected, but dopamine metabolites are reduced in *Gnao1* $^{+/G184S}$ mice

In an intact animal model, alterations in many other signaling pathways may result in the observed reduction in novelty induced locomotion. To more directly assess changes in dopamine release levels of dopamine and various metabolites were measured. Lower release of dopamine should result in a reduction of its metabolites. This was found to be the case with mutant mice having a reduced amount of the dopamine metabolite homo-vanilic acid (HVA). (Figure 2.8).

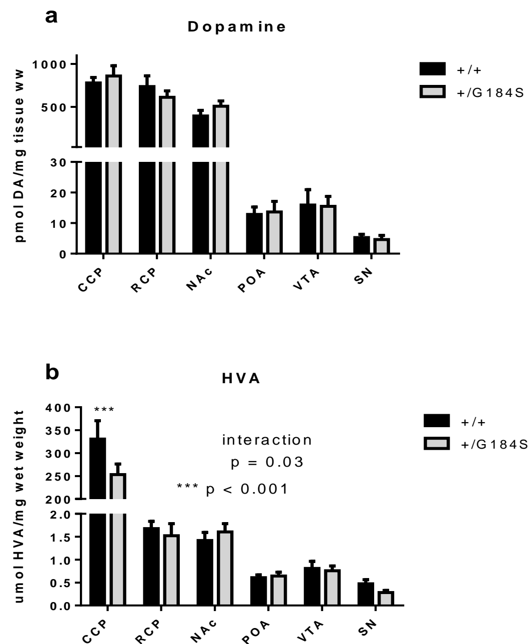


Figure 2.8 The level of HVA is reduced in *Gnao1* $^{+/RGSi}$ mice
Brains of drug naïve mice were rapidly dissected using a brain matrix to harvest the indicated regions. a) Total dopamine levels were unaffected by mutation [n=7] b) A common metabolite of dopamine, HVA was decreased within the CCP of mutant mice [n=7]. Abbreviations are as follows - CCP – caudal slide of caudate putamen. RCP – rostral slice of caudate putamen. NAc – nucleus accumbens. POA – preoptic area. VTA – ventral tegmental area. SN – substantia nigra.

Motor coordination and dopamine-induced motor activity is normal in *Gnao1* $^{+/G184S}$ mice

Alternative hypotheses to explain a reduction in novelty induced locomotion include: less downstream response to dopamine despite equivalent release or defective motor coordination. To rule this out in in *Gnao1* $^{+/G184S}$ mice the ability of a D_1 agonist to stimulate locomotion was tested. Here no difference was detected at roughly an EC_{50} dose suggesting that response to dopamine is not outside of the physiological range (Figure 2.9). Additional testing of motor coordination on a rotarod did not demonstrate a significant decrease in motor function due to the mutation (

Figure 2.10).

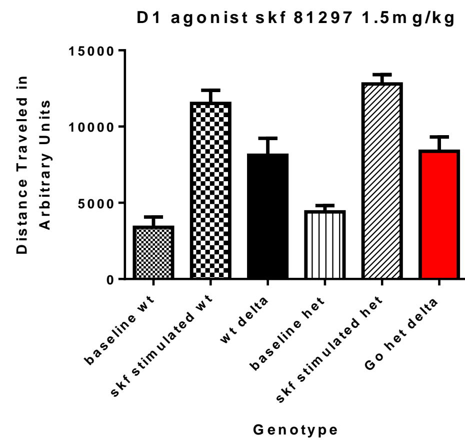


Figure 2.9 D_1 function is not altered in *Gnao1* $^{+/G184S}$ mice
Mice were acclimated to the recording chamber followed by a saline injection. Locomotion recorded following saline injection is "baseline." D_1 agonist was then injected and locomotion recorded as "stimulated." Differences in baseline locomotion were compensated for by determining magnitude of increase in locomotion from saline. See "delta." The mutant did not influence the ability of the D_1 agonist to increase locomotion.

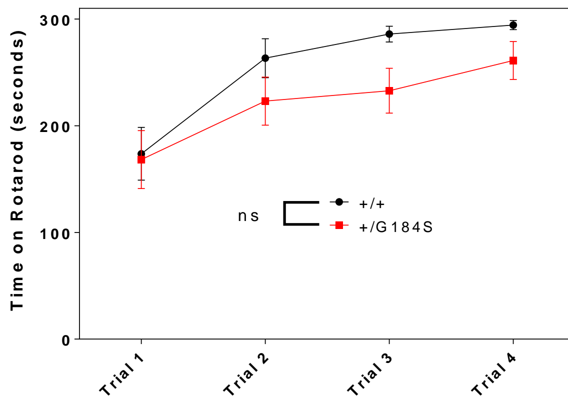


Figure 2.10 *Gnao1* $^{+/G184S}$ mice do not have baseline motor deficits
Gnao1 $^{+/G184S}$ mice and controls were placed on a rotarod at 12RPM to assess motor function. *Gnao1* $^{+/G184S}$ mice have a similar baseline to control but slightly underperformed control with repeated trials. This difference however was not significant despite a robust sample size. [n=19]

Discussion

In this chapter I demonstrate that *Gnao1* $^{+/G184S}$ mutants experience spontaneous adult lethality, enhanced frequency of IEDs, and marked sensitization to PTZ kindling. This is in contrast to previous work focusing on acute inhibition of G_o signaling which indicated a protective role for the protein. This suggests that *GNAO1* should be considered as a potential candidate gene in human epilepsy studies as either gain or reduction of function appears to be detrimental. Indeed, Nakamura *et al.* recently found *de novo* mutations likely to cause loss-of-function or gain-of-function are associated with epileptic encephalopathy (Nakamura *et al.* 2013).

It was surprising that enhanced G_o signaling would lead to PTZ kindling sensitization. G_o suppresses neurotransmitter release (Campbell *et al.* 1993; Chen & Lambert 2000) and $G_{i/o}$ proteins have been shown to protect against seizure activity (Goldenstein *et al.* 2009; Ormandy & Jope 1991). Given the role for G_o in the inhibition of calcium channels, this gain-of-function mutation may mimic the paradoxical results of high-dose levetiracetam treatment. Levetiracetam, which is used in therapy of partial onset seizures, also acts on presynaptic calcium channels (Lee *et al.* 2009; Vogl *et al.* 2012). While it has anticonvulsant effects at low doses, it can become proconvulsant at high doses (Shorvon 2010).

It is also possible that developmental changes due to mutant G_o are responsible for our observations. This is supported by the prior findings that $G\alpha_o$ activation can stimulate neurite outgrowth in cell culture models (He *et al.* 2006) and the related $G\alpha_{i2}$ G184S mutant alters self-renewal and differentiation in neural progenitor cells (Murai *et al.* 2010). However, prior work in *Gnao1* $^{-/-}$ mice lacking functional G_o has failed to identify

any defects in brain development (Valenzuela *et al.* 1997).

Understanding the relationship between IEDs and PTZ kindling within this model may also provide key insights into epileptogenesis. Currently, the role of IEDs in the development of epilepsy is under debate. Some researchers believe that IEDs serve as a protective mechanism to reduce the likelihood of future ictal events (Avoli 2001). Others have postulated that the enhancement in interictal firing promotes mossy fiber sprouting and enhances development of epilepsy (Staley *et al.* 2011). As this debate continues, this mouse line should serve as a tool to validate novel anti-IED drugs and assist in the determination of how these novel therapeutics impact outcomes.

The initial phenotype observed in this mouse model was sudden death and not an overt epilepsy syndrome. We were, however, able to observe seizures prior to death in a few mice with one documented on video. The *Gnao1* G184S mutation also enhances bradycardia to muscarinic and adenosine signaling (Fu *et al.* 2006). One proposed mechanism of SUDEP is post-ictal bradycardia or hypoventilation (Surges & Sander 2012) so a mutation in GNAO1 could exacerbate bradycardia in the setting of seizures which might contribute to SUDEP. Also, hypoventilation may underlie the perinatal lethality of the homozygous *Gnao1*$^{G184S/G184S}$ mutants given the large number of non-viable births (74%). With the combination of seizures and enhanced bradycardia, it will be of significant interest to determine whether humans with SUDEP might carry such a mutant *GNAO1* allele.

One potential mechanism behind the enhancement of *Gnao1* $^{+/G184S}$ kindling susceptibility is enhanced inhibition of GABA release. While this was not directly assessed, the dopamine system was used as a model for assessing the mutation's effect on

neurotransmitter release. Here my data are in line with the hypothesis that *Gnao1* $^{+/G184S}$ mice have reduced neurotransmitter release. Normally in response to a novel environment dopamine is released which promotes locomotion. Enhanced suppression of neurotransmitter release through D_2 activity would be expected to reduce novelty-induced locomotion (Benoit-Marand *et al.* 2001). Mutants have decreased novelty-induced locomotion potentially indicating less dopamine release or less responsiveness to dopamine. This was further tested through analysis of dopamine metabolites at baseline which were found to be lower in mutant mice. Additionally, responsiveness to dopamine was assessed through direct activation of dopamine receptors known to stimulate locomotion. In this case the mutation had no impact on outcome. Thus, decreased responsiveness to dopamine and motor impairment are unlikely to be major contributing factors. The reduction in novelty-induced locomotion among mutants could, however, be due to decreased stimulus input in the novel environment. This alternative hypothesis will need to be tested through further experimentation. Connecting these observations to epilepsy, preliminary data generated by Dr. Yuan suggests that GABA release is also inhibited in *Gnao1* $^{+/G184S}$ mice. Figure A.3 illustrates that the mice experience both a lower probability and lower amplitude of spontaneous inhibitory post synaptic currents within the hippocampus which is indicative of presynaptic changes. Future work will need to be done to confirm these results and identify the mechanism.

Materials and Methods

Generation of *Gnao1* $^{+/G184S}$ mice and perinatal viability experiments

We previously reported (Fu *et al.* 2007; Fu *et al.* 2006) enhanced bradycardia in studies of ES-cell derived cardiocytes carrying a *Gnao1* G184S knock-in. The *tm1Rneu* allele in those reports was generated in DE3 ES cells but never resulted in germline transmission. Subsequently, we generated a similar *Gnao1* G184S knock-in (*tm2Rneu*) allele in the 129-steel derived ES line, CJ7. This allele is referred to as G184S or RGSi throughout this thesis. The mutant allele construction from a CJ7 bac library was detailed previously (Goldenstein *et al.* 2009). After germline transmission of the mutant allele, pure agouti offspring were crossed to a 129-steel derived mouse expressing Cre (129S/Sv-Tg Prm-cre 58Og/J stock # 003328 from Jackson Labs, Bar Harbor, ME) and tested for excision of the loxP-flanked Neo cassette which was previously found to reduce G_o expression (Fu *et al.* 2007; Fu *et al.* 2006). Subsequent breeding on all genetic backgrounds was performed with a *Gnao1* $^{+/G184S}$ male and *Gnao1* $^{+/+}$ females with the exception of heterozygote x heterozygote crosses. Mice lacking the Neo cassette were backcrossed to the 129-steel line 129S1/SvImJ (Jackson Labs stock # 002448). After reaching N8, brother-sister crosses were performed. To limit the effect of genetic drift associated with repeated brother-sister crosses in our 129S1/SvImJ breeding we bred G184S carriers to female 129S1/SvImJ mice directly obtained from Jackson Labs roughly once after every 4 brother-sister crosses.

To obtain mutant mice on the C57BL/6J background, the *tm2Rneu* allele, after Neo excision, was successively backcrossed onto the C57BL/6J background (Jackson Labs stock # 000664).

Perinatal viability was established through timed pregnancies testing for plugs every morning which, if found, were designated E0.5. Eighteen mice were used at E18.5 and 33 at P0.

Genotyping

All mice had eartags inserted and tail biopsies taken prior to weaning. DNA was extracted from tails by an alkaline lysis method (Truett *et al.* 2000). The G184S allele of *Gnao1* was identified by PCR with primers GoFlankLoxP.A CGC AGG CTC TGA GGG CCT AAG & GoFlankLoxP.B TGC CTC ACC TCT CCG TCT CC. Reaction conditions were as follows: 1µl template, 4µl 5x Promega PCR buffer, 0.4µl 10mM dNTPs, 0.4µl 20µM GoFlankLoxP.A, 0.4µl 20µM GoFlankLoxP.B, 0.2µl Promega GoTaq and 13.6 µl DNase free water (Promega catalog # M3005, Madison WI). Reaction mixtures were denatured for 4 minutes at 95°C and then cycled 32 times through denaturing at 95°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension of 8 minutes at 72°C was then performed. The wildtype allele generates a 181bp product and the G184S allele produces a 361bp product.

Western blot

Brain homogenates were collected from group housed B6129F1 female mice 8-12 weeks of age. Mice were anesthetized by an overdose of sodium phenobarbital (University of Michigan Pharmacy) and the right hemisphere was collected in a microcentrifuge tube filled with 100ul of 0.5 mm glass beads (Next Advance, Averill Park, NY) and 600ul RIPA buffer. Brains were then homogenized and tissue lysed via a bullet blender (Next Advance) following the manufacturer's recommended settings. Samples were then centrifuged at ~10,000 RCF for 10 minutes. Protein in the supernatant

was quantified by a BCA kit (Pierce, Rockford, IL) and diluted to 2ug/ul protein in Laemmli buffer (Biorad, Hercules, CA). Western blots were then performed following standard protocols using 10% acrylamide gels transferred to PVDF membrane (Millipore, Billerica, MA). $G\alpha_o$ antibody was provided by Dr. Thomas Gettys (Louisiana State University) and used at a dilution of 1:1,000. TuJ1 antibody PRB_435P (Covance, Battle Creek, MI) was used at 1:5,000. Goat anti rabbit-HRP (Sigma, St. Louis, MO) was used at 1:10,000. Chemiluminescent substrate (Pierce) was applied for 5 minutes and blots were visualized and analyzed using a LiCor Odyssey imager using Image Studio 1.1.

Video EEG

Female mutant and control mice approximately 24 weeks old were implanted with electrodes, given one week to recover, and monitored under video EEG as described previously with a sampling frequency 256 Hz, sensitivity 30 uV/mm, and low frequency, high frequency and notch filter settings of 1 Hz, 100 Hz and 60 Hz, respectively (Lee *et al.* 2012). Older females were chosen due to the progressive nature of epilepsy and because females of inbred strains generally have a lower seizure threshold, increasing the likelihood of detection if seizures were the cause of spontaneous death (Frankel *et al.* 2001). The animals were continuously recorded for 96-206 h.

Automated spike detection analysis gave a high proportion of false positive and false negative calls based on manual confirmation. Therefore the results of automated detection are not reported. Semi-quantitative assessment of interictal epileptiform discharges (IEDs) was performed. The total number of IEDs was counted during 24 discrete and random 5 min epochs (Research Randomizer Version 3.0 retrieved on April 29, 2013 from http://www.randomizer.org/) and presented as aggregate count of IEDs/h.

IEDs were defined as waveforms lasting 20-200 ms, at least 3 times the background activity in amplitude, disturbing the background and not better described as waveforms of normal sleep or wakefulness. Seizures were defined as discrete events characterized by appearance of continuous repetitive IEDs or rhythmic high-amplitude activity, lasting at least 10 s and with unequivocal evolution in frequency, morphology or distribution.

Seizure susceptibility and epileptogenesis susceptibility

To test susceptibility to epileptogenesis, a PTZ kindling protocol was performed on both male and female mice. PTZ was administered 3 times per week at 35mg/kg starting at 8-12 weeks of age; mice were monitored for 30 minutes for behavioral signs of seizures as described by (Wilczynski *et al.* 2008), (Grecksch *et al.* 2004) and (Dhir 2012). This dose does not typically elicit effects greater than behavioral arrest on the first dose in wildtype mice regardless of gender or strain (lab observation). Behavior was recorded and then scaled to a modified Racine scale where 0 indicates no response, 1 is behavioral arrest, 2 is a tail flick, twitch, or body tremble, 3 is a forepaw press, 4 is a tonic clonic seizure involving loss of upright posture, and 5 is wild jumping or death (Racine 1972). Loss of upright posture was defined as having at least one front and one rear paw off the cage floor. Sensitization is defined as death or having 2 sequential sessions with a score of 4 or greater. The number of injections for each mouse to reach a sensitized state is reported in a survival curve in the results section. Survival curves for time to first tail flick and time to first forepaw press are also presented in Appendix A. Mice that died between observational periods were excluded from the survival curve at the last dose for which they could be observed since seizure could not be confirmed as cause of death. These exclusions were rare (less than 5%). In females, kindling could be influenced by

estrus cycle. To control for this, females were group housed with mutant and wildtype genotypes in equal numbers in each box to obtain synchronization of estrus cycle (Jemiolo *et al.* 1986). All mice in a given box entered kindling on the same day to ensure that an equal number of females per genotype were started at the same point in their cycle.

Novelty induced locomotion

Mice were habituated to the testing room first thing in the morning for a minimum of an hour. All tests were completed in the morning due to the circadian influence on dopaminergic stimuli (Abarca *et al.* 2002). Activity was recorded for two hours by an automated camera and computer system in collaboration with the Murphy laboratory.

Motor coordination and learning

Mice were placed onto a rotarod at 12RPM and time on rotarod was scored out of a maximum of 300 seconds. Time spent clinging to the rotarod was excluded, similar to prior studies (Jacobson & Cryan 2005). 30 minutes was provided between trials.

Metabolite analysis

Mice were cervically dislocated and then decapitated. Brains were rapidly dissected on ice and placed into a 30g mouse coronal brain matrix. 1mm slices containing specific regions were isolated and anatomically confirmed through use of a mouse atlas. Wet weight of each brain region isolated was recorded for normalization. Tissues were suspended in 100ul 2N percholric acid and homogenized followed by a spin at max rcf in a bench top centrifuge for 20 mins at 4 degrees. Samples were then diluted in ISS at the following concentrations - 1 / 100 for CP, 2/100 for Nac, and 20/100 for POA, SN, and VTA.

Diluted samples were then filtered with a 0.22um pore size syringe filter and then injected at 300mV with 10nA sensitivity for 13 minute runs except the CP, Nac and POA which was run with 20nA sensitivity because the dopamine signal was being clipped.

For quantification a standard curve with HVA, DPOAC, and dopamine in ISS was run at 10 & 20nA. DOPAC standards ranged from 100nM to 0.1nM. HVA standards ranged from 100nM to 1nM.

D_1 stimulated locomotion

To test D_1 mediated stimulation of locomotor activity mice were habituated to the automated activity recorder. Once mice displayed baseline locomotion within the chamber (no novelty induced locomotion) mice were injected with saline and recorded for 2 hours to quantify baseline activity. Mice were then injected with the D_1 agonist SKF81297 and their activity measured for another two hours (Usiello *et al.* 2000).

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 and GraphPad QuickCalcs (GraphPad; LaJolla, CA). Chi squared analysis was used to evaluate deviation from Mendelian ratios. Proportionality of hazards for survival curves were analyzed using SAS 9.3 (SAS Institute; Cary, NC). The Gehan-Breslow-Wilcoxon test was applied to survival and kindling data as opposed to logrank because the survival curves failed to meet the proportional hazard assumption required for valid application of logrank (Machin *et al.* 2006). In analysis of EEG data, a parametric unpaired t-test was performed assuming populations with different standard deviations using Welch's correction. Two tailed P values less than 0.05 were considered significant.

Chapter 3

Genetics of strain-dependent lethality and kindling

Summary

Chapter 2 introduced the *Gnao1* $^{+/G184S}$ mouse model and identified their enhanced response to PTZ kindling, abnormal EEG and spontaneous death. Here I identify a novel Chr17 modifier region that affects both PTZ kindling and spontaneous death in *Gnao1* $^{+/G184S}$ mutant mice. The initial region identified spanned from 41-70Mb. However this was further refined through use of recombinant mice to 41-51Mb. Consequently, I have identified a novel genomic locus, the Chr17 modifier region, which we term *Mogs1* (modifier of G-protein seizures), that should be examined as candidates in human epilepsy and sudden unexplained death in epilepsy (SUDEP).

I designed all experiments in collaboration with my mentor Dr. Neubig. I instructed undergraduates on how to perform genotyping and kindling which was predominately completed by them. Mouse breeding and final assembly and interpretation of data were performed by me. The genome-wide sequencing was performed by the DNA sequencing core based on a design by Dr. Neubig. Past and current undergraduates who meaningfully contributed to this work include Kevin Kohut, Kristen Gilbert, Madeline Pelz and Hans Dalton.

Introduction

The majority of epilepsy cases are not due to a known environmental cause, leaving genetic factors to play an important role (Michelucci *et al.* 2012). Rare mutations in ion channels (Ryan 1999), metabolic mechanisms (Suls *et al.* 2009), and neurodevelopmental pathways (Eksioglu *et al.* 1996) produce genetic epilepsies with strong Mendelian inheritance. Also, a catch-all category of "general genetic epilepsy" accounts for about 30% of patients (Michelucci *et al.* 2012) but the specific genes affected are not known. Indeed, most of the genes from well-defined familial epilepsies have been ruled out in those patients (Klassen *et al.* 2011). A role for more than one gene in individual patients (Leu *et al.* 2012) or modifier loci (Bergren *et al.* 2009; Meisler & Kearney 2005) has been proposed as an explanation for why the genetic basis has been difficult to discern.

Beyond causation, treatment response and prognosis are also highly variable among patients and are probably influenced by genetic determinants. In severe myoclonic epilepsy of infancy (SMEI), which is caused by loss of function in the sodium channel SCN1A (Claes *et al.* 2001), patients do not respond to typical anti-epileptic treatments that further decrease sodium channel activity. Efficacy of treatment response in the general epilepsy population is also determined in part by genetic modifiers (Loscher *et al.* 2009; Szoeke *et al.* 2006). Genetic factors also appear to contribute to the phenomenon of sudden unexplained death in epilepsy (SUDEP) (Sperling 2001). About 10-15% of SUDEP patients have mutations related to long QT syndrome that could lead to cardiac arrhythmias (Surges & Sander 2012) but the majority of cases remain unexplained.

Many labs have worked to understand the influence of genetics on modifying outcome in various seizure models. Loci on mouse Chr 1, 2, 3, 4, 5, and 6 have all been previously identified to modify seizures induced by acute Pentylenetetrazol (PTZ) (Ferraro *et al.* 1999; Legare *et al.* 2000). Mouse Chr 4, 15 and 8 have also been implicated in the Kainic Acid (KA) model (Schauwecker *et al.* 2004). Unfortunately due to the acute nature of these models, they do not provide insight into genetic modifiers of the remodeling process leading to many human epilepsy syndromes. Therefore better therapy for refractory cases relies upon a better understanding of the complex genetic interactions leading to epilepsy and identification of novel targets.

Here I identify a novel Chr17 modifier region that affects both PTZ kindling and spontaneous death in *Gnao1* $^{+/G184S}$ mutant mice. The initial region identified spanned from 41-70Mb. However this was further refined through use of recombinant mice to 41-51Mb. Consequently, we have identified two genomic loci, *Gnao1* and the Chr17 modifier which we term *Mogs1* (modifier of G-protein seizures), that should be examined as candidates in human epilepsy.

Results

Heterozygous *Gnao1*$^{+/G184S}$ mice experience strain-dependent spontaneous death

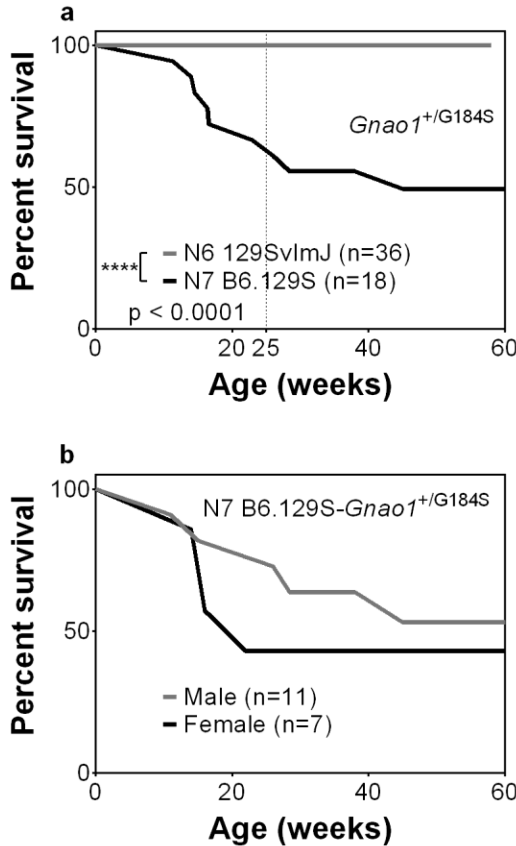


Figure 3.1 *Gnao1* $^{+/G184S}$ mice experience strain-dependent lethality
a) N6 129S1/SvImJ-*Gnao1* $^{+/G184S}$ and N7 B6 wildtype mice experience a relatively normal lifespan compared to N7 B6-*Gnao1* $^{+/G184S}$ (Gehan-Breslow-Wilcoxon df(1)=12.8 p<0.001) b) N7 B6-*Gnao1* $^{+/G184S}$ mice die prematurely regardless of gender

In contrast to the spontaneous death in B6 *Gnao1* $^{+/G184S}$ mice reported in Chapter 2, heterozygous *Gnao1* $^{+/G184S}$ on the 129S1/SvImJ background live a relatively normal lifespan with 100% surviving >1 year (Figure 3.1). The difference in outcome, based upon genetic background, suggests that a genetic approach could provide insights into the mechanism behind the *Gnao1* $^{+/G184S}$ mediated premature death and sensitivity to PTZ kindling.

Gnao1 $^{+/G184S}$ spontaneous death is modified by a region within 41-70 Mb on Chromosome 17

No deaths were observed up to 47 weeks in mutant B6129SF1 (F1) mice regardless of their gender (n=8 per sex per genotype). Thus 129S alleles appear to provide a dominant protective effect against the spontaneous death phenotype of *Gnao1*$^{+/G184S}$ mice or, conversely, the C57BL/6J background effect is recessive.

To define the genetic locus underlying the strain differences, the 11 male and 7 female N7 B6 *Gnao1*$^{+/G184S}$ mice from Figure 3.1b were split into two groups, the short-lived group was defined as those that died prior to the survival curve leveling off at ~ 25 weeks of age. The long-lived group included those that lived past the 25 week cutoff. Females were equally represented in both the short and long-lived cohorts. Males were skewed towards the long lived cohort, but this was not significant. The frequency and location of 129S alleles were compared between the short-lived and long-lived groups. Given the dominant effect of 129S alleles in F1 mice, we expected that a single copy of the 129S alleles in B6 mutant mice would be protective and therefore be found at a greater frequency in the long lived cohort. All mice retained 129S alleles on Chr 8 that are near the *Gnao1* locus as expected (Figure 3.2a). The only region that preferentially retained 129S alleles in the long-lived cohort was on Chr17 after 39Mb through the end of the chromosome (Figure 3.2 a & b). The x axis labels in Figure 3.2b represent the locations tested where SNPs differ between 129S and C57BL/6J mice. This initial analysis was not sufficiently powered to statistically validate the modifier due to the required correction for multiple comparisons.

To both confirm and reduce the size of the modifier, we obtained recombinant mice that only maintained 129S alleles at 47Mb and 62Mb but were homozygous for B6 alleles at 41Mb & 70Mb allowing us to roughly split the putative modifier region in half. In an independent experiment with these 44 mice, we tested the ability of the 41-70Mb subregion to afford protection, thus eliminating the need for the multiple comparison correction. The Chr17 129S 41-70Mb subregion protected B6-*Gnao1* $^{+/G184S}$ mice from spontaneous death in the independent cohort (Figure 3.2c Gehan-Breslow-Wilcoxon $df(1)=4.9$ $p < 0.05$).

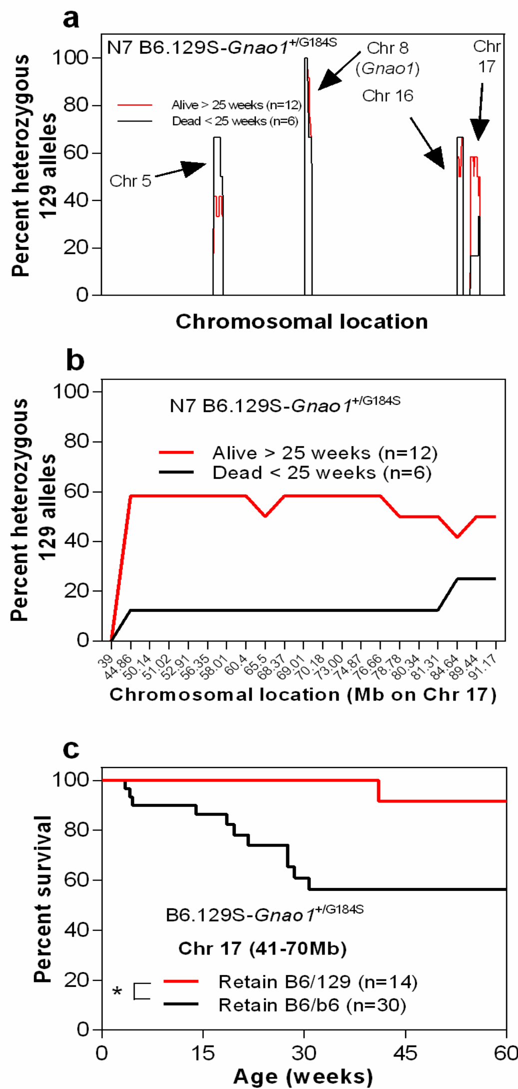


Figure 3.2 A locus on chromosome 17 modifies the strain-dependent lethality
a) Whole genome SNP analysis was done and retained 129S alleles were assessed. Plots show the percentage of the B6 *Gnao1* $^{+/G184S}$ mice carrying 129S alleles at a particular location in the genome. Those that lived past 25 weeks of age are in red and those that died prior are in black. b) Only one region on Chr 17 showed preferential retention of 129S alleles in the population that lived past 25 weeks of age. c) In an independent cohort we tested for the ability of the first half of this putative modifier region to enhance viability of B6-*Gnao1* $^{+/G184S}$ mice. Those mice retaining 129S alleles at both 47Mb and 62Mb (but homozygous B6 at 41Mb & 70Mb) on Chr 17 showed increased survival (Gehan-Breslow-Wilcoxon df(1)=4.9 $p < 0.05$).

Heterozygous *Gnao1* $^{+/G184S}$ mice also have a strain-dependent sensitization to PTZ kindling

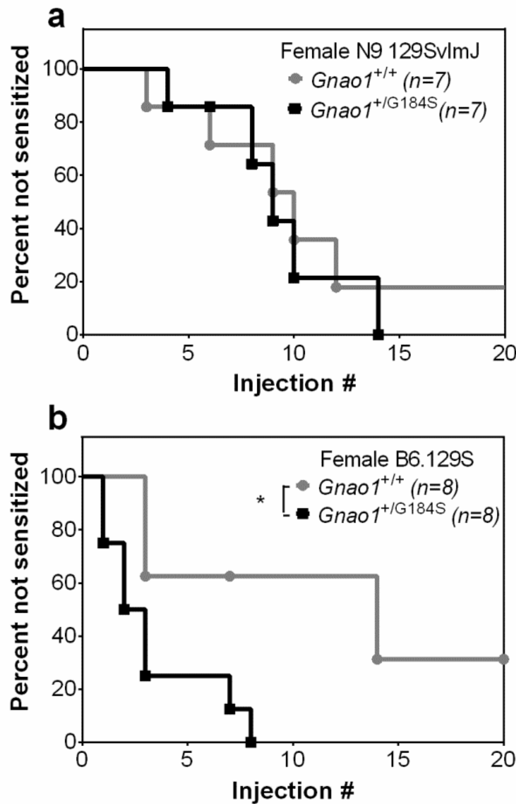


Figure 3.3 *Gnao1* $^{+/G184S}$ mice have a strain-dependent reduction in time to sensitize to repeated low dose PTZ
The time to undergo sensitization to PTZ kindling (see Methods for definition) was recorded. a) N7 129S-*Gnao1* $^{+/G184S}$ female mice sensitize in response to PTZ similar to control. b) B6-*Gnao1* $^{+/G184S}$ females sensitize more rapidly than littermate controls (Gehan-Breslow-Wilcoxon df(1)=5.74 $p<0.05$).

In the PTZ kindling model, paralleling the strain-dependent nature of the adult lethality, female N7 129S1/SvImJ *Gnao1*$^{+/G184S}$ mice generally showed no difference in sensitization to PTZ kindling compared to wild-type littermates (Figure 3.3a), while as reported in Chapter 2 female B6 *Gnao1*$^{+/G184S}$ mutants were more sensitive to PTZ kindling than control (Figure 3.3b Gehan-Breslow-Wilcoxon df(1)=5.74 $p<0.02$).

Chr 17 locus modifies PTZ susceptibility

We next tested the hypothesis that the Chr 17 spontaneous death modifier protected against the enhanced PTZ susceptibility. In this case, all experiments were restricted to females for reasons discussed in methods. Female F1 *Gnao1* $^{+/G184S}$ mice were protected from PTZ kindling sensitization compared to B6 *Gnao1* $^{+/G184S}$ female mice (Figure 3.4a, Gehan-Breslow-Wilcoxon df(1)=7.9 $p < 0.01$). This indicates, as in the spontaneous lethality, that a single copy of 129S alleles was sufficient for protection. 129S alleles within just the Chr 17 41-70Mb modifier region also protected B6 female *Gnao1* $^{+/G184S}$ from sensitization to kindling (Figure 3.4b Gehan-Breslow-Wilcoxon df(1)=4.28 $p<0.05$).We then asked whether the Chr 17-mediated protection against kindling depended on the presence of the *Gnao1* G184S allele. If so, that could indicate a specific interaction with G_o and a gene product from the modifier region. If not, the region is likely to be a general modifier of kindling unrelated to our G protein mutant model. To address this, we compared female B6 *Gnao1* $^{+/+}$ mice both with and without the Chr 17 modifier for the rate at which they were sensitized. The region did not afford robust protection against PTZ sensitization (Figure 3.4c). While there may be a modest effect without the *Gnao1* G184S allele, the striking effect in its presence prompted us to name this Chr17 locus *Mogs1* (modifier of G protein-induced PTZ susceptibility 1) in parallel with the *Moe1* and *Moe2* loci which have been previously described for protection against spontaneous seizures induced by sodium channel mutants (Bergren *et al.* 2005; Bergren *et al.* 2009).

The modifier *Mogs1*, however, does not appear to be the sole modifier locus. Analysis of F2 offspring from intercrosses of B6129SF1-*Gnao1* $^{+/G184S}$ mice showed that

only 4 out of 60 mice died before one year of age. Given that 25% of the F2 genome should be homozygous for B6 alleles at any given locus, 15 mice should have died if there was a single high-penetrance modifier region. Thus *Mogs1* is likely to be one of two or more dominant or additive modifier regions.

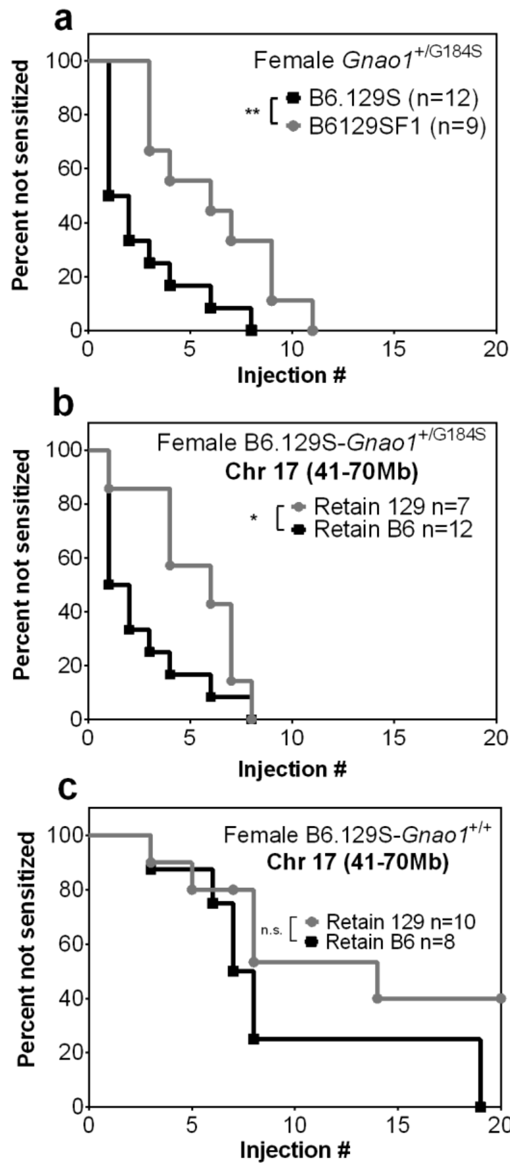


Figure 3.4 The locus on chromosome 17 also protects B6-*Gnao1* $^{+/G184S}$ mice from sensitization to kindling. a) Female B6-*Gnao1* $^{+/G184S}$ female mice sensitize to kindling faster than female F1-*Gnao1* $^{+/G184S}$ mice demonstrating that a single copy of 129 alleles is sufficient to delay sensitization to PTZ kindling (Gehan-Breslow-Wilcoxon df(1)=7.9 $p < 0.01$). b) The onset of sensitivity to kindling for B6-*Gnao1* $^{+/G184S}$ mutants is delayed by the presence of a single copy of 129S alleles in the 41-70Mb Chr 17 modifier region. c) In a sample of similar size to that in b, the Chr 17 modifier region did not significantly alter sensitization to PTZ kindling in B6-*Gnao1* $^{+/+}$ mice.

One modifier is likely linked to 47Mb on Chr17

Given the size of the modifier region and large number of genes present therein I have developed several *Gnao1* $^{+/RGSi}$ lines that contain subregions of the 41-70Mb modifier. Regions currently developed include markers at 47Mb, 62Mb, 51-62Mb, and 51-57Mb. Each of these lines contains 129 alleles at the locations listed in their name but are pure B6 at all the other test sites on Chr17. They are, however, not defined by whole genome mapping. Some still have 129 alleles on Chr5 or Chr16. Subsequent breeding will be required to produce true congenic mice containing the various Chr17 129 allele sets. Preliminary results using offspring of the 62Mb line, which has a single copy of 129 alleles surrounding 62Mb on Chr17, rule out this region as a strong modifier. The Chr17 47Mb line demonstrated significant protection for those mice retaining 129 alleles at 47Mb alone (Figure 3.5b, Gehan-Breslow-Wilcoxon df(1)=.3.68 $p < 0.05$). Thus at least one modifier gene from *Mogs1* is likely to occur between 41-51Mb as these mice are pure B6 outside of the 41 and 51Mb region on Chr 17.

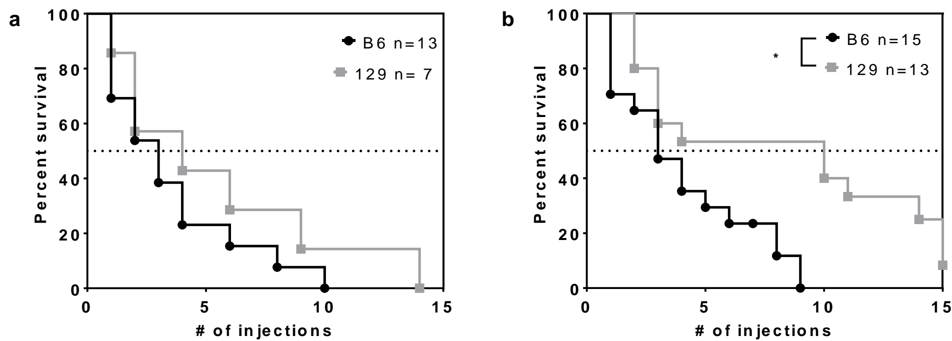


Figure 3.5 One modifier within *Mogs1* occurs between 41-51Mb
Female *Gnao1* $^{+/G184S}$ mice were generated from a male carrying both a subregion of the 41-70Mb and the *Gnao1*G184S allele. a) 129S alleles around 62Mb on Chr17 appear unable to protect from kindling b) 129S alleles between 41-51Mb are protective from *Gnao1* $^{+/G184S}$ induced sensitization to PTZ kindling

Discussion

In this Chapter I demonstrated that the PTZ kindling and spontaneous death were strain-dependent. This provided an experimental handle allowing us to identify a genetic modifier locus. The modifier region *Mogs1* (Chr 17, 41-70 Mb) alters both the spontaneous death and PTZ-induced kindling phenotype in the *Gnao1* $^{+/G184S}$ mice. This region has been further refined by demonstrating that 41-51Mb is sufficient to afford protection (*Mogs1a*). However, the rest of the region has not been conclusively eliminated yet. Interestingly, the protective modifier alleles on Chr17 come from the 129S strain, which is more sensitive to PTZ kindling compared to C57BL/6J. The modifier region also appears to have its effect on PTZ susceptibility in G_o mutant mice but not control mice. *Mogs1* also may not influence response to acute PTZ administration as it was not found in a genomic analysis of acute PTZ-induced seizures (high dose - 80 mg/kg) in B6 vs DBA mice (Ferraro *et al.* 1999). These two observations suggest that this modifier region (*Mogs1*) may have a unique interaction with G_o function in PTZ kindling-type remodeling mechanisms.

The *Mogs1* region (41-70Mb) includes the coding sequences of ~ 130 known proteins. One that was an obvious candidate was Rab5a protein which has been proposed as a novel G_o effector in studies in Drosophila (Purvanov *et al.* 2010). Loss of an effector molecule could easily suppress a gain-of-function mutation in G_o. There are no non-synonymous coding SNPs in *Rab5a* and despite several SNPs in the 5' and 3' UTR, there were no changes in mRNA expression as detected by quantitative real-time PCR (data not shown). Thus it is unlikely that *Rab5a* is the modifier gene. To develop a list of other possible candidates, we looked for non-synonymous SNPs or splice site variants

identified in the Sanger informatics website (Table 3.1). Three proteins have altered stop codons. Two genes, *9130008F23Rik* K55* and *Trem2* 149*, have premature stops while *Yipf3* lost a stop codon, *202W. The gene *Polh* has a variant in a splice site while *Ttbk1, Tbc1d5* and *Lrfn2* have in-frame dels in the 129S sequence. For non-synonymous SNPs, the predicted effect on protein function was assessed using Provean (Choi *et al.* 2012). Seven genes *Pgk2*, *Gpr115*, *Gm5093*, *BC048355*, *Lrfn2*, *Kcnh8* and *Vmn2r118* were predicted to have non-synonymous SNPs disruptive of protein function in the 129S strain. Given that the informatics databases that I had access to may be incomplete and that distal SNPs may influence gene expression, a high-priority candidate list of 20 genes with known CNS function or a role in GPCR signaling was generated regardless of Sanger SNP status (Table 3.2). These lists represent a starting point for future candidate analysis in conjunction with higher resolution experimental mapping of *Mogs1*. Whole exome and targeted exome sequencing is currently underway.

The approach that we took to find this modifier region started from N7 mice in which significant selection for loci affecting survival of the mutants may have already occurred. Consequently, it cannot be concluded that *Mogs1* is the only modifier. Indeed, a more unbiased approach suggests that it is not. Analysis of F2 crosses from (C57BL/6J x 129/SvImJ *Gnao1* $^{+/G184S}$)F1 mice showed that only 4 out of 60 mice died before one year of age. Given that 25% of the F2 genome should be pure B6, 15 mice should have died if there was a single high-penetrance suppressor region. Thus *Mogs1* is likely one of two or more modifier regions.

The present results buttress recent work implicating alleles encoding GPCRs, RGS proteins, and G_o as candidates in future human genetic analyses of epilepsy. Only

12 non-synonymous SNPs were reported in *GNAO1* in the exome variant server database out of ~13,000 alleles tested (evs.gs.washington.edu). Most were conservative, suggesting that damaging mutations may not be tolerated for medallion inheritance. However paralleling the *de novo* mutations found in severe early onset epilepsies, a recent report by Nakamura *et al.* documented *de novo* mutations in *GNAO1* in four patients with epileptic encephalopathy (Nakamura *et al.* 2013). Interestingly one of the *de novo* mutations also occurs within the switch II region of G_o, as our G184S mutation does, and involves a similar substitution of a bulky amino acid for glycine. Thus, while not likely to contribute to inherited epilepsies, *GNAO1* may be a particularly important candidate to consider when structural abnormalities of the brain are comorbid with epilepsy in human patients. Studies of our animal model should also provide an attractive opportunity to learn more about brain function and treatment options in these patients.

In this chapter we defined a novel epilepsy modifier locus, *Mogs1*, on Chr 17. The mechanism underlying this model, enhanced G_o signaling, is a surprising cause of a seizure disorder as $G_{i/o}$ signaling has typically been thought to have neuroinhibitory actions. This could, however, result from an overall greater inhibition of inhibitory signaling leading to a net gain in excitatory signaling. Our results thus suggest new candidate genes for genetic epilepsies. Also the modifier effect is consistent with an emerging recognition that complex diseases may depend on multiple rare mutations, preventing their detection in large GWA studies (Sisodiya & Mefford 2011; Tan *et al.* 2004). A better understanding of G_o signaling in seizure disorders may facilitate the identification of novel GPCR drug targets for epilepsy.

Materials and Methods

Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health; all experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Experiments were performed using male and female mice as indicated in methods below. All experimental mice were group-housed with unlimited access to food and water until experimentation. Lights were maintained on a 12 hour light/dark cycle with lights on at 5:00/6am depending on time of year. All experiments were conducted during the light phase with the exception of the EEG video recordings which were throughout the light cycle.

Generation of *Gnao1* $^{+/G184S}$ mice and perinatal viability experiments

Most details were reported in Chapter 2. Only additional details are provided here. A single N6 B6-*Gnao1*$^{+/G184S}$ male ancestor gave rise to all B6-*Gnao1*$^{+/G184S}$ mice presented in this study. The reduced viability and premature lethality of the *Gnao1*$^{+/G184S}$ mutants reported in chapter 2 precluded congenic development due to strain collapse by N10. Thus, data presented in this paper are from N7, N8, and N9 which will collectively be referred to as B6. To control for the genetic variability within the generations, we tested regions that had 129S alleles present in the N6 founder to ensure that retention of other 129S regions did not influence the results. All B6129SF1-*Gnao1*$^{+/G184S}$ and littermate control mice were generated from crosses with *Gnao1*$^{+/G184S}$ 129S1/SvImJ male mice and C57BL/6J females to ensure genetic homogeneity.

Genotyping

See Chapter 2 for details on genotyping. For genome-wide SNP analysis mouse

tail DNA was purified using proteinase K (Invitrogen) digestion followed by ethanol precipitation.

Viability

Data on survival to weaning was determined by observed genotype frequencies of male and female mice at 2-3 weeks of age using 41 N4 129S1/SvImJ (25 males and 16 females) and 55 N4 B6;129 (29 males and 26 females) offspring from heterozygote x heterozygote crosses. For adult survival in Figure 3.1 data were collected from 36 N6 129S1/SvImJ-*Gnao1*$^{+/G184S}$ (10 males 26 females) and 18 N7 B6.129 -*Gnao1*$^{+/G814S}$ (11 males and 7 females) group-housed mice. All N7 B6 mice in this figure were sired by the single N6 B6 founder male crossed to C57BL/6J females that were within F4 generations from mice purchased directly from Jackson Labs.

Identification of modifier region

To determine whether the protective effect of the 129S1/SvImJ background on spontaneous lethality was dominant, we generated mutant B6129SF1 mice from an N7 129S1/SvImJ male mouse carrying the *Gnao1* G184S allele and assessed them for deaths out to 47 weeks of age (see results). After determining that 129S alleles were dominant in protecting from premature lethality we employed a genome-wide scan on the 18 mice from the survival curve study in Figure 3.1b (11 N7F0 B6 -*Gnao1*$^{+/tm2Rneu}$ males & 7 females) to identify regions where 129S alleles were preferentially retained in mice that lived past the early phase of mortality (i.e. 25 weeks). The scan was performed utilizing an Illumina GoldenGate medium density mouse panel at the University of Michigan Sequencing core. A confirmation study on Chr 17 was then done to overcome the low power of the initial screen and reduce the modifier's size using an independent cohort of

drug naïve, group-housed B6-Gnao1$^{+/G184S}$ mice (10 males & 4 females retain 129 on Chr17, 12 females & 18 males lack 129 on Chr17). They carried 129S alleles at 47Mb and 62Mb on Chr17 but had only B6 alleles by 41Mb and 70Mb. Tests were also performed on Chr 5 and Chr16 as covariates to ensure they did not influence the results as these mice were not congenic for 129S only on Chr 17.

Each primer pair amplified two discrete SNPs identified on the Jackson lab informatics website (informatics.jax.org) to limit sequencing errors. DNA sequences surrounding the two SNPs were obtained from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway NCBI37/mm9 NCBI37/mm9 build). Primers were then designed using Primer 3 (http://frodo.wi.mit.edu/primer3/ v.0.4.0) using the rodent mispriming database and only primers that were a minimum of 60 bases and a maximum of 600 bases from the desired SNPs were selected. PCR primers and the SNPs tested are listed in Table 3.3. All PCR reactions were performed with the same cycling parameters and reagent concentrations used for genotyping except the annealing temperature was 59°C. PCR products were isolated by ethanol precipitation and sequenced by the University of Michigan DNA Sequencing Core. Chromatograms were analyzed to determine which alleles were carried within the modifier region.

Seizure susceptibility and epileptogenesis susceptibility

See Chapter 2 methods for details on the kindling protocol. For comparison across strains, we relied on the fact that kindling is a repeated process and thus should sample various points within the estrus cycle as different strains of mice must be housed separately. To assess the ability of the Chr17 modifier to influence response to PTZ, we only analyzed mutant B6 females. Females were group housed together a minimum of

two weeks prior to initiation of kindling. Experiments were limited to females due to the limited number of mutant males available for breeding, and animal use protocol requirements that preclude combining males from separate litters which would have increased environmental variability.

Candidate gene identification

The exhaustive list of candidate genes was generated with Biomart (http://www.ensembl.org/biomart/martview*)*. A list of known non-synonymous SNPs within the modifier region was generated from the Sanger informatics website (http://www.sanger.ac.uk/resources/mouse/genomes/). These were then translated into relative amino acid substitutions for specific splice variants using Ensembl. Provean, a computational method with predictive power similar to SIFT and PolyPhen-2 was then used to determine likely functional consequences (http://provean.jcvi.org/index.php) (Choi *et al.* 2012).

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 and GraphPad QuickCalcs (GraphPad; LaJolla, CA). Chi squared analysis was used to evaluate deviation from Mendelian ratios. Proportionality of hazards for survival curves were analyzed using SAS 9.3 (SAS Institute; Cary, NC). The Gehan-Breslow-Wilcoxon test was applied to survival and kindling data as opposed to logrank because the survival curves failed to meet the proportional hazard assumption required for valid application of logrank (Machin *et al.* 2006). In analysis of EEG data, a parametric unpaired t-test was performed assuming populations with different standard deviations using Welch's correction. Two-tailed P values less than 0.05 were considered significant.

Informatics Tables

Ensembl Gene ID	*Gene*	Number of SNPs			Predicted Effect of coding nsSNPs
		5'UTR	NSC	3'UTR	
ENSMUSG00000031233	*Pgk2*		1		Deleterious
ENSMUSG00000090747	*Gm17495*	1	3*		Neutral
ENSMUSG00000073396	*Gm6084*		1	1	Neutral
ENSMUSG00000023930	*Crisp2*	1		1	
ENSMUSG00000023926	*Rhag*	1		1	
ENSMUSG00000054951	*9130008F23Rik*		6		Premature stop
ENSMUSG00000091043	*Glyatl3*		2*		Neutral
ENSMUSG00000023919	*Cenpq*	4	3		Neutral
ENSMUSG00000023921	*Mut*			15	
ENSMUSG00000042256	*3110082D06Rik*			29	
ENSMUSG00000023918	*Gpr115*	3	4	10	Deleterious
ENSMUSG00000057899	*Gpr111*		3	3	Neutral
ENSMUSG00000061665	*CD2ap*	1	2	5	Neutral
ENSMUSG00000023915	*Tnfrsf21*	3	1	6	Neutral
ENSMUSG00000041293	*Gpr110*	2	3	5	Neutral
ENSMUSG00000056492	*Gpr116*			44	
ENSMUSG00000023914	*Mep1a*			1	
ENSMUSG00000023913	*Pla2g7*	1	1	3	Neutral
ENSMUSG00000040140	*Tdrd6*		8		Neutral
ENSMUSG00000023912	*Slc25a27*			1	
ENSMUSG00000023963	*Cyp39a1*		2	3	Neutral
ENSMUSG00000039601	*Rcan2*	1			
ENSMUSG00000023960	*Enpp5*	2	2	1	Neutral
ENSMUSG00000023961	*Enpp4*			20	
ENSMUSG00000023959	*Clic5*			22	

ENSMUSG00000039153	*Runx2*	4		9	
ENSMUSG00000038954	*Supt3h*	1			
ENSMUSG00000023932	*Cdc5l*		2#	1	All NSC low confidence
ENSMUSG00000023949	*Tcte1*			1	
ENSMUSG00000096847	*Tmem151b*		1#		All NSC low confidence
ENSMUSG00000037089	*Slc35b2*	1		1	
ENSMUSG00000023951	*Vegfa*			1	
ENSMUSG00000034509	*Ma*D_{2L}*1bp*		1		Neutral
ENSMUSG00000023952	*Gtpbp2*	1		3	
ENSMUSG00000023953	*Polh*	2	2*		Neutral
ENSMUSG00000067150	*Xpo5*		2		Neutral
ENSMUSG00000071074	*Yipf3*		1	3	Premature stop
ENSMUSG00000071073	*Gm88*		1*		
ENSMUSG00000012296	*Tjap1*	2	1	1	Neutral
ENSMUSG00000032842	*Abcc10*	3			
ENSMUSG00000015597	*Zfp318*	2	2	1	Neutral
ENSMUSG00000067144	*Slc22a7*		2		Neutral
ENSMUSG00000091742	*Gm5093*		6*		Deleterious
ENSMUSG00000015599	*Ttbk1*		3#	8	Neutral
ENSMUSG00000040658	*BC048355*		3	1	Deleterious
ENSMUSG00000040327	*Cul9*	1	6*		Neutral
ENSMUSG00000015605	*Srf*	1		3	
ENSMUSG00000023972	*Ptk7*		2	2	Neutral
ENSMUSG00000003546	*Klc4*			3	
ENSMUSG00000038545	*Cul7*	1	1		Neutral
ENSMUSG00000063576	*Klhdc3*		1		Neutral
ENSMUSG00000002768	*Mea1*			2	

ENSMUSG00000059409	*Ppp2r5d*	2		4	
ENSMUSG00000002763	*Pex6*	1	1	1	Neutral
ENSMUSG00000023973	*Cnpy3*		1	5	Neutral
ENSMUSG00000062619	*2310039H08Rik*	1			
ENSMUSG00000063888	*Rpl7l1*			3	
ENSMUSG00000036568	*BC032203*			1	
ENSMUSG00000036430	*Tbcc*			1	
ENSMUSG00000023978	*Prph2*			2	
ENSMUSG00000023977	*Ubr2*			1	
ENSMUSG00000064043	*Trerf1*		2*		Neutral
ENSMUSG00000034729	*Mrps10*	6	3		Neutral
ENSMUSG00000023979	*Guca1b*		2	13	Neutral
ENSMUSG00000023982	*Guca1a*	3	1		Neutral
ENSMUSG00000047150	*1700001C19Rik*		1	5	Neutral
ENSMUSG00000034382	*AI661453*		1*	1	
ENSMUSG00000034165	*Ccnd3*	1			
ENSMUSG00000090115	*Usp49*			1	
ENSMUSG00000032717	*Mdfi*		2	2	Neutral
ENSMUSG00000073386	*9830107B12Rik*			4	
ENSMUSG00000042265	*Trem1*			6	
ENSMUSG00000051682	*Treml4*		3	3	Neutral
ENSMUSG00000071068	*Treml2*	2	2	4	Neutral
ENSMUSG00000023992	*Trem2*		6	3	Premature Stop
ENSMUSG00000023994	*Nfya*	2		5	
ENSMUSG00000040771	*AI314976*	2		1	
ENSMUSG00000040694	*Apobec2*		1	1	Neutral
ENSMUSG00000023995	*Tspo2*			1	
ENSMUSG00000043592	*Unc5cl*		1	7	Neutral
ENSMUSG00000040490	*Lrfn2*		1		Deleterious

ENSMUSG00000023999	*Kif6*			22	
ENSMUSG00000039316	*Rftn1*		1	2	Neutral
ENSMUSG00000067103	*AY702103*			16	
ENSMUSG00000010592	*Dazl*	1		2	
ENSMUSG00000044645	*Gm7334*		1*		
ENSMUSG00000023923	*Tbc1d5*	1	1#	23	
ENSMUSG00000035580	*Kcnh8*		1		Deleterious
ENSMUSG00000017831	*Rab5a*	1		4	
ENSMUSG00000044957	*4921523A10Rik*		1		Neutral
ENSMUSG00000000708	*Kat2b*	1		1	
ENSMUSG00000023940	*Sgol1*	4	5	4	Neutral
ENSMUSG00000023122	*Sult1c2*	2			
ENSMUSG00000023945	*Slc5a7*	1		16	
	Gm17474		4^		
ENSMUSG00000024172	*St6gal2*	1	1	36	Neutral
ENSMUSG00000091504	*Vmn2r118*		7		Deleterious
ENSMUSG00000024174	*Pot1b*		1	9	Neutral
ENSMUSG00000032915	*Emr4*			1	
ENSMUSG00000002831	*Plin4*	1	1	1	Neutral
ENSMUSG00000001228	*Uhrf1*			1	
ENSMUSG00000013236	*Ptprs*			1	
ENSMUSG00000042625	*Safb2*		3*	6	Neutral
ENSMUSG00000071054	*Safb*			2	
ENSMUSG00000049760	*2410015M20Rik*			1	
ENSMUSG00000041168	*Lonp1*		1		Neutral
ENSMUSG00000054723	*Vmac*			1	

ENSMUSG00000002379	*Ndufa11*	1			
ENSMUSG00000024207	*Acsbg2*	1	1	1	Neutral
ENSMUSG00000024209	*1700061G19Rik*			1	
ENSMUSG00000024212	*Mllt1*			2	
ENSMUSG00000045019	*Acer1*			5	
ENSMUSG00000002660	*Clpp*			1	
ENSMUSG00000002664	*Pspn*		1		Neutral
ENSMUSG00000007670	*Khsrp*	1	1*	4	
ENSMUSG00000046329	*Slc25a23*			1	
ENSMUSG00000044279	*Crb3*	1	1	2	Neutral
ENSMUSG00000002668	*Dennd1c*		1		Neutral
ENSMUSG00000038048	*Cntnap5c*		2		Neutral
ENSMUSG00000024227	*2610034M16Rik*			6	
ENSMUSG00000023965	*Fbxl17*			2	

Table 3.1 Variants between C57BL/6J and 129S1/SvImJ between 41-70Mb on Chromosome 17
Non-synonymous SNPs (nsSNPs) in the coding region and SNPs in 5' or 3' UTRs were determined between the C57BL/6J and 129S1/SvImJ strain using the Sanger Mouse Genomes Project website. Effects of non-synonymous SNPs were predicted using Provean with a cutoff score of -2.5. See methods for details. * Indicates not all SNPs could be identified in Ensembl and thus the prediction is only based on those SNPs that were found in both Sanger and Ensembl. # Indicates some or all of the predicted SNPs are low confidence and were not used to compute potential changes in protein function. ^ Indicates that the protein was not found in ensemble and therefore no prediction could be made.

Gene	Location on Chr 17 (Mb)	Function	Reference
Gpr115	42.79	Orphan GPCR	
Gpr111	42.84	Orphan GPCR	
Gpr110	43.4	Orphan GPCR	
Gpr116	43.5	Orphan GPCR	
Rcan2	43.94	Regulates calcineurin activity in brain	(Porta *et al.* 2007)
Slc29a1	45.72	Transporter that regulates adenosine levels and controls sensitivity to ethanol withdrawal induced seizures	(Kim *et al.* 2011)
Gtpbp2	46.29	GTPase of unknown function	
Ubr2	47.1	Influences neural stem cell development	(Naujokat 2009)
Foxp4	48	transcription factor involved in brain development	(Takahashi *et al.* 2008)
Mocs1	49.6	Deficiency leads to altered EEGs	(Sie *et al.* 2010)
Satb1	51.9	Controls dendritic spine density during development	(Balamotis *et al.* 2012)
KcnH8	52.7	Potassium channel in a region linked to temporal lobe epilepsy	(Combi *et al.* 2005)
Rab5a	53.6	Putative G_o effector	(Purvanov *et al.* 2010)
Slc5a7	54.4	choline transporter	(Sales *et al.* 2010)
St6gal2	55.6	Sialylation affects neural plasticity	(Okabe *et al.* 2001)
Sema6b	56.3	maturation of dendritic spines	(Pasterkamp & Giger 2009)
Nrtn	56.9	KO has reduced hippocampal kindling	(Nanobashvili *et al.* 2000)
Pspn	57.1	neuronal survival and outgrowth	(Yang *et al.* 2004)
Gpr108	57.4	Orphan GPCR	

Table 3.2 Candidate Genes in the extended Chr17 region (41-70Mb) based upon function
The gene list for the entire conservative estimate of the modifier region from 39-65.5Mb on Chr 17 was examined using Biomart. A literature search was performed for each gene for a connection to epilepsy, GPCR signaling, seizure threshold and sudden death. Those genes with a connection to one of the search terms are provided in the table.

SNP names	Chromosome location	Forward Primer	Reverse Primer
rs29685319 rs29546941	5 – 5.9Mb	Tcaggaaaccctactcccta	tcattgcaagaaagccaaact
rs4221298 rs4221299	16 - 96Mb	Tccaaggaggccaaagatact	aaaaacagggtacaggcatcc
rs33479194 rs13482982	17 – 41Mb	Ctgtgttcccaaccacctct	tctagtcagtgcctcgctca
rs29605468 rs33703249	17 - 47Mb	Tggcaagacgtgttcattttc	tcagagggcttttgaggagag
rs33050836 rs29523235	17 - 62Mb	Ggtcatctggaggagcaacc	gaacgcgagtgacttgatgc
rs33464243 rs33400606	17 – 70Mb	Catggcctcaggtaggtgata	tttgaactgcttgaaggaagc

Table 3.3 Primer sequences for each SNP tested
Regions of retained 129S alleles on each chromosome were analyzed using the Jackson Lab informatics website to identify a test site with two SNPs within a 100 bp region. DNA sequences flanking this region were then obtained from the UCSC Genome Browser and subjected to primer design using the primer3 website. Validated primers are listed in the table above. See methods for further detail.

Chapter 4

Disruption of GIRK channel activation in G_o gain-of-function mice

Summary

Chapter 2 provided an initial characterization of the *Gnao1* $^{+/RGSi}$ mice. The chapter also highlighted a conundrum in that mutant mice are susceptible to PTZ kindling despite initial expectations they would be protected. Chapter 3 worked towards dissecting the mechanism behind the enhanced spontaneous death and susceptibility to PTZ kindling using a genetic approach. Now I will shift gears to approach the problem from a receptor-centric view by working to better understand signaling through three receptor systems implicated in epilepsy, $GABA_{B,}$, μ-opioid, and 5-HT_{1A}.

Here I demonstrate that *Gnao1* $^{+/RGSi}$ exhibit normal motor impairment due to baclofen, but exhibit substantially less hypothermia. *Gnao1* $^{+/RGSi}$ mice also experience diminished hypothermia due to a variety of pharmacological treatments known to depend upon GIRK channel function. The altered response of *Gnao1* $^{+/ RGSi}$ mice may be due to diminished GIRK signaling within the Periaqueductal Gray (PAG), a key region in thermoregulation. The decrease in GIRK channel activity is unlikely to be due to a simple loss-of-function of the mutant Gα subunit as mice lacking 50% of G_o do not have equivalent changes.

This work was highly collaborative. I designed all experiments and then instructed and supervised undergraduates who performed the work with the following exceptions: 1) Nick Senese generated the morphine hypothermia data following my protocols 2) Susan Ingram and Minghua Li designed and planned all electrophysiology involving the PAG. Past and current undergraduates who meaningfully contributed to this work by running behavioral experiments include Kevin Kohut, Matthew Stern, and Hans Dalton.

Introduction

Side-effects of clinically prescribed drugs are a major health problem. Roughly 30% of patients on medication for a chronic condition stop treatment due to side-effects (Dusing *et al.* 1998; Hugtenburg *et al.* 2006). This and other issues of non-compliance with prescription drugs are associated with $289 billion in annual healthcare costs and lead to over 100,000 deaths per year within the US alone (Viswanathan *et al.* 2012). With many current therapies focused on activation of G-protein Coupled Receptors (GPCR), it may be impossible to pharmacologically separate on-target effects from side-effects. For example baclofen, a GPCR agonist, induces both muscle relaxation and disrupts thermoregulation through $GABA_B$ receptor activation (Costa *et al.* 2005; Pravetoni & Wickman 2008).

Through modulation of G-protein activity downstream of GPCR activation it may be possible to potentiate desired effects while minimizing side-effects. This is a result of the region specific localization of G-proteins and their negative regulators, the Regulators of G-protein Signaling (Blazer & Neubig 2009). For example, we recently demonstrated

that mice with enhanced G_{i2} signaling have enhanced 5-HT_{1A} mediated antidepressant action without altering 5-HT_{1A} mediated hypothermia (Talbot *et al.* 2010).

In this work I specifically chose to focus on one clinically relevant side-effect, disruption of thermoregulation. Two to three percent of patients treated with anti-psychotics will develop hyperthermia associated with Neuroleptic Malignant Syndrome (NMS) (Mann & Lazarus 2003). NMS has also been observed in the treatment of Parkinson's disease and with the use of the anti-spastic agent baclofen (Mann & Lazarus 2003; Turner & Gainsborough 2001). A host of other drugs, including anti-depressants (SSRIs, MOIs) and ecstasy (MDMA), can induce CNS-mediated hyperthermia (Eyer & Zilker 2007). As of 2004, ecstasy-induced hyperthermia was treated by ice baths (Fantegrossi *et al.* 2004). With treatment, the aforementioned hyperthermia syndromes are still lethal in 40% of patients (Mann & Lazarus 2003). With several distinct drug classes potentially leading to fatal hyperthermia further research in how these drugs alter thermoregulation is warranted.

The inhibitory G-protein family, $G_{i/o}$ is known to mediate actions of a variety of GPCRs that disrupt thermoregulation through G-protein-coupled Inwardly-Rectifying Potassium (GIRK) channel activation, including – 5-HT_{1A}, $GABA_B$, and the μ opioid receptors (Albert & Robillard 2002; Costa *et al.* 2005). Unfortunately, *in vitro* transfected systems are typically unable to identify which $G_{i/o}$ family member is responsible for a given signaling output (Fernandez-Fernandez *et al.* 2001). This is compounded by the difficulty of extrapolating from a cellular response to a physiological one.

I hypothesize that distinct $G_{i/o}$ family members are involved in specific subsets of the therapeutic and/or side-effects of many clinically relevant drugs.

Here I demonstrate that mice with enhanced G_o signaling experience expected motor impairment due to baclofen, but exhibit substantially less hypothermia. *Gnao1* $^{+/RGSi}$ mice also experience diminished hypothermia due to a variety of pharmacological treatments known to depend upon GIRK channel function. The altered response of *Gnao1* $^{+/RGSi}$ mice may be due to diminished GIRK signaling within the Periaqueductal Gray (PAG), a key region in thermoregulation (Romanovsky 2007); (Bachtell *et al.* 2003; Yoshida *et al.* 2005); (de Menezes *et al.* 2006). The decrease in GIRK channel activity is unlikely to be due to a simple loss-of-function of the mutant Gα subunit as mice lacking 50% of G_o do not have equivalent changes. Thus we have demonstrated, for the first time, the ability to selectively modulate a subset of baclofen's effects by targeting downstream effectors.

Results

Gnao1 $^{+/RGSi}$ mice have decreased hypothermia but equivalent or slightly enhanced motor impairment due to baclofen

Given that $GABA_B$ receptors are thought to signal through G_o (Campbell *et al.* 1993; Greif *et al.* 2000), we tested two physiological outputs of $GABA_B$ receptor activation, hypothermia and motor impairment. Each of these effects is thought to be due to GIRK channel activation (Costa *et al.* 2005; Pravetoni & Wickman 2008). However, different brain regions are likely involved. The neural basis of baclofen-mediated ataxia has been localized to the cerebellum, a region not associated with thermoregulation

(Maity *et al.* 2012). B6-*Gnao1* $^{+/RGSi}$ male mice have reduced hypothermia but normal motor impairment in response to baclofen administration (Figure Figure 4.1, Two-way ANOVA: **1A** $F(1, 14)= 28.89$ $p < 0.0001$, **1B** $F(1, 19)= 16.32$ $p < 0.001$).

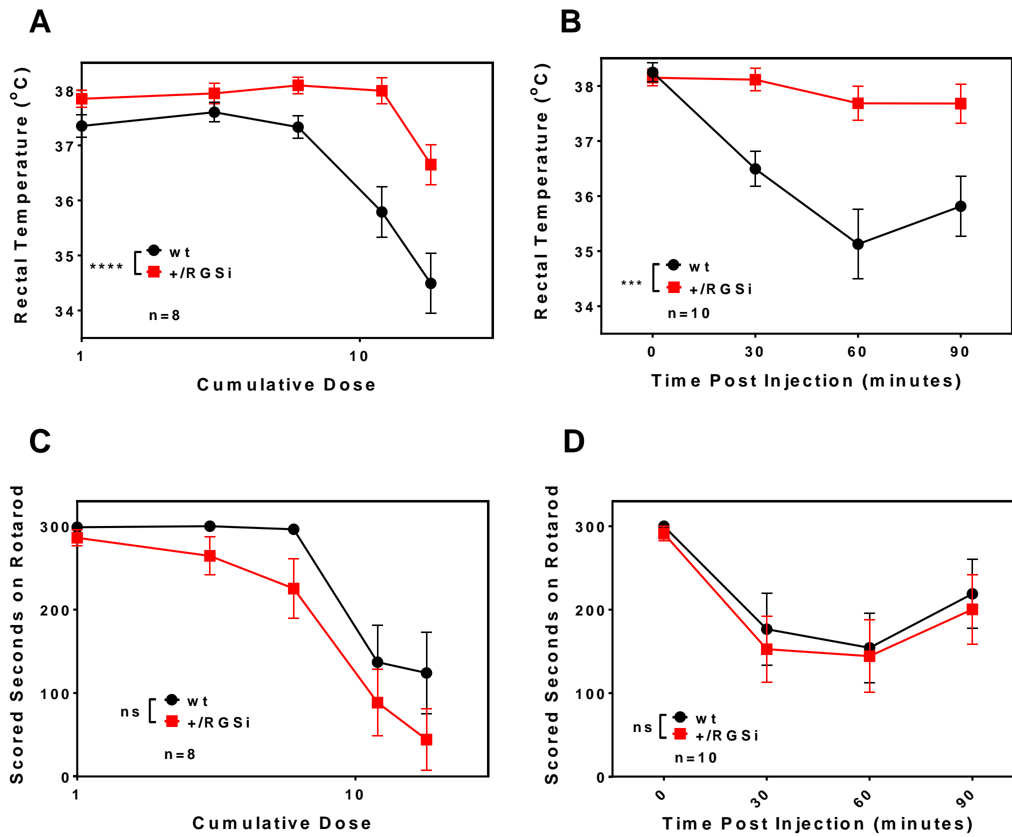


Figure 4.1 *Gnao1*$^{+/RGSi}$ mice have an altered response profile to baclofen
A) Hypothermia is reduced in response to escalating doses of baclofen in B6-*Gnao1* $^{+/RGSi}$ mice. **B)** Hypothermia is reduced in response to a 6mg/kg baclofen in B6-*Gnao1* $^{+/RGSi}$ mice. **C)** Motor impairment is equivalent across genotypes due to escalating doses of baclofen. **D)** Kinetics of motor impairment are unaffected by genotype.

Gnao1 $^{+/RGSi}$ mice have decreased pharmacologically induced hypothermia by other drugs that act through GIRK channels

Due to limited viability of mutant mice on the B6 background we switched to using B6129F1 mice (F1). In Figure 4.2A we reproduce the defect in baclofen-mediated hypothermia in F1 *Gnao1* $^{+/RGSi}$ mice indicating that the phenotype is not background-dependent (2-way ANOVA, $F(1, 7) = 8.26$ $p=0.02$). F1 *Gnao1* $^{+/RGSi}$ mice also have a reduction in hypothermia due to 5-HT_{1A} and μ-opioid receptors as assessed by their respective agonists, 8-OH-DPAT and morphine. Each receptor is known to induce hypothermia, in part, through GIRK channels (Figure 4.2B morphine single-dose $F(1,8)= 88.97$ $p<0.0001$; Figure 4.2C single-dose 8-OH-DPAT 2-way ANOVA $F(1,14)=43.41$ $p< 0.0001$, Figure 4.2D escalating-dose $F(1, 7)= 15.44$ $p<0.01$) (Costa *et al.* 2005).

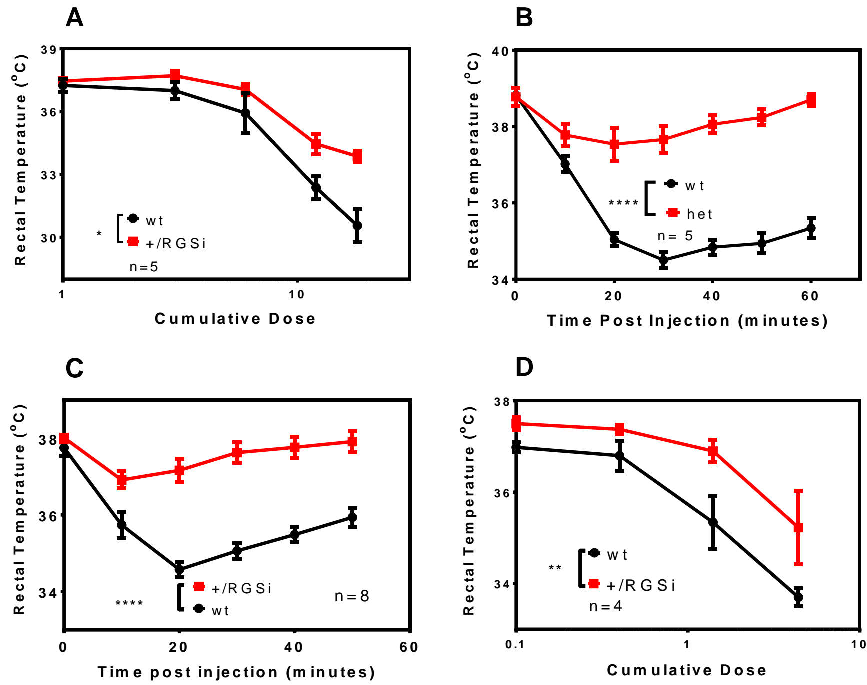


Figure 4.2 GIRK mediated hypothermia is disrupted in F1 *Gnao1* +/RGSi mice
A) Hypothermia due to baclofen is attenuated in F1 *Gnao1* +/RGSi mice as assayed by an escalating dose response curve. B) Both the maximal response and time course of 30 mg/kg morphine mediated hypothermia is altered in F1 *Gnao1* +/RGSi mice as assayed by a single dose time course. C) Both the maximal response and time course of 1mg/kg 8-OH-DPAT mediated hypothermia is altered in F1 *Gnao1* +/RGSi mice as assayed by a single dose time course. D) 8-OH-DPAT mediated hypothermia is reduced in F1 *Gnao1* +/RGSi mice as assayed by an escalating dose response curve.

F1 *Gnao1* $^{+/RGSi}$ mice have a normal hypothermia response to drugs that act independent of GIRK channel activity

The reduced hypothermia in Figure 4.2 could be due to a global disruption of thermoregulation. Therefore we tested D_2-like receptor-mediated hypothermia which is known to be independent of GIRK channel function (Costa *et al.* 2005). It has been postulated to be mediated through a presynaptic mechanism (Zarrindast & Tabatabai 1992). In Figure 4.3, it can be seen that the G_o mutation has no statistically significant influence on the hypothermia response in an equivalently powered experiment. Thus the defect in hypothermia is pathway-dependent and not a universal downstream defect.

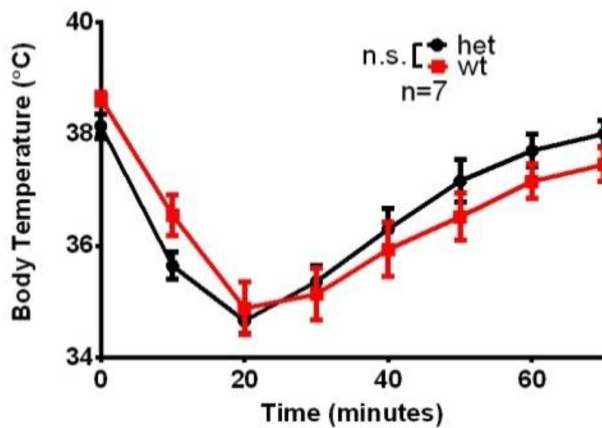


Figure 4.3 Non-GIRK channel mediated hypothermia is preserved in F1 *Gnao1* $^{+/RGSi}$ mice
Non-GIRK channel dependent hypothermia was tested using 0.5 mg/kg of the D_2-like agonist quinpirole. Here hypothermia was indistinguishable among genotypes.

Gnao1 $^{+/RGSi}$ mice have decreased $GABA_B$ and μ-opioid receptor (MOR) activation of GIRK channel activity in the PAG

While the *in vivo* changes in hypothermia are indicative of decreased GIRK channel activation, this could also have been due to effects up-or downstream of the GIRK channel in the hypothermia pathway. Therefore to reduce the level of system complexity, I collaborated with the Ingram laboratory to directly test GIRK activity within the periaqueductal gray area (PAG), a region known to play a central role in thermoregulation and to contain both $GABA_B$ and MOR receptors (Romanovsky 2007). Here we found a decrease in the magnitude of both $GABA_B$ and the MOR receptor-mediated stimulation of GIRK channel currents (Figure 4.4). These data indicate that GPCR-mediated activation of GIRK channels is disrupted in the PAG of mutant mice.

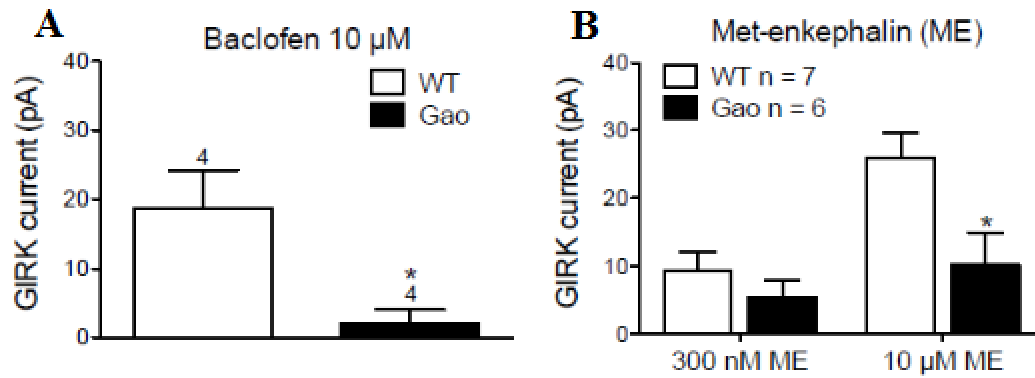


Figure 4.4 GPCR activation of GIRK channel currents is reduced in *Gnao1* $^{+/RGSi}$ mice

A) Baclofen (10 μM) activates GIRK channel currents in PAG neurons in control mice but had a minimal response in neurons of the *Gnao1* $^{+/RGSi}$ mice (t-test, $t(6) = 2.906$, $p < 0.05$). B) The μ-opioid receptor agonist met-enkephalin (ME) activates GIRK channel currents in control mice but the magnitude of the current is substantially reduced in *Gnao1* $^{+/RGSi}$ mice ($F(1,11) = 6.354$, $p < 0.05$).

Reduced G_o function does not explain altered hypothermia

Given that the mutation was designed as a gain-of-function mutation, it was unexpected to see the loss of signaling. One potential way this mutation may result in a loss of function is through a reduction in protein level. It has been previously reported that 129S1/SvImJ *Gnao1* $^{+/RGSi}$ mice exhibit a slight reduction in G_o protein level (Lamberts *et al.* 2013). This was not found in our own hands on the F1 background (Figure 2.2). To further assess whether reduced G_o protein could cause this effect, we tested *Gnao1* $^{+/-}$ mice which have a 50% reduction in protein levels (Lamberts *et al.* 2011). They still, however, exhibited normal 8-OH-DPAT induced hypothermia (Figure 4.5).

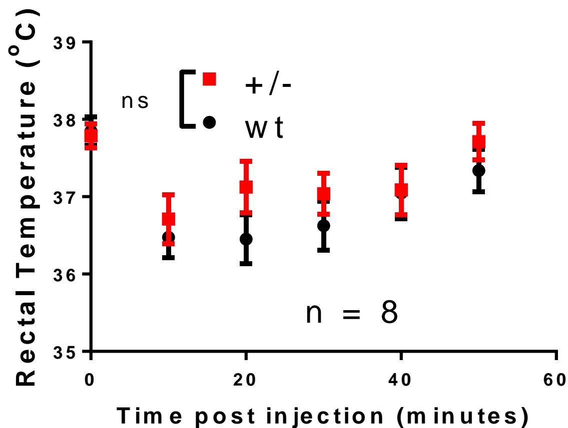


Figure 4.5 A 50% reduction in G_o protein does not impair GIRK-dependent hypothermia
1mg/kg of 8-OH-DPAT was administered to mice lacking 50% of G_o. All mice responded equivalently

Transcriptional regulation does not explain decreased GIRK channel activation

Compensatory mechanisms may reduce transcription of key components within the signaling cascade. Therefore we checked GIRK and several other CNS proteins in both the hippocampus and cortex of F1 *Gnao1* $^{+/RGSi}$ males. Unfortunately, while an area of interest, the PAG could not be isolated due to technical limitations. Figure 4.6 illustrates that despite seeing the expected increase of RGS4 within the cortex, no difference in GIRK was detected due to the mutation in either brain region.

Discussion

Here we support the hypothesis that modulating G-protein signaling is able to selectively influence a subset of responses to a given pharmaceutical. Specifically, in this Chapter, I demonstrate *Gnao1* $^{+/RGSi}$ mice to have reduced baclofen-mediated hypothermia while the baclofen-mediated motor impairment is comparable to control. Further, this chapter demonstrates that morphine-induced hypothermia is reduced in mutant mice, despite previous reports demonstrating that the mutation enhances morphine-induced supraspinal antinociception (Lamberts *et al.* 2013). The reduction in hypothermia appears to be due to a disruption of GIRK channel activation within the PAG. These findings do

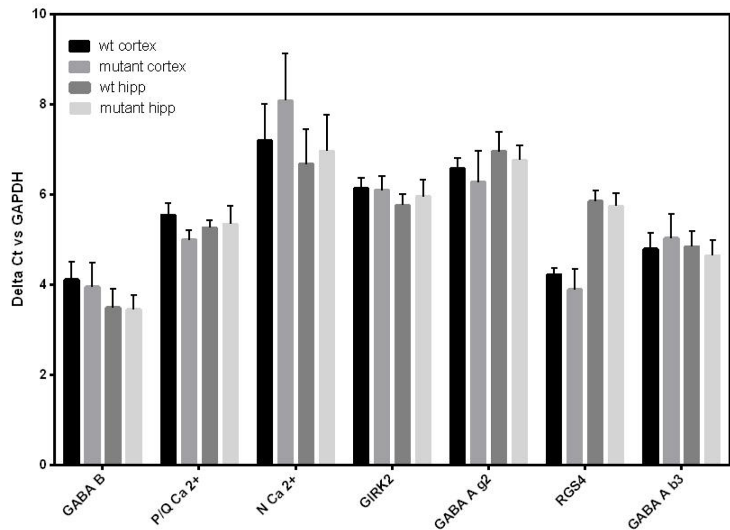


Figure 4.6 GIRK transcription in cortex and hippocampus is unaltered
mRNA levels for several excitatory and inhibitory signaling components were quantified using GAPDH for normalization. Cortex and hippocampus of mutant and control mice were assayed. The mutation did not influence mRNA levels.

not appear to be due to a loss of G_o function nor does it appear to due to disruption of transcriptional control of GIRK channels.

Historically, many labs have had difficulty in differentially modulating baclofen's hypothermia and motor-impairing effects. *Gabbr1* $^{-/-}$ mice, which lack $GABA_B$ receptors, lose both effects indicating that a single receptor is responsible for both actions of the drug (Schuler *et al.* 2001). It also appears that both effects, in addition to being through the same receptor, are also due to the same effector, GIRK2 containing channels. *Kcnj6* $^{-/-}$ mice have a significant loss in both measures of baclofen action (Costa *et al.* 2005); (Pravetoni & Wickman 2008). However Maity *et al.* recently identified the pathways upon which baclofen acts to induce muscle relaxation within the cerebellum (Maity *et al.* 2012). Within the signaling cascade they identified RGS6 as a key modulator and confirmed that *Rgs6* $^{-/-}$ mice were dramatically sensitized to the motor-impairing effects. Conspicuously missing, however, is the impact that RGS6 has on other baclofen-mediated effects such as sedation or hypothermia.

The selective disruption in baclofen-mediated hypothermia in *Gnao1* $^{+/RGSi}$ mice appears to be due to altered GIRK channel activity. *Gnao1* $^{+/RGSi}$ mice have significantly reduced hypothermia in response to several drugs which induce hypothermia in a GIRK channel-dependent manner (Figure 4.1 & Figure 4.2). However quinpirole-mediated hypothermia, which acts independently of GIRK channels, is intact. This likely implies that central downstream thermoregulatory pathways are still intact.

One may argue that by providing a large dose of quinpirole we have masked an effect on a central downstream hypothermia pathway, however this is unlikely. The dose

of quinpirole administered generated a comparable magnitude drop in core temperature as compared to those drugs that were GIRK channel-dependent. Further, it was not a maximal response as a dose of 1 mg/kg generated a two degree larger drop and the mutation had no effect on that response either (data not shown).

Another interesting conclusion to be drawn from these data is that enhanced G_o activity must not universally impact GIRK channel signaling. *Girk2* $^{-/-}$ mice and *Girk1* $^{-/-}$ mice lack a baclofen-mediated motor impairment. Therefore if the disruption in GIRK channel activity was universal across the brain of *Gnao1* $^{+/RGSi}$ mice they should have a blunted motor impairment responses as well. Yet based upon Figure 4.1 this is not the case.

It was quite challenging to understand how a gain-of-function mutation would lead to a reduction in physiological signaling. Therefore we looked at the electrophysiological level which further confirmed that, at least within the PAG, GIRK channel activity was significantly reduced. There are at least four possible explanations for this finding including: reduced expression of G-protein, reduced GIRK channel, reduced receptor levels, or a role for RGS action to enhance G-protein-mediated GIRK signaling. Unfortunately, in light of data presented here and in other works, none of these hypotheses appear reasonable. First, loss of G_o function appears unlikely as we tested if a 50% reduction in protein level could phenocopy the blunted hypothermia but found a normal hypothermic response to 8-OH-DPAT in heterozygote *Gnao1* $^{+/-}$ mice. Second, we explored the potential role for transcriptional control of *Kcnj6* to be a mediator of our observations. This is based upon a prior report indicating that G_o signaling can suppress transcription of N-type calcium channel activity (Kim *et al.* 2003). However, I found the

mutation to have no effect within the cortex and the hippocampus. As mentioned above though, a brain region selective impact may be responsible; therefore a more robust analysis of mRNA from other brain regions, including the PAG, will be needed to fully eliminate this mechanism. The third potential explanation for the blunted hypothermia is that loss of RGS/G protein interactions diminishes GIRK channel activation. Previous reports, however, typically indicate a negative role for RGS proteins in GIRK channel activity (Labouebe *et al.* 2007; Mutneja *et al.* 2005). Work presented elsewhere also makes loss of receptor function an unlikely explanation. Dr. Lydic's laboratory has conducted GTPγS binding on a variety of receptors in *Gnao1* $^{+/RGSi}$ brains and found little change indicating both relatively normal receptor levels and G-protein coupling. Work by Lamberts *et al.* demonstrated enhanced activity of μ-opioid receptors within the PAG of *Gnao1* $^{+/RGSi}$ mice by assaying μ-opioid mediated suppression of calcium channels. Thus the molecular mechanism behind the loss of GIRK channel activity remains elusive but may involve trafficking of GIRK channels or signaling complex assembly.

Based upon the discussion thus far we are left with one alternative hypothesis, despite prior *in vitro* data, G_o may function *in vivo* to reduce GIRK activation. This actually fits with some prior *in vivo* work where *Gnao1* $^{-/-}$ mice demonstrated enhanced GIRK channel activity within the hippocampus (Greif *et al.* 2000) which they attributed to disruption of the normal G_o /RGS coupling leaving another $G_{i/o}$ family member to compensate that was not subject to RGS regulation by the RGS proteins present within the hippocampus. However, I think this is actually quite an unlikely explanation due to two major findings. One, relatively few RGS proteins demonstrate G_o selectively and mRNA from several non-selective of RGS family members is abundant within the

hippocampus (Gold *et al.* 1997), Chapter 1). Additionally, I have now demonstrated that *Gnao1* $^{+/RGSi}$ mice have reduced GIRK channel activity which would not be expected based upon the rational presented in the *Gnao1* $^{-/-}$ paper. Therefore a possible explanation is that Go's function in vivo may be to reduce GIRK signaling. The biochemical mechanism could be through a dominant negative effect whereby G_o activation generates second messengers which serve to block other $G_{i/o}$ family members from activating GIRK. Further experiments will be necessary to test this hypothesis.

In summary we have identified that introduction of a *Gnao1* RGSi allele *in vivo* selectively disrupts GIRK channel-dependent hypothermia pathways including those within the PAG. Further analysis centered upon the PAG in our mouse system should yield insights to the role of G_o and RGS proteins in controlling the function of GIRK channels within this region.

Materials and Methods

Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health; all experimental protocols were approved by the relevant University Committee on the Use and Care of Animals. Experiments were performed using male mice. All experimental mice were group-housed with unlimited access to food and water until experimentation. Lights were maintained on a 12-hour light/dark cycle with lights on at 5:00/6am depending on time of year. All experiments were conducted during the light phase.

Generation of mice and genotyping

The *Gnao1* RGSi mutation was initially generated on the 129S1/SvImJ background which was used to produce RGSi mice on the B6129F1 and C57BL/6J

background as described in previous chapters. All mice had eartags inserted and tail biopsies taken prior to weaning. DNA was extracted from tails by an alkaline lysis method (Truett *et al.* 2000). The RGSi allele of *Gnao1* was identified by PCR as described in chapter 2. *Gnao1* $^{-/-}$ mice were a generous gift of Dr. Mortenson and genotyped as previously reported (Duan *et al.* 2007).

Hypothermia

Experimenter was blinded to genotype. Mice were moved from group housing to individual cages with free access to food and water for 2 hours prior to experimentation. Starting at 30 minutes prior to drug administration baseline temperatures were recorded using a Tcat 2df controller rectal thermometer (Physitemp Clifton, NJ) which was placed at a 20 mm probe depth. This was repeated every 10 minutes until just prior to i.p. drug injection. The temperature taken just prior to drug injection was used as the baseline value (t=0). For single-dose studies, no further injections were administered and temperature was taken by rectal thermometer at the times indicated. For cumulative-dose response curves the first physiological read outs post-injection were taken at the time of peak effect in the respective single-dose vs time curve (i.e. 60 minutes for baclofen and 20 minutes for 8-OH-DPAT). Following physiological read outs an i.p. injection of the relevant drug was administered and the same amount of time was allowed to pass prior to taking readings and injecting the next dose. 8-OH-DPAT (Sigma), morphine, and baclofen (Sigma) were dissolved in saline. Drugs were made as stocks and used over multiple days. Control and mutant mice were tested in equal numbers each day.

Motor Impairment

For those hypothermia experiments that also involved testing for motor

impairment, mice were trained on a rotarod set at 12 RPM in four sessions spaced 30 minutes apart the day before the experiment. On the day of the experiment baseline performance was measured. Only mice that fell once or less during a 300 second test were used. Mice were then injected with baclofen, had their temperature taken, and then time on the rotarod was determined. Time spent clinging to the rotarod was excluded, similar to prior studies (Jacobson & Cryan 2005).

Electrophysiology

Mice were deeply anesthetized with isoflurane and the brains rapidly removed and placed in ice cold "cutting buffer" containing (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 D-dextrose, 50 sucrose. Coronal slices (~230 µm) containing the periaqueductal gray area (PAG) were sliced in 95% O_2 and 5% CO_2 oxygenated "cutting buffer". Slices were incubated in warm (35 ^{0}C) oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 D-Dextrose, pH 7.4, and the osmolarity was adjusted to 300-310 mOsm.

Whole cell patch clamp recordings were made from visually identified PAG neurons. Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) on a two-stage puller (Narishige, Tokyo, Japan). Pipettes had a resistance of 2-4 Mohm and intracellular solutions contained (in mM): 138 potassium methylsulfate, 10 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 30 D-dextrose, 4 Mg-ATP, 1 Na-GTP; pH 7.3, 280-290 mOsm. Whole cell series resistance was compensated ~80%. Neurons were held at -70 mV. Currents were collected at 2 kHz and digitized at 5 kHz using an Axopatch 200B amplifier controlled by Axograph Data Acquisition software (AxographX, Sydney,

Australia). Recordings in which access resistance or capacitance changed by >15% during the experiment were excluded from data analysis.

Quantitative Real Time PCR

F1 males 8 weeks of age were decapitated and heads were placed in liquid nitrogen for 6 seconds to prevent mRNA degradation. Cortex and hippocampus dissected using a 30g coronal mouse brain matrix (Electron Microscopy Sciences) and homogenized in a bullet blender (Next Advance) per the manufacturer's directions using homogenization buffer from an RNeasy Mini kit (QIAGEN, Valencia, CA). RNA was extracted using the RNeasy Mini kit following the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed after digestion with DNase using a TaqMan cDNA reverse transcription kit with random hexemer primers (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in 20-µl reactions containing 1 µl of the cDNA sample and 0.3 µM forward and reverse primers with the RT2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD).

Statistics

Data were analyzed using GraphPad Prism 5.0 (GraphPad; LaJolla, CA). All data are reported as mean ± SEM. Two-way ANOVA was employed in the analysis of thermal and motor impairment figures. Student's t-test and ANOVA were used where appropriate for electrophysiology. Two-tailed p values less than 0.05 were considered significant.

Chapter 5

Synthesis

This thesis elucidated a role for G_o in modulating kindling susceptibility. One method was a pharmacological approach assessing altered receptor signaling in a novel G_o gain-of-function mouse model. The second was a genetic approach to identify unique modifiers of the pro-seizure phenotype found in mutant mice. The work presented in this thesis has further highlighted the critical role for G_o signaling in models of epilepsy and modulation of specific outcomes in GPCR signaling. Future work building upon this foundation will be of critical importance in the development of therapies for the recently recognized role of mutations within *GNAO1* in epileptic encephalopathy (Nakamura *et al.* 2013).

Unique role for G_o in kindling susceptibility

A variety of laboratories has previously identified activation of $G_{i/o}$-coupled GPCRs as protective from seizures (Grecksch *et al.* 2004; Pericic *et al.* 2005). However it remained to be determined which inhibitory G protein was involved in this process. Several labs generated G_o null mice and noted no overt seizure phenotype (Duan *et al.* 2007; Jiang *et al.* 1998). However my thesis suggests G_o as a key player in modifying CNS excitability. This further supports the observation of one other laboratory that did report spontaneous seizures in G_o null mice (Valenzuela *et al.* 1997).

The prior literature discussed above provided a simplistic understanding of G_o where G_o activation was considered to be protective from seizures. However recent research, including in my own thesis, has highlighted the importance of finely tuning the level of G_o activity. Nakamura *et al.* identified a mutation that is highly analogous to our own RGSi mutation in patients with epileptic encephalopathy (Nakamura *et al.* 2013). In support of this mutation likely being a gain-of-function, my thesis demonstrates, for the first time, that enhanced G_o signaling in a rodent model enhances susceptibility to kindling and promotes the development of spontaneous electrical disturbances within the brain. While other mutations found by Nakamura *et al.* were predicted to be loss-of-function, this does not match my rodent data whereby mice with a 50% reduction in G_o protein levels do not display sensitization to kindling (Figure A.2). Thus I would postulate that these mutations, might in fact, act as dominant negatives.

Prior work in cell culture experiments has implicated a role for G_o in neuronal development and migration, which is suggestive of a possible mechanism at play in the enhanced kindling susceptibility. Yet prior studies in G_o null mice were unable to detect morphological abnormalities within the CNS (Valenzuela *et al.* 1997). Here, for the first time, I report anatomical abnormalities as a reduction in spine density due to enhanced G_o signaling (Figure A.4). Though it remains to be determined if the changes are a primary result of the mutation or a secondary defect due to IEDs.

Mouse models studying G_o effectors implicate reduced GIRK activity as a likely mechanism of *Gnao1* $^{+/RGSi}$ enhancement of kindling. Historically within the literature two of the major targets of G_o activation are N-type calcium channels and GIRK channels. Enhanced suppression of N-type calcium channels in *Gnao1* $^{+/RGSi}$ mice,

however, is unlikely to result in seizures based upon animal model data. N-type calcium channel null mice are viable, and despite three different independently generated lines no mention of altered seizure susceptibility is mentioned (Hatakeyama *et al.* 2001; Kim *et al.* 2001; Saegusa *et al.* 2001). *Girk2* $^{-/-}$ mice however display spontaneous seizures and premature death like *Gnao1* $^{+/RGSi}$ mice (Signorini *et al.* 1997). While prior *in vitro* literature proposed a positive role for G_o in GIRK channel activation (Fernandez-Fernandez *et al.* 2001), Chapter 4 of my thesis demonstrates a negative role for G_o activity in GIRK channel activity. This conclusion is further supported by prior *in vivo* work which found enhanced GIRK activity in *Gnao1* $^{-/-}$ mice (Greif *et al.* 2000). One possible mechanism behind G_o's unexpected suppression of GIRK channel activity is that the specific βγ subunits that G_o releases bind, but have low efficacy to activate GIRK channels. Thus βγ from G_o may act in a dominant-negative fashion. Future studies in transfected primary neurons will be required to test this hypothesis. An additional way to test the significance of reduced GIRK channel activity in the enhanced kindling phenotype would be to see if administration of the newly discovered direct GIRK channel activator, ML297, ameliorates the phenotype (Kaufmann *et al.* 2013).

Identification of a novel modifier of epilepsy with relevance to human patients

Genetic modifiers which influence the progression of spontaneous seizures in mouse models of epilepsy have been identified (Bergren *et al.* 2009). However the model systems employed typical utilize ion channel mutations. It remains unknown if therapeutics leveraging these modifiers will improve treatment for epilepsies of other origins. Significantly, the novel work of my thesis indicates another modifier on mouse Chr17 could provide useful insights in the treatment of idiopathic epilepsies that depend

on altered GPCR signaling. This is particularly relevant in light of new data suggesting *de novo* mutations in GNAO1 can contribute to epilepsy (Nakamura *et al.* 2013).

Understanding sudden unexpected death in epilepsy (SUDEP) has proved quite challenging with few known risk factors (Surges & Sander 2012). One animal model involving a loss of Kv1.1 demonstrated that mutations increasing parasympathetic tone can be a risk factor (Glasscock *et al.* 2010). Modifiers protective from this phenotype have yet to be identified. Interestingly, our *Gnao1*$^{+/RGSi}$ mice are also likely to have enhanced parasympathetic signaling as G_o plays a major role in these signaling pathways (Duan *et al.* 2007);(Valenzuela *et al.* 1997). Therefore *Mogs1* should also be considered as a potential modifier of SUDEP. To support this theory it will be of interest to see if *Mogs1* is able to reduce the incidence of SUDEP in mice lacking Kv1.1.

Future directions

Model system development

The model used in my thesis has a major limitation. Temporal or spacial dissection of G_o signaling is not possible due to the global nature of the mutation. This is particularly significant in light of the IED activity found in the B6.129 *Gnao1* $^{+/RGSi}$ mice. Further dissecting the molecular mechanisms of any phenotype will prove challenging as the phenotype may be secondary to the IED activity. Time course experiments to find primary defects will be required to determine the origin of the IED activity. However to study the role of G_o in other neurotransmitter systems within the mature brain alternative models will be required to overcome this limitation.

To avoid complications due to IED activity *Gnao1* $^{+/RGSi}$ mice could be studied on a different genetic background. The two alternative backgrounds currently available include B6x129 F1 and 129S1/SvImJ. Mutant F1 mice may not display spontaneous IEDs as, in contrast to their B6 counterparts, they do not experience spontaneous death. Normal baseline EEG activity may, however, be an unrealistic assumption because mutant F1 mice parallel their B6 counterparts in rapid PTZ kindling (Figure A.1). Studying mutants on the 129 background is a second option. The fact that they kindle at the same rate as control makes their having normal baseline EEG activity more likely. However the 129 strain has several disadvantages including poor breeding and minimal locomotion (Labs 1998). The minimal locomotion presents a problem due to the behavioral nature of many assays. Thus studying the mutation on the 129 genetic background may not prove fruitful.

An alternate approach would be to develop a mouse model that could be temporally and spacially controlled. This could be accomplished through the development of a conditional knock-in. However, based upon the Neubig lab's experience in developing the G_{i2} conditional knock-in, the use of a minigene can dramatically reduce protein expression. Reduced protein expression was also a complicating factor for one researcher who attempted to create a conditional *Gnao1* $^{+/RGSi}$ mouse (personal communcation). However, the cause of reduced protein expression can not be conclusively tied to the mutation as the neo cassette was not removed, which can also reduce protein expression.

One approach to overcome the limitations of a conditional knock-in is the use of a transgenic BAC containing a Cre inducible *Gnao1* RGSi allele. Gain-of-function

mutations typically lead to dramatic phenotypes as highlighted in the introductory chapter. Thus even if only 1/3 of the G_o is from the RGSi allele it should be sufficient for experimental purposes.

Future research areas

The mechanism of IED development should be further pursued as IEDs are recognized as a clinically significant problem leading to deficits in cognitive function (Jaseja 2007) (Pressler *et al.* 2005). Despite their significance, the effectiveness of current medications in reducing IED frequency is under debate (D'Antuono *et al.* 2010; Pressler *et al.* 2005); (Stodieck *et al.* 2001). Despite the purported benefits of suppressing IEDs, evidence has also been presented for a protective role of IEDs to reduce frequency of seizures (Avoli 2001). Understanding the development of IEDs in *Gnao1* $^{+/RGSi}$ mice should yield insights into how best to suppress IEDs and the long term consequences of doing so.

Understanding excitatory signaling within the piriform cortex and hippocampus of *Gnao1* $^{+/RGSi}$ mice is likely a key step in understanding the mechanism of IED development. This is a result of several lines of data. IEDs occur within the piriform cortex early in the kindling process and only later propagate to the hippocampus (de Curtis *et al.* 1999). B6.129 *Gnao1* $^{+/RGSi}$ mice kindle more quickly potentially indicating alterations in the propagation of IEDs within these mice. Yukun Yuan in our lab has generated preliminary data that spontaneous inhibitory post synaptic events are reduced both in frequency and amplitude (Figure A.3). This data could support the hypothesis that suppression of GABA release in *Gnao1* $^{+/RGSi}$ mice contributes to IED development.

There are other alternative explanations for the changes observed by Yukun, including either death of inhibitory neurons, which commonly occurs in epileptic brains (Buckmaster & Jongen-Relo 1999), or a lower density of inhibitory synapses. The viability of interneurons in the adult brains should be further explored. A decrease in inhibitory cell number would support the hypothesis that G_o $^{+/RGSi}$ mice experience spontaneous seizures, which is still under debate. If no changes were observed it would rule out a decrease in inhibitory neurons as an explanation of Yukun's data.

Future experimentation should also focus on cardiac function in *Gnao1* $^{+/RGSi}$ mice. G_o, beyond being expressed in the brain, is also present in the heart. Here it is responsible for controlling the bradycardia response to muscarinic agonists (Duan *et al.* 2007). Thus I would hypothesize that *Gnao1* $^{+/RGSi}$ mice should have enhanced muscarinic induced bradycardia. This could have two significant implications for the findings of my thesis. First, cardiac dysfunction may precipitate seizures through hypoxia-induced neuronal cell death. Secondly, the strain-dependent spontaneous death may be a model for sudden unexpected death in epilepsy (SUDEP). 10-15% of those who experience SUDEP have long QT intervals, indicating cardiac dysfunction (Surges & Sander 2012). Further, one proposed mechanism of SUDEP is post-ictal bradycardia or hypoventilation (Surges & Sander 2012). Testing both a brain and cardiac specific *Gnao1* $^{+/RGSi}$ mouse line in kindling and spontaneous death would prove critical to ruling out the role of cardiac dysfunction in *Gnao1* $^{+/RGSi}$ enhanced kindling and spontaneous death.

Appendix

Additional Data

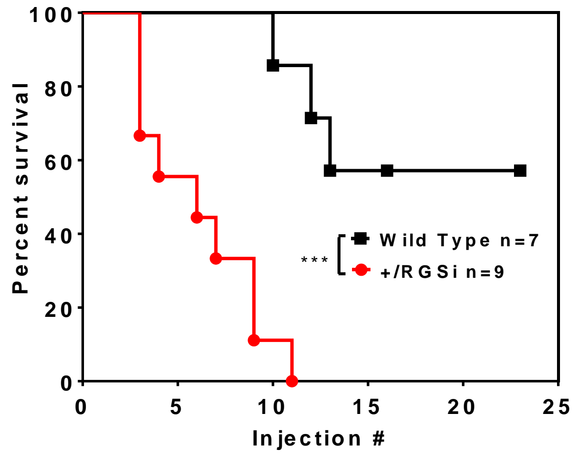


Figure A.1 F1 *Gnao1* $^{+/RGSi}$ mice kindle faster than control
Female F1 mice were subjected to the kindling protocol in Chapter 2. Mutant mice kindle faster than control. $p < 0.001$

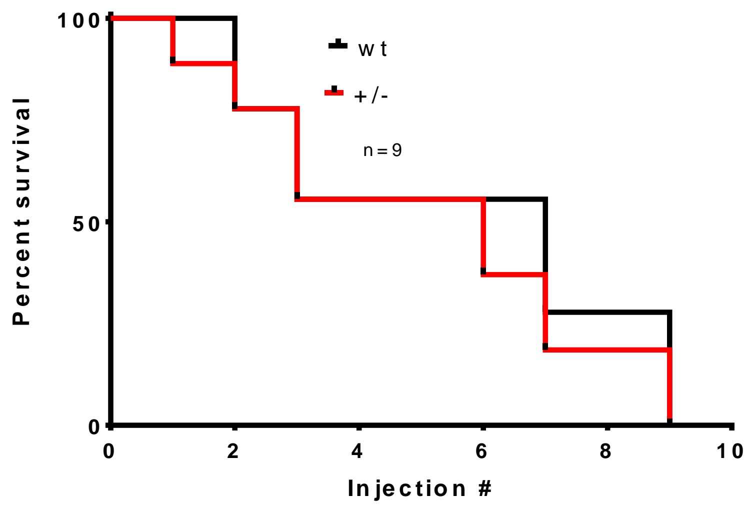


Figure A.2 A 50% reduction in G_o protein levels does not alter kindling
Female *Gnao1* $^{+/-}$ mice on the B6 background were subjected to kindling following the protocol in Chapter 2. Loss of protein did not influence rate of kindling.

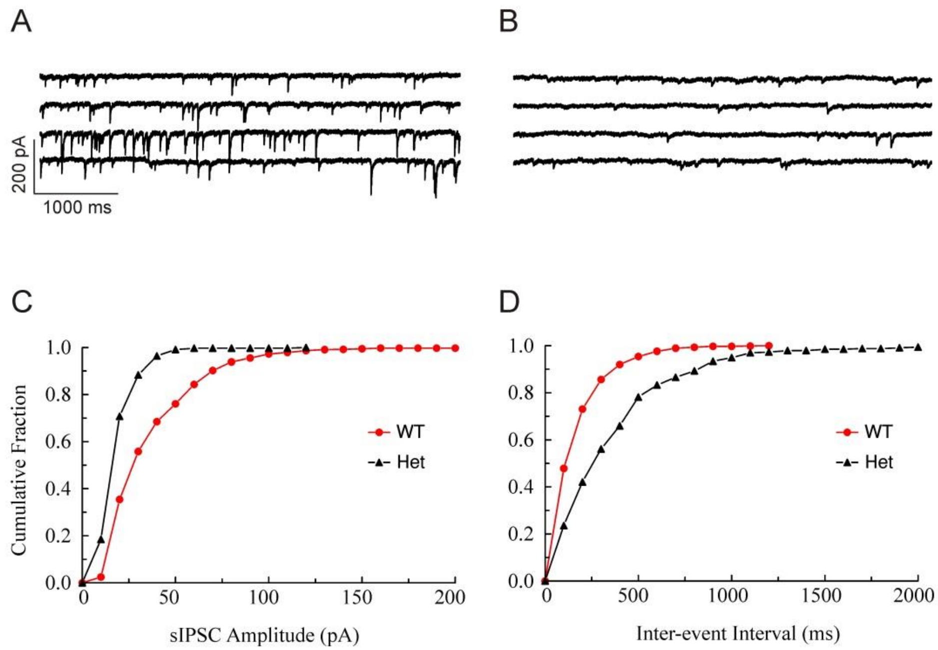


Figure A.3 *Gnao1* $^{+/RGSi}$ mice have lower amplitude and less frequent sIPSC
Dr Yuan tested adult B6 mice for changes in inhibitory signaling. These findings are indicative of mutant mice having less inhibitory signaling.

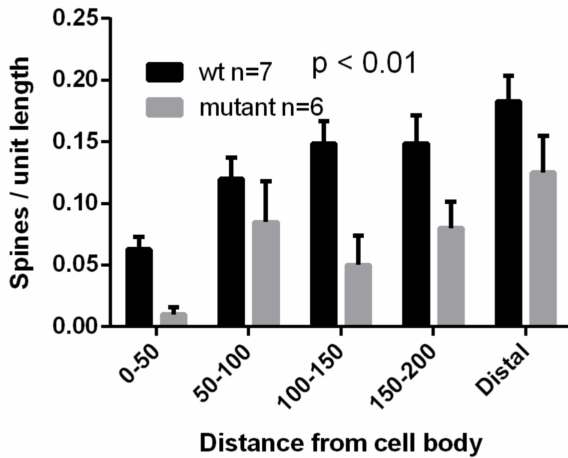


Figure A.4 *Gnao1* $^{+/RGSi}$ mice have a lower CA1 spine density by 4 weeks of age
Gnao1 $^{+/RGSi}$ mice were bred with GFPm mice. At 4 weeks of age brains were harvested and spine density of CA1 pyramidal cells was calculated. Mutant mice have a lower spine density at several distances from the soma.

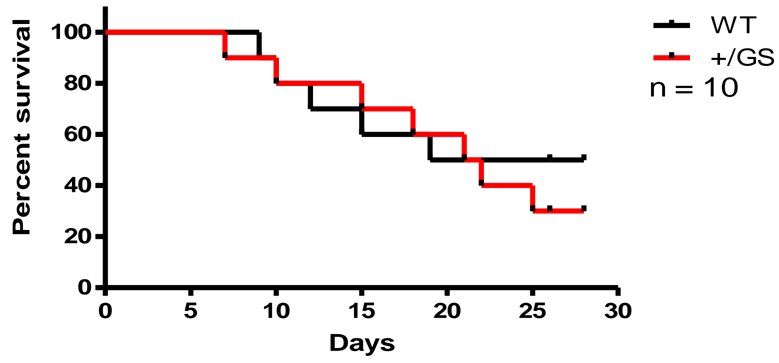


Figure A.5 *Gnai2* $^{+/RGSi}$ mutant mice kindle at a rate comparable to control
The RGSi mutation in the G_{i2} protein has no impact on the rate of kindling even on the B6 background.

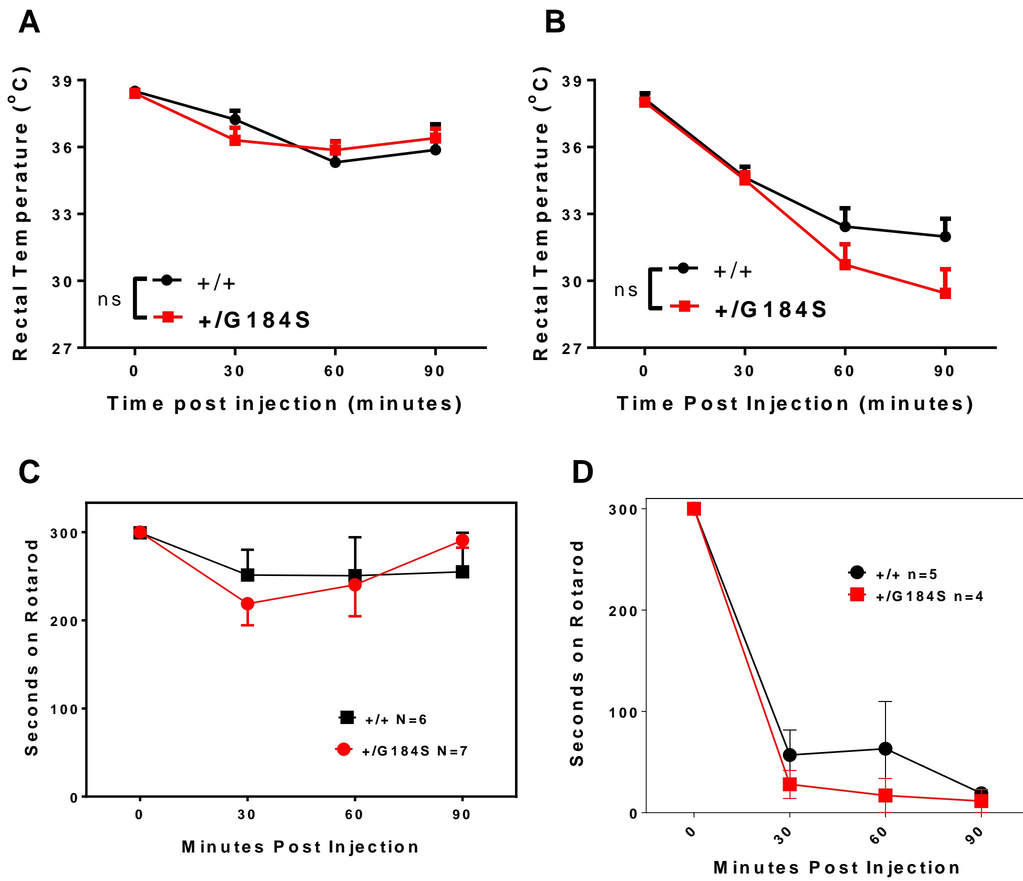


Figure A.6 ***Gnai2*** **+/G184S mice display responses to baclofen similar to that of control**

A) 6 mg/kg hypothermia response B) 10 mg/kg hypothermia response C) 6mg/kg motor impairment D) 10mg/kg motor impairment

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