ALTERATIONS IN $GABA_A$ RECEPTOR EXPRESSION AND PHYSIOLOGY IN A MOUSE MODEL OF IDIOPATHIC GENERALIZED EPILEPSY

By

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I would like to dedicate this work to my parents, the foundation of my life.
As a child, they taught me that I could do anything I wanted as long as I was willing to work for it. Their love and guidance has always given me the strength and confidence necessary to pursue my dreams fearlessly. I love them more than words can describe and I am grateful every day for the blessing of their love.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
α1HetKO	heterozygous α1 subunit knockout
aCSF	artificial cerebrospinal fluid
CAE	childhood absence epilepsy
cDNA	complimentary deoxyribonucleic acid
EEG	electroencephalogram
ERAD	endoplasmic reticulum associated degradation
GABA	γ aminobutyric acid
$GABA_A$	γ aminobutyric acid type A
IPSC	inhibitory post synaptic current
mIPSC	miniature inhibitory post synaptic current
mRNA	messenger ribonucleic acid
NMD	nonsense mediated decay
PCR	polymerase chain reaction
PTC	premature termination codon
qRT-PCR	quantitative real time polymerase chain reaction
SWD	spike and wave discharge
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
WT	wild type

CHAPTER I

EPILEPSY

Introduction

Epilepsy is a collection of neurological disorders which is diagnosed upon the occurrence of two or more unprovoked seizures separated by at least 24 hours. Seizures arise from excessive and hypersynchronous discharges from large neuronal populations in the brain. This is generally thought to occur due to an imbalance between excitation and inhibition in the brain. Epilepsy is one of the most common neurological disorders and is estimated to affect 0.5-1%of the population worldwide (Hauser 1994). In the United States, epilepsy affects nearly 2 million people and costs approximately $15.5 billion annually. Despite the fact that there is a wide variety of treatment options available, over 30% of epilepsy patients are refractory to treatment and continue to experience seizures. Additionally, it has been postulated that nearly 10% of Americans will experience a seizure at some point in their life. For these reasons, the U.S. Centers for Disease Control has deemed epilepsy a public health problem (National Center for Chronic Disease Prevention and Health Promotion 2011).

Epilepsies can be separated into two major classes: partial and generalized. In partial epilepsies, seizures begin focally at a cortical site and are typically confined to a specific region or neural network but if the aberrant activity spreads widely enough, the seizures can become secondarily generalized and involve the entire brain. Partial seizures can be further divided depending on whether or not they are accompanied by impairments in consciousness; complex partial seizures elicit impairments of

consciousness while simple partial seizures do not. Partial epilepsies often result from injuries, lesions, or malformations in the brain. In contrast with partial seizures, generalized seizures initiate across the entire brain simultaneously with no obvious focal origin. Generalized epilepsies can be symptomatic, meaning that they result from a known cause such as Lennox-Gastaut syndrome, or they can be idiopathic, meaning that there is no known cause. Idiopathic generalized epilepsies are widely believed to have a genetic etiology.

The first line of treatment for epilepsy is typically the use of pharmaceutical drugs. There are several anti-epileptic drugs available, but their effectiveness and utility vary depending on the type of epilepsy and seizure manifestation. A drug that is particularly effective in the treatment of partial seizures could have no effect, or even exacerbate absence seizures, while some treatments for absence seizures have no effect on partial seizures. Further, there is extensive individual heterogeneity in the effectiveness of specific drugs even among patients with the same type of epilepsy and seizure manifestations. Alternative treatments for patients who are refractory to medication exist and include the ketogenic diet, vagal nerve stimulation, and surgical resection of the seizure focus. The ketogenic diet can be effective in both generalized and partial epilepsies, but vagal nerve stimulation and surgical resection are typically only employed in cases of partial epilepsy.

Childhood Absence Epilepsy

Childhood Absence Epilepsy (CAE) is a form of idiopathic generalized epilepsy characterized by the occurrence of non-convulsive absence (formerly referred to as petit mal) seizures which are sudden and brief lapses in consciousness typically accompanied

by behavioral arrest and a blank stare. In the most severe cases children experience hundreds of absence seizures per day which is incredibly disruptive to daily activities—especially in school aged children. CAE onset is typically between 3 and 12 years of age and the disorder is present in 2-8 per 100,000 children under the age of 15 with females disproportionately affected (Crunelli 2002; Carney 2005). Interestingly, approximately 70% of all cases of CAE remit in adolescence although the reason for this is currently unclear (Crunelli 2002).

Absence seizures are accompanied by a characteristic signature on the electroencephalogram (EEG)—a bilateral and synchronous spike and wave discharge (SWD) with a frequency of ~3 Hz. The interictal EEG does not show any generalized or regional slowing, but oftentimes demonstrates brief (1-3 second) runs of generalized SWD without any associated clinical changes. Based on extensive studies performed in multiple animal models of absence epilepsy, the 3 Hz SWD is thought to arise from aberrant oscillations within the thalamocortical circuitry. Rodent models of SWD have implicated three major brain regions associated with these aberrant oscillations: the somatosensory area of the cerebral cortex and the reticular and ventrobasal nuclei of the thalamus. These three structures form an interconnected circuit in which normal sleep spindle oscillations occur during sleep (Beenhakker 2009). A schematic of this circuit is shown in Figure 1.1 with excitatory projections in green and inhibitory projections in red. The mechanism by which these normal oscillations occur is complex and beyond the scope of this report, but has been reviewed extensively elsewhere: Beenhakker and Huguenard 2009, *Neuron.* It is thought that the thalamocortical circuitry involved in sleep spindle-associated oscillations also pathologically support SWDs and absence

seizures that occur during waking. Interestingly, the first line of treatment for CAE, a drug called ethosuximide, functions by blocking T-type calcium channels which are known to play an integral role in producing the aforementioned thalamocortical oscillations. The exact mechanism by which the circuitry malfunctions to produce aberrant oscillations is unknown; based on the complexity and interconnectedness of the circuitry, several different dysfunctions could feasibly compromise the circuit in a way that would support aberrant oscillations. Thus it is possible and maybe even likely that multiple different etiologies can lead to absence seizures and there is not one universal cause.

Because seizures are thought to arise from an imbalance in excitatory and inhibitory activity in the brain, any mutation that could potentially enhance excitatory activity or limit inhibitory activity could lead to a predisposition for epilepsy. While most idiopathic generalized epilepsies are assumed to be polygenic, monogenic forms have been identified. To date, 17 different mutations in various subunits of the γ amino-butyric acid type A ($GABA_A$) receptor have been identified in patients with idiopathic generalized epilepsy syndromes. At least seven of these mutations have been primarily associated with CAE while still more are secondarily associated with absence seizures. Because $GABA_A$ receptors mediate the majority of fast inhibition in the adult central nervous system, it is not surprising that $GABA_A$ receptor dysfunction is associated with epilepsy. The subsequent chapter will provide significant background information on $GABA_A$Rs and outline our particular focus within the field of $GABA_A$ receptor-associated idiopathic generalized epilepsies.

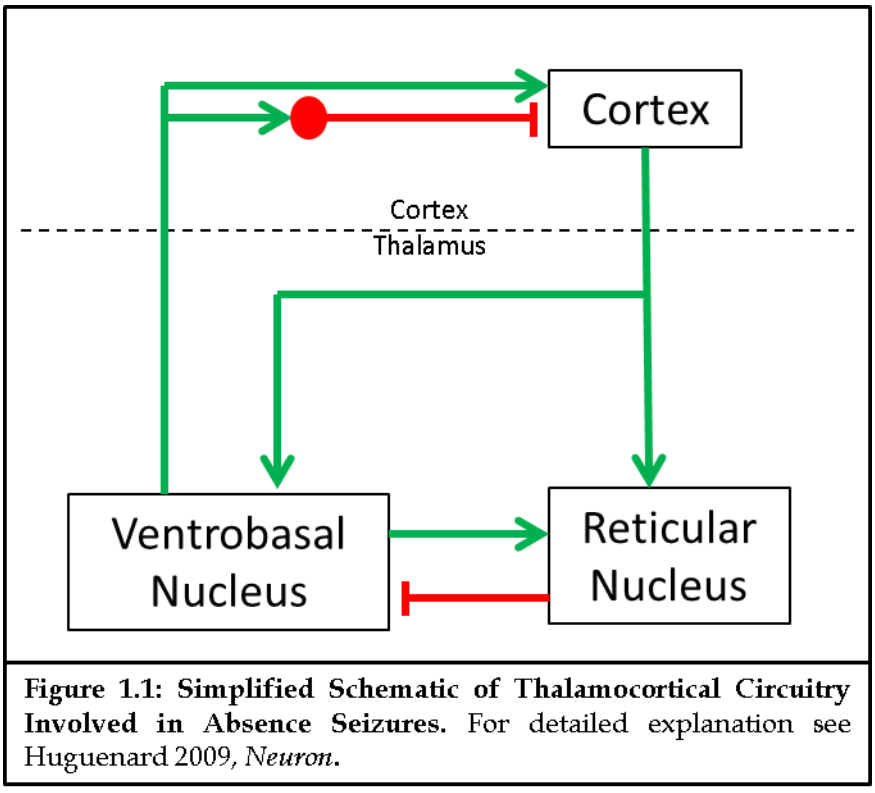

Figure 1.1: Simplified Schematic of Thalamocortical Circuitry Involved in Absence Seizures. For detailed explanation see Huguenard 2009, *Neuron*.

CHAPTER II

$GABA_A$ RECEPTORS AND EPILEPSY

Introduction

$GABA_A$ receptors are a family of chloride selective ligand gated ion channels that mediate the majority of fast inhibition in the adult central nervous system (Sieghart 2006). $GABA_A$ receptors belong to a larger superfamily of ligand gated ion channels called Cys-loop receptors which also includes nicotinic acetylcholine receptors, glycine receptors, and serotonin type III receptors (Connolly 2004; Unwin 1993). The $GABA_A$ receptor gene family is comprised of at least 19 different subunits classified by sequence homology into 8 subtypes that can assemble in various combinations to produce functional $GABA_A$Rs: α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3 (Sieghart 2002). Based on the large number of different $GABA_A$ receptor subunits, there are seemingly myriad different subunit combinations possible, although only a subset of these theoretical combinations has been identified *in vivo* (Mckernan 1996). The cache of possible subunit combinations coupled with differing spatial and temporal expression patterns provides considerable structural and functional heterogeneity to $GABA_A$ receptors. Distinct subunit combinations produce distinct receptor isoforms which display highly variable properties throughout development and into adulthood (Laurie 1992; Olsen 2009; Möhler 2006; Hevers 1998). This topic will be discussed in further detail below. The vast majority of $GABA_A$ receptors exist as a combination of two α subunits, two β subunits, and either a γ or δ (Mckernan 1996; Baumann 2001) subunit arranged as shown in Figure 2.1A-B. The ε, π, and ρ1-3 subunits are far less common and are generally positioned in place of the γ or δ subunit while the θ subunit can assume the position of the β subunit.

The morphology of each $GABA_A$ receptor subunit includes: a large extracellular domain at the N-terminus containing the characteristic disulfide bridge between two cysteine residues that creates the "Cys-loop" for which the receptor family is named, four helical transmembrane domains termed M1-M4 with the M2 of each subunit lining the ion pore, a large intracellular domain between the third and fourth transmembrane domains which serves as a site for phospho-regulation, and a very small extracellular C-terminal domain (Macdonald 1994; Sieghart 2006; Connolly 2004) as shown in Figure 1C.

Full activation of $GABA_A$ receptors requires the binding of two molecules of the neurotransmitter γ-amino butyric acid (GABA)—one at each α/β subunit interface (Baumann 2003; Kash 2003). Upon activation of the receptor, the channel opens and chloride ions flow down their electrochemical gradient through the pore. In the mature brain, this leads to an influx of chloride ions which causes the membrane potential of the cell to hyperpolarize and thus serves to inhibit the generation of action potentials. As previously mentioned, the wide variety of $GABA_A$ receptor isoforms supports extensive functional heterogeneity, perhaps best demonstrated by the existence of two distinct forms of $GABA_A$ receptor mediated inhibition: tonic and phasic (Mody 2001). Tonic inhibition is mediated by extrasynaptically localized $GABA_A$ receptors largely comprised of a δ subunit with an α4 and/or an α6 subunit (Sur 1999), though α5 containing receptors lacking a δ subunit are known to function extrasynaptically as well (Caraiscos 2004). These receptors are continuously activated by low levels (1 μM) of ambient GABA typically overflowing from the synaptic cleft. The properties of these receptors are conducive to persistent extrasynaptic activation as they are highly sensitive to GABA, activate relatively slowly, and desensitize minimally (Mtchedlishvili 2006).

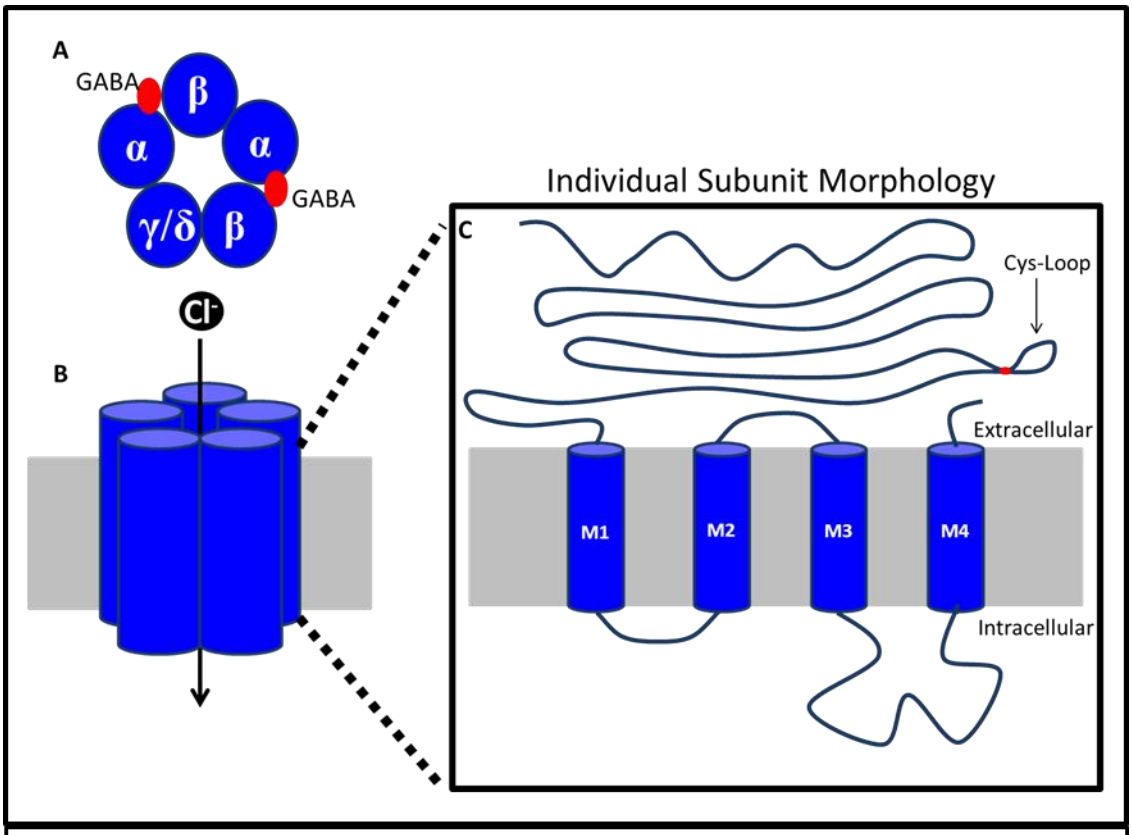

Figure 2.1: Structure of $GABA_A$ Receptors. A: Top down view of typical subunit arrangement with GABA binding site indicated by red ovals. B: Side view of receptor depicting chloride flowing through the pore. C: Morphology of an individual subunit.

The functional role of tonic inhibition is the regulation of neuronal excitability. Conversely, phasic inhibition is mediated by synaptic $GABA_A$ receptors most often containing a γ subunit with α1, α2, and/or α3 subunits. Unlike their extrasynaptic counterparts, synaptic $GABA_A$ receptors are transiently activated by much higher (1 mM) concentrations of GABA released from the presynaptic neuron into the synaptic cleft. These receptors differ from those mediating tonic inhibition to allow for fast transmission of a fleeting signal and are thus less sensitive to GABA, activate rapidly, and desensitize extensively (Haas 1999). The short-lived activation of these receptors produces a transient inhibitory post-synaptic current (IPSC) which functions to transform presynaptic GABA release into a post-synaptic signal (Farrant 2005; Nusser 1998). The remainder of this chapter will focus on the most predominant subtype of synaptic receptors: those containing the α1 subunit. The distinct receptor properties and known roles of the α1 subunit as well as important findings from α1 subunit knockout mice and recently discovered epilepsy associated mutations in the α1 subunit are all discussed at length below.

Biophysical Properties of the α1 Subunit Relative to Other Synaptic α Subunits

The α1 subunit is the most predominant α subunit in the adult brain and is most often assembled into the α1β2γ2 isoform comprising 60% of all $GABA_A$ receptors (Möhler 2006). Further heterogeneity exists among synaptic receptors as the specific α subtype influences biophysical properties of $GABA_A$ receptors including GABA sensitivity and the rates of activation, deactivation, and desensitization (Gingrich 1995; Eyre 2012; Picton 2007; Lavoie 1997; Böhme 2004). The primary strategy employed to determine the contribution of each α subunit to a particular property is to express

different α subunits with the same β and γ subunit partners in heterologous cells and explore the various properties. One such property, GABA sensitivity, is defined by the concentration of GABA that is required to produce a given response. A common measure of GABA sensitivity is the concentration of GABA required to elicit a half-maximal response in a given receptor subtype, known as the EC50. A low EC50 indicates higher sensitivity and vice versa. The activation rate of a particular $GABA_A$ receptor is the rate at which receptor current increases from 10% to 90% of the maximal or peak current and the deactivation rate is the rate at which current amplitude decreases after GABA is removed. The desensitized state of the receptor is a high affinity state in which GABA is bound but the ion channel is closed, thus the desensitization rate is the rate at which the response diminishes in continued presence of GABA. Each of the biophysical properties defined above influence the shape and time course of $GABA_A$ receptor mediated IPSCs, thus the α subunit within the receptor in large part dictates the properties of inhibitory currents. The biophysical properties of α1 containing $GABA_A$ receptors relative to other synaptic α subunits are summarized in Table 2.1 (Gingrich 1995; Eyre 2012; Picton 2007; Lavoie 1997).

Interestingly, the properties of $GABA_A$ receptor mediated IPSCs are known to change throughout development (Okada 2000; Dunning 1999)—namely the decay kinetics which are heavily influenced by the deactivation and desensitization rates of the receptors (Jones 1995; Ortinski 2004). In fetal and early post-natal development, $GABA_A$ receptor mediated IPSCs decay relatively slowly; later in development, the IPSCs decay much faster. The timing of this change coincides with the timing of a well-established

Table 2.1: Relative Biophysical Properties Conferred by Synaptic α Subunits	
GABA Sensitivity	α1 > α2 > α3
Activation Rate	α2 > α1 > α3
Deactivation Rate	α1 > α2 > α3
Desensitization Rate	α1 = α2 > α3

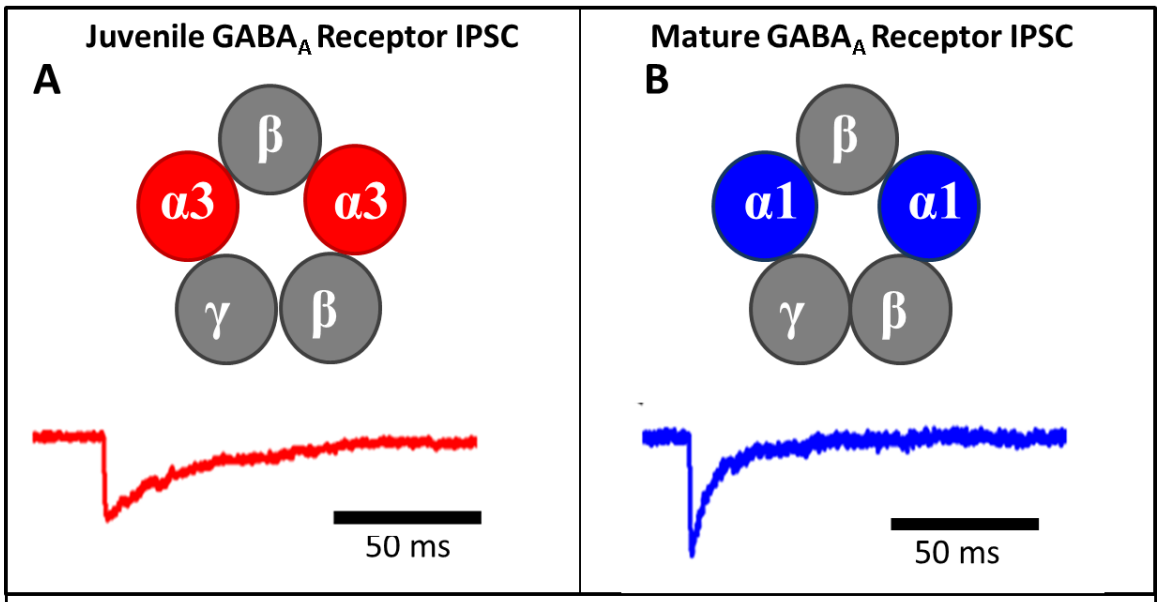

Figure 2.2: Developmental Change in α Subunit Expression and IPSC Kinetics
A: Juvenile α3 containing receptor with much slower IPSC decay kinetics.
B: Mature α1 containing receptor with fast IPSC decay kinetics.

developmental change in $GABA_A$ receptor α subunit expression (Laurie 1992; Hashimoto 2009; Fritschy 1994). Early in development, the α2 and α3 subunits predominate but soon after birth their expression begins to wane while the expression of the α1 subunit steadily increases to become the most abundant α subunit by postnatal day 12 in mice (Laurie 1992). A comparison of juvenile and mature $GABA_A$ receptor mediated IPSCs is shown in Figure 2.2. Given that the identity of the α subunit impacts IPSC properties, it is feasible that the developmental changes in α subunit expression and IPSC decay causally linked. Indeed, it has been shown that in mice lacking the α1 subunit juvenile IPSC kinetics persist into adulthood (Bosman 2005; Vicini 2001; Goldstein 2002). The functional role of this developmental switch in α subunit expression and the concomitant change in IPSC kinetics is currently not well understood.

Findings From α1 Subunit Knockout Mice

In 2001, transgenic mice lacking the α1 subunit of the $GABA_A$ receptor were generated and studies have shown that the loss of the α1 subunit results in a 50-60% decrease in the total number of $GABA_A$ receptors in the brain (Vicini 2001; Sur 2001). Consistent with this finding, the expression of the β2/3 and γ2 subunits—the most common binding partners of the α1 subunit—is also decreased in α1 knockout mice (Kralic 2006; Kralic 2002). Given that the α1 subunit is the most abundant subunit and its loss results in a loss of the majority of $GABA_A$ receptors in the brain, it is very surprising that these animals are viable and lack any obvious phenotypic abnormalities aside from a slight handling-induced tremor. The fact that the mice are overtly normal could suggest that changes occur within the $GABA_A$ receptor system to compensate for the

loss of α1. Indeed, post-transcriptional increases in the total expression of the other α subunits have been observed (Kralic 2002; Kralic 2006), but changes in surface expression have not been explored. The nature and extent of the compensation in total expression seems to vary among brain regions and has not been systematically quantified in the entire brain. One study suggests that neurons upregulate the subunits they normally express rather than expanding their subunit repertoire (Kralic 2006), which is consistent with a post-transcriptional mode of upregulation. The consequences of these compensatory changes are not completely understood, but α1 knockout mice fail to develop mature IPSC kinetics (Bosman 2005; Vicini 2001; Goldstein 2002) and exhibit a lower threshold for pharmacologically induced seizures (Kralic 2002). This could indicate a decrease in inhibitory tone, although it was reported that α1 knockout mice did not experience spontaneous seizures (Sur 2001). However, it is important to note that these conclusions were drawn by visual inspection only rather than EEG analysis. Certain types of seizures, such as absence seizures, result in very subtle alterations in behavior that are difficult to detect even in a human, much less a mouse. Thus it is possible that these animals did have seizures but did not display any easily detectable seizure behavior.

The α1 Subunit and Epilepsy

Because $GABA_A$ receptors are the primary source of inhibition in the central nervous system, it is not surprising that several mutations in various $GABA_A$ receptor subunits have been identified in patients afflicted with idiopathic generalized epilepsy syndromes such as childhood absence epilepsy and juvenile myoclonic epilepsy (Macdonald 2006; Macdonald 2004; Macdonald 2010a). Epilepsy is a large collection of

syndromes diagnosed upon the occurrence of two or more unprovoked seizures and may be classified as idiopathic generalized epilepsy if the cause is thought to be genetic and the seizures appear to involve the entire brain simultaneously with no obvious focal origin. Most idiopathic generalized epilepsies are thought to be multigenic which renders animal modeling fairly difficult, but the identification of monogenic forms has permitted the generation of animal models based on human disease associated mutations. To date there have been four mutations identified specifically in the α1 subunit in human patients suffering from idiopathic generalized epilepsy. Two of these mutations, K353delins18X and D219N (Lachance-Touchette 2011), were only recently reported and have not been fully characterized. The other two however, A322D (Cossette 2002) and S326fs328X (Maljevic 2006), have been studied in somewhat more detail.

a1(K353delins18X) Mutation The α1(K353delins18X) mutation was identified in 1 unaffected and 3 affected individuals with idiopathic generalized epilepsy exhibiting generalized tonic-clonic seizures. These seizures manifest as a sudden tensing of skeletal muscles followed by rapid contractions and relaxations resulting in characteristic convulsions (Pamplona 1989). This mutation involves the insertion of 25 base pairs into intron 10 which interrupts mRNA splicing and causes the retention of intron 10 in the transcript. This leads to an 18 amino acid insertion into the protein as well as the truncation of the fourth transmembrane domain due to a premature stop codon. Work in heterologous expression systems revealed that the protein is localized to the endoplasmic reticulum with complete loss of cell surface expression. In agreement with these findings, GABA mediated currents were absent in these cells (Lachance-Touchette

2011). The fate of the mutant protein and the mechanism by which the α1(K353delins18X) mutation leads to epilepsy is yet to be determined.

a1(D219N) Missense Mutation The D219N missense mutation was identified in 4 of 5 affected individuals with idiopathic generalized epilepsy in a French-Canadian family exhibiting idiopathic generalized epilepsy or febrile seizures—so named due to their coincidence with fever (Pamplona 1989). Two of the four individuals with febrile seizures also reported a single generalized tonic clonic seizure. Studies conducted in heterologous expression systems indicate that surface expression of the mutant subunit is reduced as compared to wild type (WT) α1 subunit, consistent with observations of decreased GABA evoked peak current amplitudes. Additionally, α1(D219N) subunit containing receptors were shown to desensitize more rapidly than WT α1 subunit containing receptors (Lachance-Touchette 2011). Further studies characterizing the effects of this mutation both *in vitro* and *in vivo* will be required to elucidate the mechanism by which it promotes the development of epilepsy.

a1(A322D) Missense Mutation The A322D missense mutation was identified in 8 affected individuals within a large French-Canadian family suffering from a type of idiopathic generalized epilepsy called juvenile myoclonic epilepsy. Myoclonic seizures are characterized by sudden, brief, involuntary jerks of the arms or legs (Pamplona 1989). This mutation is autosomal dominant and results in the insertion of a charged aspartate residue in place of a highly conserved alanine within the M3 domain (Cossette 2002; Macdonald 2006; Macdonald 2004; Macdonald 2010a). Experiments in heterologous cells indicate that this mutation disrupts the insertion of M3 into the lipid bilayer which results in its retention in the endoplasmic reticulum and subsequent

partial degradation through endoplasmic reticulum associated degradation (ERAD). (Gallagher 2005; Macdonald 2004; Macdonald 2006; Macdonald 2010a) However, the mutant subunit is not completely degraded as total and surface mutant protein is detectable, albeit at significantly lower levels than the WT α1 subunit (Gallagher 2005; Macdonald 2004; Macdonald 2006; Macdonald 2010a). It has also been postulated that the α1(A322D) mutation exerts a dominant negative effect by oligomerizing with and trapping WT subunits in the endoplasmic reticulum which are then subject to ERAD (Ding 2010; Macdonald 2006). Consistent with reduced surface expression, the peak GABA evoked current through receptors containing the α1(A322D) subunit was reduced 88% (Gallagher 2004). In the mutant receptors, open time of the channel was considerably reduced. Additionally, α1(A322D) subunit containing receptors exhibited substantially reduced sensitivity to GABA with a nearly 100-fold increase in GABA EC50 (Gallagher 2004).

a1(S326fs328X) Frameshift Mutation The α1(S326fs328X) mutation is an autosomal dominant de novo mutation identified in a patient with childhood absence epilepsy. In contrast to the previously mentioned seizure types, absence seizures are not associated with any sort of convulsions or motor movements. Rather, they are characterized by sudden brief lapses in consciousness often accompanied by a blank stare (Pamplona 1989). A single base pair deletion leads to a frameshift and premature termination codon (PTC) in the eighth exon which corresponds to the third transmembrane domain of the protein (Maljevic 2006). The PTC has been shown to induce nonsense mediated decay (NMD) of the mutant mRNA, albeit incomplete. Mutant mRNA that escapes NMD is subsequently translated into truncated protein

which is retained in the ER and subjected to ERAD (Kang 2009; Macdonald 2010b). Thus, the α1(S326fs328X) mutant subunit is not incorporated into the cell membrane and GABA evoked currents are absent (Kang 2009; Maljevic 2006). Based on these findings, it is thought that the epilepsy phenotype is a result of haploinsufficiency in the WT α1 gene. This raises the intriguing possibility that heterozygous α1 knockout animals could serve as a model for this disease. Indeed, we recently revealed through EEG analysis that heterozygous α1 knockout mice do in fact experience absence seizures (Arain 2012). This may explain why it was previously reported that α1 knockout mice did not exhibit an epileptic phenotype as mentioned above. The seizures were greatly attenuated by treatment with ethosuximide, the most commonly prescribed drug for absence seizures in human patients. This represents a novel model of absence epilepsy based on a mutation identified in a human epilepsy patient that is face, construct, and predictively valid.

Conclusion

$GABA_A$Rs are a heterogeneous population of receptors and their properties are heavily influenced by their α subunit composition. The α1 subunit is the most predominant subunit in the adult brain and is largely responsible for the maintenance of inhibitory tone in the CNS. As evidenced by the consequences of the dysfunction or loss of the α1 subunit, it also seems to be involved in epilepsy susceptibility. The transgenic α1(A322D) knock-in and α1 knockout mouse lines that our group focuses on represent the first mouse models of genetic epilepsy based on mutations in the α1 subunit identified in human epilepsy patients. These two mutations were both identified in patients suffering from generalized epilepsy, but their seizure phenotypes were distinct.

Based on our preliminary analyses, the epileptic phenotypes of the two mouse lines also differ. By dissecting the similarities and differences in pathogenesis behind these two models, we aim to identify common themes among all generalized epilepsies and also delineate important differences that contribute to distinct disease manifestations. While epilepsy is an exceptionally complicated and heterogeneous condition, the advent of animal models reflecting specific disease-associated mutations in $GABA_ARs$ represents a promising new avenue for advancing our understanding of the pathogenesis of generalized epilepsy. The majority of my work has been in the heterozygous α1 knockout model of CAE; this work will be discussed in detail in subsequent chapters.

CHAPTER III

EXPERIMENTAL STRATEGY

Rationale

In 2006, a heterozygous de novo frameshift mutation in the α1 subunit of the $GABA_AR$ was identified in a patient with childhood absence epilepsy (Maljevic 2006). Subsequent analysis of this mutation *in vitro* revealed that the mutation caused complete elimination of the mutant α1 subunit protein via nonsense mediated decay of the mutant mRNA and endoplasmic reticulum associated degradation of the mutant protein (Kang 2009). Thus, it is thought that the patient's epileptic phenotype arose as a result of haploinsufficiency of the wild type $GABA_A$ receptor α1 subunit. This indicated that heterozygous α1 knockout mice may serve as a model for studying CAE. Thus we generated α1 knockout mice and characterized the heterozygous animals to determine if they recapitulate an epilepsy phenotype. Indeed, EEG analysis revealed that the heterozygous α1 knockout animals exhibit an absence epilepsy phenotype characterized by brief 6-8 Hz SWD often accompanied by behavioral arrest (Arain 2012). Aberrant oscillations within thalamocortical circuitry are most widely associated with absence seizures and SWDs and the cortex is postulated to play a leading role (Polack 2007). Interestingly, the epileptiform events in the heterozygous α1 knockout mice were greatly attenuated by treatment with ethosuximide, the most commonly prescribed drug for the treatment of absence seizures in humans. Thus, heterozygous α1 knockout mice serve as a model for absence epilepsy that is construct, face, and predictively valid (Arain 2012).

It has been reported that homozygous α1 knockout animals express 60% less $GABA_A$Rs than wild type mice (Kralic 2002). Additionally, multiple studies have shown that the complete loss of the α1 subunit is compensated for, in part, by increased expression of other $GABA_A$ receptor subunits—namely α2 and α3 (Kralic 2006; Kralic 2002). However, few studies have assessed whether or not compensation occurs in heterozygous α1 subunit knockout mice, nor has there been any work specifically measuring levels of $GABA_A$ receptor subunit proteins expressed on the cell surface. Because only receptors expressed on the surface of cells mediate GABAergic currents, surface expression of $GABA_A$ receptor subunit proteins is very important to measure; this is the receptor population that dictates cellular GABAergic physiology. Likewise, it is well established that different α subunits confer distinct receptor properties and distinct inhibition (Eyre 2012). Thus, if compensation occurs in the heterozygous animals, physiological properties of the cells could be altered which may in turn offer insight into the pathophysiology of absence epilepsy.

Our overarching goal was to investigate the basis for the absence phenotype in the heterozygous α1 knockout animals by assessing the expression of various $GABA_A$ receptor subunit proteins using biochemical techniques and to determine whether or not the heterozygous α1 knockout animals exhibited alterations in cortical GABAergic physiology.

Experimental Strategy

Using heterozygous α1 knockout (α1HetKO) mice and wild type mice as controls, we performed the following experiments. Employing brain slice biotinylation and quantitative Western blotting, we measured both surface and total expression of

$GABA_A$ receptor proteins. We first determined the effect of α1HetKO on the total and surface expression of *functional* $GABA_A$ receptors Using β subunit expression—all known functional $GABA_A$ receptors contain two β subunits. Next we quantified the surface and total expression of the α1 subunit as well as the other synaptic α subunits, α2 and α3.

Upon observing changes in expression of subunit proteins, we then attempted to identify the mechanism by which these changes occurred. We determined if the changes were a result of altered gene expression by measuring mRNA via quantitative real time polymerase chain reaction (qRT-PCR). We also used a recently described electrophysiological technique (Kotowski 2011) in combination with the dynamin-mediated endocytosis inhibitor, dynasore, to assess whether or not α1HetKO animals exhibited altered endocytosis or recycling of their surface-resident $GABA_A$ receptors.

Lastly, we sought to determine if the changes we observed in $GABA_A$ receptor expression using biochemical techniques had any effect on neuronal GABAergic physiology in layer VI of the cortex. This is the layer that projects to the thalamus and is thought to play an integral role in aberrant thalamocortical oscillations.

In these studies, we identified changes in $GABA_A$ receptor expression and physiology in animals exhibiting an absence epilepsy phenotype. The results reported and discussed in the following chapters may serve to elucidate pathophysiological mechanisms by which $GABA_A$ receptor dysfunction could lead to the development of absence epilepsy.

CHAPTER IV

METHODOLOGY

Animals

All procedures and protocols performed here were approved by the Vanderbilt University Animal Care and Use Committee. Mice were housed in a temperature and humidity controlled environment with ad libitum access to food and water on a 12:12 light:dark cycle. In 2001, Vicini et al. produced a line of transgenic mice with loxP sequences flanking exon 9 of the $GABA_A$ receptor α1 subunit (Vicini 2001). Using these mice, we subsequently generated global α1 subunit deletion mice and bred them into a congenic C57BL/6J background (Arain 2012). We crossed heterozygous α1 knockout mice with wild type mice and used the female offspring between ages P33-P37. These mice were shown to have frequent absence seizures in our previous EEG studies. The genotypes of the mice used for experiments were determined using PCR.

Brain Slice Preparation

Solutions used for brain slice preparation and incubation are listed in Table 4.1.The mice were anesthetized using isoflurane and then sacrificed. The brains were rapidly dissected and moved to ice cold cutting solution for sectioning. In the biotinylation experiments, three to four 300 μm midline sagittal slices were made using a vibratome (Leica VT1200S). The slices were then incubated in artificial cerebrospinal fluid (aCSF) and bubbled with 100% O_2 at 0°C and biotinylated less than one hour after slices were cut. For electrophysiology experiments, 300 μm coronal slices containing the somatosensory cortex were sectioned in cutting buffer and then incubated in aCSF

which contained 26 mM $NaHCO_3$ and 2 mM $MgCl_2$ and bubbled with 95%O_2/5%CO_2 at 36°C for 30 minutes (Schofield 2009; Zhou 2011; Moyer 1998). The slices were then incubated at room temperature for at least one hour before recording.

Table 4.1: Brain Slice Solutions					
Biotinylation				Electrophysiology	
Cutting		aCSF		Cutting	
Reagent	mM	Reagent	mM	Reagent	mM
Sucrose	210	NaCl	126	Sucrose	214
NaCl	20	KCl	2.5	CaCl2	0.05
KCl	2.5	NaH_2PO_4	1.25	KCl	2.5
NaH_2PO_4	1.2	$CaCl_2$	2	NaH2PO4	1.25
$MgCl_2$	1	$MgCl_2$	1	MgSO4	10
D-glucose	10	D-glucose	10	NaHCO3	24
pH	7.4	pH	7.4	D-glucose	11
				pH	7.4

Brain Slice Biotinylation

Brain slice biotinylation has been described previously for the quantification of protein expressed on the cell surface in intact brain slices (Goodkin 2008; Robertson 2010; Vithlani 2011). After cutting, the brain slices were incubated at 4°C for 45minutes in aCSF containing 1 mg/mL of the membrane-impermeable biotinylation reagent sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3'-dithiopropionate (Sulfo-NHS-SS-biotin, Thermo Scientific). Next, the biotinylation reaction was quenched by washing the slices with aCSF containing 0.1 M glycine. The cortices were dissected from the slices and

subsequently sonicated in radioimmunoassay solution (RIPA, 20 mM Tris pH 7.4, 1% TritonX-100, 250 mM NaCl) containing 1:100 protease inhibitor cocktail (Sigma Aldrich) and 0.5% deoxycholate and 0.1%SDS.

Protein concentrations were measured using a bicinchoninic acid-based assay (Thermo Scientific). 150 μg of cortical protein lysate was incubated with 100 μL neutravidin beads (Thermo Scientific) in a total volume of 500 μL RIPA overnight at 4°C in order to isolate the biotinylated proteins from the cell surface. In order to confirm that the neutravidin beads did not become saturated with biotinylated protein, we incubated the beads with increasing concentrations of cortical protein lysate and subsequently observed proportional increases in the amount of biotinylated material recovered from the beads. After the overnight incubation, the beads were centrifuged to form a pellet which was then washed three times with RIPA. The biotinylated protein was then eluted from the beads using 60-80 μL of Laemmli sample buffer (BioRad) containing 5% β-mercaptoethanol to yield the surface protein fraction. The surface fraction was then analyzed using Western blot. To verify that the biotinylation reagent was selective for surface membrane proteins, we determined the amount of the cytoplasmic protein GAPDH present in samples purified with the neutravidin beads versus unpurified samples.

Antibodies and Western Blots

Primary and secondary antibodies used in the analysis of Western blots are listed in Table 4.2 and Table 4.3, respectively. Total and surface proteins were separated on 10% SDS-PAGE gels and subsequently electrotransferred onto nitrocellulose membranes. To verify linearity of detection, increasing concentrations or volumes of

protein were added to the gel (5-10-20 µg from the total fraction, and 5-10-20 µL of surface fraction eluted from either the neutravidin or protein G beads). We excluded blots in which the signal from each protein did not increase proportionally with the amount loaded from our analyses. Likewise, we confirmed that α1HetKO did not affect the total or surface expression of our loading control protein, the α subunit of the Na^+/K^+ ATPase. Before exposing the membrane to antibody, nonspecific binding was blocked using 5% nonfat dry milk in Tris buffered saline containing 0.1%Tween at pH 7.4. Blots were incubated in primary antibody overnight at 4°C and then with secondary antibody at room temperature for one hour. The blots were then imaged on an infrared fluorescent imaging system (Licor).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Relative abundances of the α1 and α3 subunits of the $GABA_A$ receptor mRNA were measured in mouse cortices from wild type and heterozygous α1 subunit knockout mice as described previously (Zhang 2011). We isolated total RNA from freshly dissected cortices using a silica membrane column (Purelink). We produced corresponding cDNA from 200 ng of RNA using reverse transcriptase and random hexamers (Applied Biosystems). qRT-PCR was performed with an Applied Biosystems 7900 system with TaqMan universal Master Mix, FAM-labeled probes (Applied Biosystems) for the α1 subunit (Mm00439046, spanning Exon 9 which is deleted in the transgene) and the α3 subunit (Mm01294271). We used a VIC-labeled probe (Applied Biosystems) for beta-actin, our endogenous control (4352341E). The following RT-PCR conditions were used for these experiments: 10 minutes at 90°C, 40 cycles of: 15 seconds at 95°C for denaturation and 60 seconds at 60°C for annealing and extension. After

verifying that the heterozygous loss of the α1 subunit did not alter the expression of the endogenous control, we used the ΔΔCt cycle threshold method to determine the effect of the heterozygous α1 subunit knockout on the expression of α1 and α3 subunit mRNA when normalized to expression of beta-actin mRNA.

Electrophysiology

Using an upright Nikon eclipse FN-1 IR-DIC microscope, pyramidal neurons residing in layer VI in the S1/S2 region of the somatosensory cortex were identified for whole-cell patch clamp recordings. Neurons located in the cortical layer above the white matter and exhibiting a characteristic apical dendrite and large soma were chosen for these recordings. The solution within the patch pipette contained the following in mM: 135 CsCl, 10 EGTA, 10 HEPES, 5 ATP-Mg, and 5 QX-314 at a pH of 7.3 and osmolarity within the range of 290-295 mOsm (Schofield 2009). The bath solution was aCSF which also contained 20 μM NBQX to block AMPA and kainite receptors. Filled electrodes exhibited 2-4 MΩ resistances; serial resistance was monitored throughout the experiments and we discarded recordings that had greater than 25 MΩ or 20% change in serial resistance during the recording. The data were collected with a MultiClamp 700B amplifier and Clampexx 10.2 software, filtered at 2 kHz, and digitized at 20 kHz using a Digidata 1440A analog to digital converter (Molecular Devices Inc.). All data were recorded with compensation for series resistance (70%) and cell capacitance.

Miniature inhibitory post synaptic currents (mIPSCs) were recorded in bath solution also containing 1 μM tetrodotoxin (Sigma-Aldrich) to block sodium channels and thus action potentials. Recordings were made in voltage clamp mode with the cells clamped at -60 mV for at least 20 minutes. The mIPSCs were identified offline

automatically using Clampfit 10.2 and confirmed visually. Peak mIPSC amplitudes, inter-event intervals, and 10-90% rise times were also identified. We fit the current decay of each mIPSC to a single exponential and calculated the decay constant (τ). mIPSCs having peak amplitudes less than 1 pA, τ less than 1 ms or greater than 1000 ms, or rise time greater than 10 ms were excluded from our analyses. Cumulative histograms were constructed for all mIPSCs from all neurons of the same genotype for peak amplitudes, event intervals, rise times, and decay constants. We also calculated the mean peak amplitude, event interval, rise time, and decay constant from each individual neuron and averaged these among cells of the same genotype. We then compared these averaged values from heterozygous α1 knockout and wild type neurons using a two tailed t-test. Additionally, averaged mIPSC tracings were created for each neuron and the current decay of these average traces were fit with one or two time constants (τ_1 and τ_2) and then calculated the weighted decay constant (τ_w) using the following equation: $(\tau_1 \times A_1 + \tau_2 \times A_2) \div (A_1 + A_2)$ where A_1 and A_2 are the amplitudes corresponding to τ_1 and τ_2, respectively.

Receptor Endocytosis and Recycling

To determine the effect of heterozygous loss of the $GABA_A$ receptor α1 subunit on $GABA_A$ receptor endocytosis and recycling from the plasma membrane in intact brain slices, we adapted a previously published protocol used to measure dopamine receptor endocytosis (Kotowski 2011). First, we recorded mIPSCs as described above for a five minute baseline period. Next, we added dynasore (3-hydroxy-naphthalene-2-carboxylic acid), an effective inhibitor of dynamin-dependent endocytosis, and recorded mIPSCs for an additional 20 minutes. The final concentrations of dynasore and DMSO

were 80 μM and 0.2%, respectively. mIPSC peak amplitudes were averaged in one minute epochs before and after treatment with dynasore, and time-dependent changes in mIPSC amplitudes were compared between wild type and heterozygous $GABA_A$ receptor α1 subunit knockout neurons.

Data Analysis and Statistics

Statistical analyses were performed with the R 2.12.2 Statistical Package for Windows (R Foundation for Statistical Computing). All of the results presented herein are the mean ± standard error. The statistical significance of averaged values was assessed using single sample or independent sample t-test as appropriate. Additionally, we used the two sample Kolmogorov—Smirnov (K-S) test to compare distributions of wild type and heterozygous α1 knockout mIPSC peak amplitudes, event intervals, 10-90% rise times, and decay constants. P values less than 0.05 were considered to be statistically significant.

Table 4.2: Primary Antibodies					
Protein	Clone/Catalog No.	Dilution	Raised In Species	Mono/Polyclonal	Source
$GABA_AR$ α1 Subunit	N95/35	1:250	mouse	monoclonal	UC Davis/NIH NeuroMab
$GABA_AR$ β1 Subunit	N96/55	1:100	mouse	monoclonal	UC Davis/NIH NeuroMab
$GABA_AR$ β3 Subunit	N87/25	1:100	mouse	monoclonal	UC Davis/NIH NeuroMab
$GABA_AR$ β2 Subunit	AB5561	1:100	rabbit	polyclonal	Millipore
$GABA_AR$ α2 Subunit	AB72445	1:100	rabbit	polyclonal	Abcam
$GABA_AR$ α3 Subunit	AGA-003	1:500	rabbit	polyclonal	Alomone
Na^+/K^+ ATPase α subunit	A6F	1:100	mouse	monoclonal	The Developmental Studies Hybridoma Databank
GAPDH	AB9485	1:2000	rabbit	polyclonal	Abcam

Table 4.3: Fluorescently Conjugated Secondary Antibodies			
Antibody	Catalog No.	Dilution	Source
Goat Anti-rabbit-680	926-32221	1:10,000	Licor
Goat Anti-mouse-800	926-32210	1:10,000	Licor

CHAPTER V

RESULTS

α1HetKO Causes a Modest Reduction in Functional $GABA_A$ Receptor Expression

Because all known functional $GABA_A$ receptors contain two β subunits, β subunit expression can be used as a measure of functional $GABA_A$ receptor expression. We measured the total and surface expression of the β1, β2, and β3 subunits in the cortex of WT and α1HetKO mice. We did not detect any total or surface expression of the β1 subunit in the WT or α1HetKO samples (not shown). However, we were able to reliably detect recombinantly expressed β1 subunits with our immunoblots; this indicates that our methods for detecting the β1 subunit protein are effective and that the β1 subunit is not abundantly expressed in the cortex of mice at this age. α1HetKO caused a reduction to 75 ± 8% as compared to WT in total β2 expression (N=8, P=0.019 vs. 100%) and a reduction to 78 ± 5% in surface expression of β2 as compared to WT (N=8, P=0.003 vs. 100%, Figure 5.1 A). Conversely, α1HetKO did not alter the total (102 ± 8%, N=6, P=0.796 vs. 100%) or surface (96 ± 5%, N=8, P=0.458 vs. 100%) expression of the β3 subunit when compared to WT (Figure 5.1 B). α1HetKO did not alter β3 expression and caused only a modest reduction in β2 expression, suggesting only a small reduction in functional $GABA_A$ receptor expression despite the heterozygous loss of the most abundant subunit. This finding is consistent with previous reports of compensatory upregulation of the expression of other $GABA_A$ receptor subunits in homozygous α1 subunit knockout mice. We next examine the expression of synaptic α subunits to determine if compensation occurs in the heterozygous condition as well.

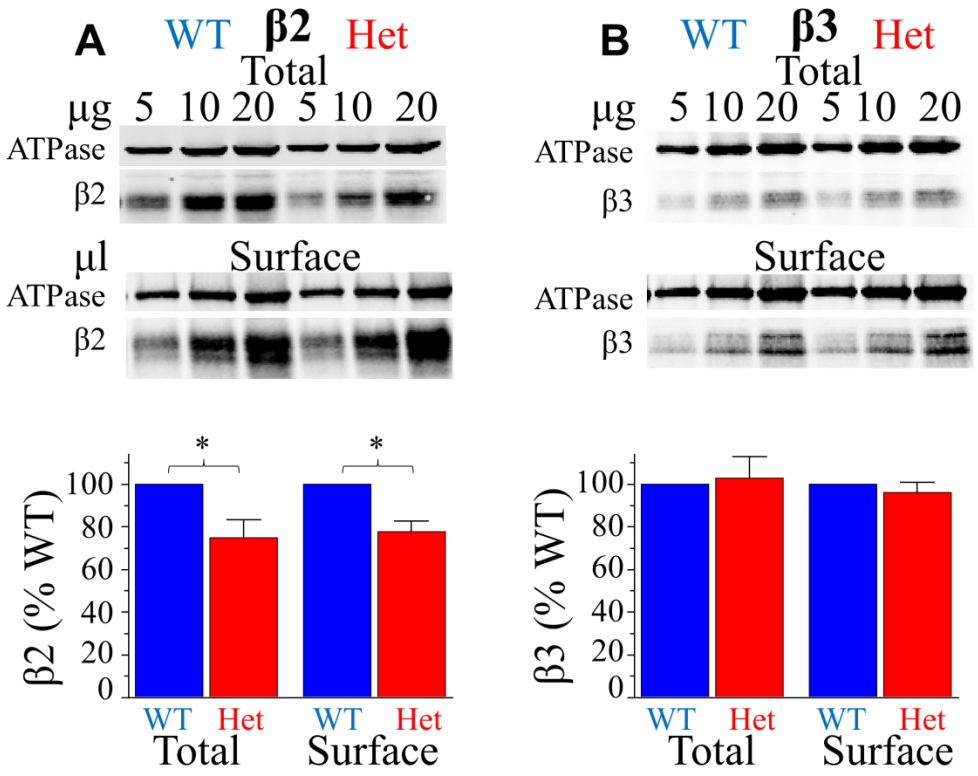

Figure 5.1: α1HetKO Causes a Modest Reduction in Functional $GABA_A$ Receptor Expression. We analyzed 5, 10, and 20μg of total and 5, 10, and 20μL surface protein from biotinylated cortical brain slices on Western blot and stained for the Na^+/K^+ ATPase α subunit, GAPDH (not shown), the β2 subunit (A), and the β3 subunit (B). The graphs display the relative amounts of β2 and β3 subunit proteins normalized to the Na^+/K^+ ATPase α subunit loading control from α1HetKO mice as compared to wild type. A: α1HetKO caused a 75 ± 8% reduction in total β2 expression (N=8, P=0.019 vs. 100%) and a 78 ± 5% reduction in surface expression of β2 as compared to WT (N=8, P=0.003 vs. 100%). B: α1HetKO did not alter the total (102 ± 8%, N=6, P=0.796 vs. 100%) or surface (96 ± 5%, N=8, P=0.458 vs. 100%) expression of the β3 subunit as compared to WT. Data acquired by Li Ding and M.E. Deel.

α1HetKO Increases the Surface : Total Expression Ratio of the α1 Subunit

A previous report indicated that α1HetKO decreased total expression of the α1 subunit protein (Kralic 2006), but there have been no studies investigating the effect of α1HetKO on the surface expression of the α1 subunit. Because only receptors expressed on the surface of cells mediate GABAergic currents, this is the receptor population that dictates cellular GABAergic physiology. Thus, in order to understand how α1HetKO could alter cellular physiology and precipitate epilepsy, it is vital to measure the effects of α1HetKO on surface expression of $GABA_A$ receptor subunits. Consistent with previous results (Kralic 2002), we found that α1HetKO decreased total α1 expression to 62 ± 8% of WT expression (N=8, P=0.002 vs. 100%, Figure 5.2). Yet, α1HetKO elicited a significantly smaller reduction of α1 subunit protein expressed on the cell surface (89 ± 5%, N=5, P=0.015 vs. total expression, Figure 5.2). These results indicate that the cortex of α1HetKO mice may partially compensate for the heterozygous loss of α1 by increasing the surface : total expression ratio. We next explored whether or not other synaptic α subunits—namely α2 and α3—participated in compensation in the α1HetKO mice.

Expression of the α3 But Not the α2 Subunit is Increased in α1HetKO Cortex

Wild type cortical synapses express the α2 and α3 subunits in conjunction with the α1 subunit, though α1 is by far the most abundant (Heller 2012; Hutcheon 2004; Yu 2006). Thus, we investigated whether or not α2 and α3 could compensate for the loss of α1 by measuring the total and surface levels of these two subunits in cortices from WT and α1HetKO mice.

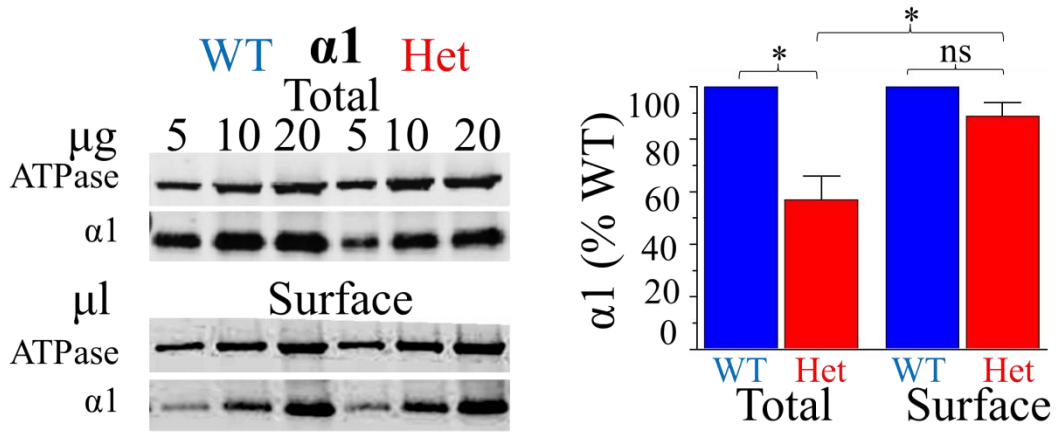

Figure 5.2: α1HetKO Increased the Surface : Total Expression Ratio of α1 Subunit Protein in the Cortex. We analyzed 5, 10, and 20µg of total and 5, 10, and 20µL surface protein from biotinylated cortical brain slices on Western blot and stained for the Na^+/K^+ ATPase α subunit, GAPDH (not shown), and the α1 subunit. The graphs display the relative amounts of α1 subunit protein normalized to the Na^+/K^+ ATPase α subunit loading control from α1HetKO mice as compared to wild type. α1HetKO decreased total α1 expression to 62 ± 8% of WT expression (N=8, P=0.002 vs. 100%). However, α1HetKO elicited a significantly smaller reduction of α1 subunit protein expressed on the cell surface (89 ± 5%, N=5, P=0.015 vs. total expression). Data acquired by Li Ding and M.E. Deel.

α1HetKO did not elicit any significant changes in the total (105 ± 4%, N=4, P=0.389, Figure 5.3 A) or surface (117 ± 7%, N=5, P=0.081, Figure 5.3 A) expression of the α2 subunit as compared to WT. Conversely, α1HetKO significantly increased the expression of the α3 subunit in both the surface and total fractions. Total α3 expression was increased to 138 ± 18% that of WT (N=10, P=0.016 vs. 100%, Figure 5.3 B) while α3 surface expression increased to 174 ± 24% that of WT (N=7, P=0.020 vs. 100%, Figure 5.3 B). These findings indicate that α1HetKO also partially compensates for the loss of the α1 subunit by increasing both the total and surface expression of the α3 subunit protein. We next attempted to identify the mechanism by which the compensatory changes identified thus far occur.

α1HetKO Does Not Alter the Expression of WT α1 or α3 Subunit mRNA

Using quantitative real time PCR (qRT-PCR), we measured levels of WT α1 and α3 subunit mRNAs in the cortices from WT and α1HetKO mice. We confirmed that α1HetKO did not alter the expression of actin, our endogenous control mRNA used for normalization. α1HetKO expressed 52 ± 7% (N=6, P=0.750 vs. 50%, not shown) that of WT α1 subunit mRNA, consistent with the heterozygous deletion of the α1 subunit gene. This indicates that α1HetKO does not compensate for the loss of α1 by upregulating the expression of the remaining WT allele. Likewise, we observed no significant changes in the expression of α3 subunit mRNA (90 ± 7%, N=6, P=0.230 vs. 100%, Figure 5.4). These results indicated a post-transcriptional mode of upregulation may be responsible for the compensatory increases in α1 and α3 protein expression. We next examined whether α1HetKO altered endocytosis and recycling of $GABA_A$ receptors from the cell surface as a possible mode of upregulation.

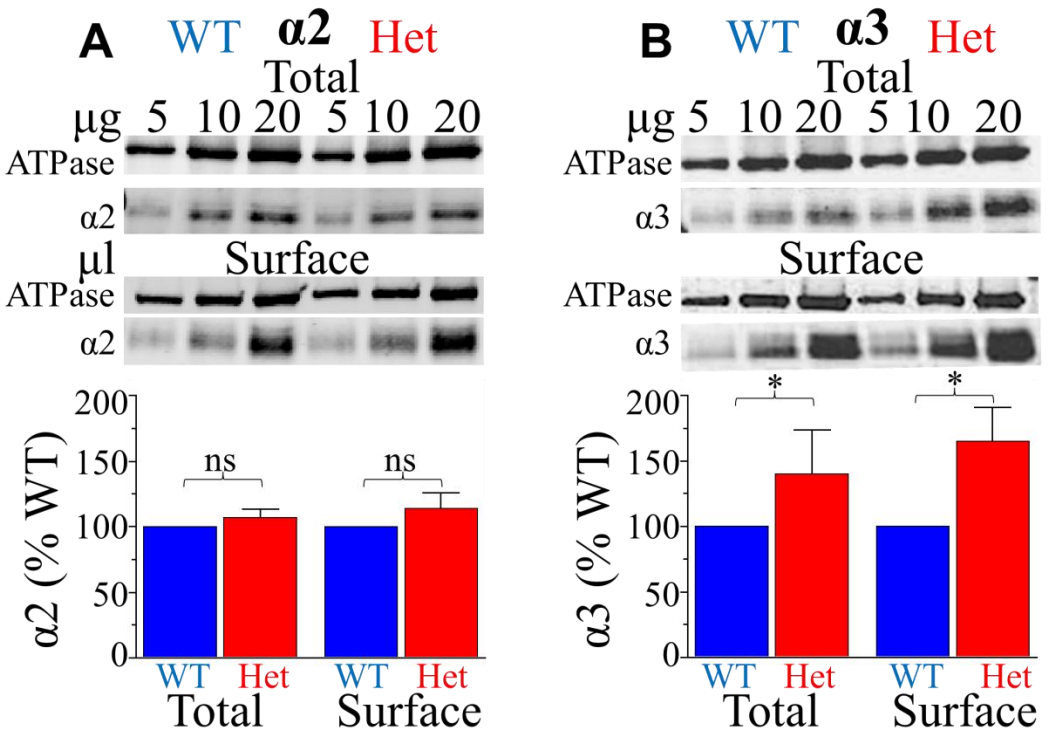

Figure 5.3: α1HetKO Increased Surface and Total Expression of α3 Subunit Protein But Did Not Alter α2 Subunit Protein Expression. We analyzed 5, 10, and 20µg of total and 5, 10, and 20µL surface protein from biotinylated cortical brain slices on Western blot and stained for the Na^+/K^+ ATPase α subunit, GAPDH (not shown), the α2 subunit (A), and the α3 subunit (B). The graphs display the relative amounts of α2 and α3 subunit proteins normalized to the Na^+/K^+ ATPase α subunit loading control from α1HetKO mice as compared to wild type. A: α1HetKO did not elicit any significant changes in the total (105 ± 4%, N=4, P=0.389) or surface (117 ± 7%, N=5, P=0.081) expression of the α2 subunit as compared to WT. B: α1HetKO significantly increased the expression of the α3 subunit in both the surface (174 ± 24%, N=7, P=0.020 vs. 100%) and total (138 ± 18%, N=10, P=0.016 vs. 100%) as compared to WT. Data acquired by Li Ding and M.E. Deel.

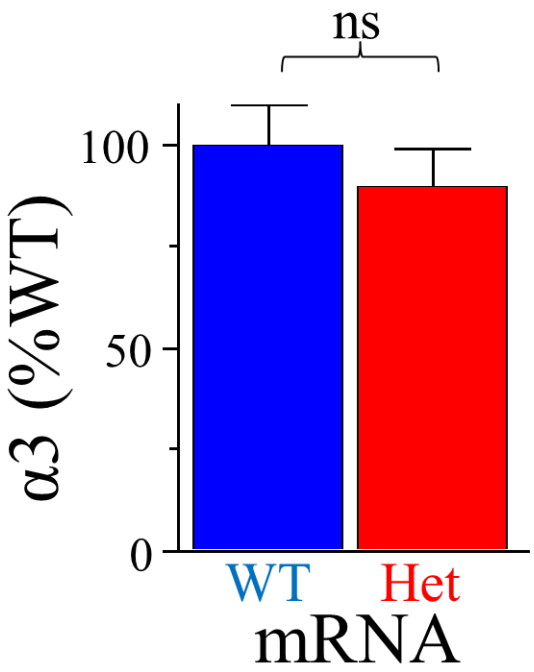

Figure 5.4: α1HetKO Does Not Alter the Expression α3 Subunit mRNA. Using quantitative real time PCR (qRT-PCR), we measured levels of α3 subunit mRNA in the cortices from WT and α1HetKO mice. We confirmed that α1HetKO did not alter the expression of actin, our endogenous control mRNA used for normalization (not shown). We observed no significant changes in the expression of α3 subunit mRNA (90 ± 7%, N=6, P=0.230 vs. 100%).

α1HetKO Alters the Endocytosis and Recycling of $GABA_A$ Receptors

Recently, dopamine receptor endocytosis and recycling was measured using brain slice patch clamp electrophysiology in conjunction with the administration of dynasore, an effective inhibitor of dynamin-dependent endocytosis (Kotowski 2011). Previous reports indicate that the surface expression of $GABA_A$ receptors is regulated by dynamin-dependent endocytosis (Kittler 2000; Kittler 2004; Kittler 2005; Kittler 2008). Therefore, we measured the effects of dynasore on GABAergic currents in brain slices from WT and α1HetKO mice. We recorded mIPSCs from layer VI pyramidal neurons for a baseline period of five minutes and then added 80 μM dynasore to the bath solution and recorded mIPSCs for an additional 20 minutes. The application of dynasore elicited a time-dependent increase in the mIPSC amplitude of WT neurons but had no significant effect on those of α1HetKO neurons (Figure 5.5 A). After 20 minutes of dynasore treatment, mIPSC peak amplitude in WT was 126 ± 9.4% of baseline (N=5) while mIPSC peak amplitude in α1HetKO neurons was only 87 ± 7.9% of baseline (N=5, P=0.014 α1HetKO vs. WT effect of dynasore, Figure 5.5 B). These data suggest that WT neurons exhibit a higher rate of $GABA_A$ receptor insertion into the membrane from the cytoplasm and that α1HetKO reduce the amount of $GABA_A$ receptors in early endosomes through a decreased rate of baseline endocytosis.

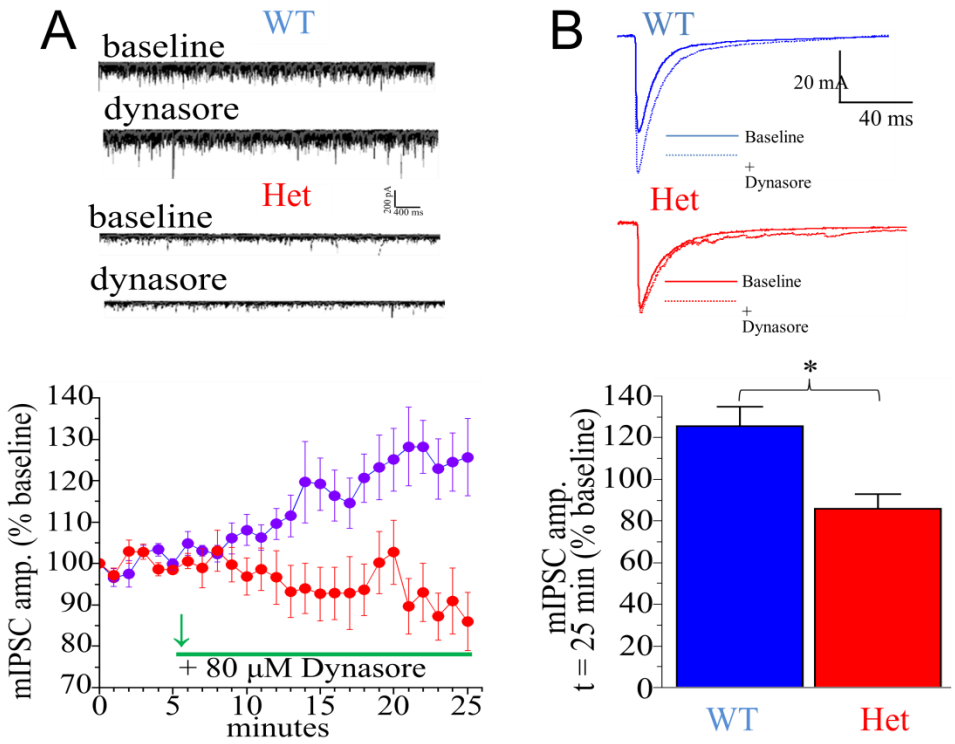

Figure 5.5: Inhibition of Dynamin-Dependent Endocytosis Using Dynasore Increased Wild Type But Did Not Alter α1HetKO mIPSC Peak Current Amplitude. We measured the effects of dynasore on GABAergic currents in brain slices from WT and α1HetKO mice. We recorded mIPSCs from layer VI pyramidal neurons for a baseline period of five minutes and then added 80µM dynasore to the bath solution and recorded mIPSCs for an additional 20 minutes. A: The application of dynasore elicited a time-dependent increase in the mIPSC amplitude of WT neurons but had no significant effect on those of α1HetKO neurons. B: After 20 minutes of dynasore treatment, mIPSC peak amplitude in WT was 126 ± 9.4% of baseline (N=5) while mIPSC peak amplitude in α1HetKO neurons was only 87 ± 7.9% of baseline (N=5, P=0.014 α1HetKO vs. WT effect of dynasore). Data acquired by Dr. Chengwen Zhou.

α1HetKO Alters Synaptic GABAergic Currents

Recall from Chapter II that the identity of the α subunit within a $GABA_A$ receptor in large part dictates the biophysical properties of the receptor and thus the properties of inhibitory currents of the neuron in which they are expressed. In particular, $GABA_A$ receptors containing the α3 subunit exhibit reduced sensitivity to GABA, increased rise times, and slower current decay kinetics as compared to α1 containing receptors. Because our biochemical experiments indicated a large increase in α3 subunit expression in the α1HetKO mice, we investigated the effects of α1HetKO on the properties of synaptic GABAergic currents in the cortex. Specifically, we recorded mIPSCs from layer VI pyramidal neurons in the somatosensory cortex of brain slices from WT and α1HetKO mice (Figure5.6 A). Recall from Figure 1.1 that this region is thought to initiate the aberrant oscillations characteristic of absence seizures (Polack 2007) and projects to the thalamus which is thought to sustain and facilitate the generalization of the oscillations (Paz 2011).

We found that α1HetKOcaused a significant reduction in the average mIPSC peak amplitude when all mIPSCs were analyzed together in a cumulative histogram as well as when averaged among individual neurons (WT: -43 ± 4.1pA, α1HetKO: -32 ± 2.5pA, P=0.032, Figure 5.6 C). α1HetKO also significantly increased the rise time of inhibitory currents (WT: 1.8 ± 0.15ms, α1HetKO: 2.4 ± 0.20ms, P=0.024). We used a single exponential to fit the time course of current decay of each mIPSC to calculate the decay time constant, τ, for each mIPSC. We found that both when analyzed in a cumulative histogram and when averaged among neurons, the α1HetKO neurons exhibited significantly longer decay time constants (WT: 24 ± 0.9ms, α1HetKO: 27 ±

1.3ms, P=0.034, Figure 5.6 B). Oftentimes, $GABA_A$ receptor current decay is fit to the sum of two exponentials. However, it is very difficult to accurately determine when applied to each individual mIPSC; thus, we averaged all the mIPSC traces from each individual neuron and then fit the current decay to one or two exponentials and calculated the weighted decay time constant, τ_w. We found that α1HetKO neurons exhibited a significantly longer τ_w as compared to WT (WT: 12.7 ± 1.0ms, α1HetKO: 17.4 ± 1.6ms, P=0.024). The changes we observed in the synaptic physiology of layer VI cortical neurons from α1HetKO mice are consistent with our biochemical findings of increased α3 subunit expression in α1HetKO mice.

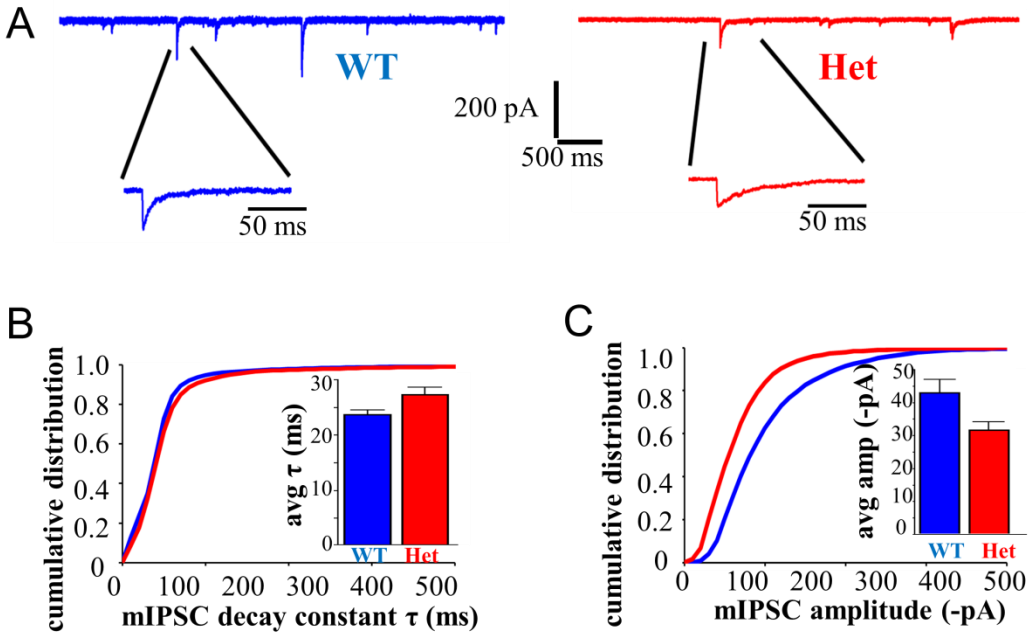

Figure 5.6: α1HetKO Decreased the mIPSC Peak Current Amplitude and Altered the Time Course of Current Kinetics in Layer VI Pyramidal Neurons. We recorded mIPSCs from layer VI pyramidal neurons in the somatosensory cortex of brain slices from WT and α1HetKO mice. A: Representative mIPSC traces from WT (blue, left) and α1HetKO (red, right) neurons. B: When analyzed in a cumulative histogram and when averaged among neurons, the α1HetKO neurons exhibited significantly longer decay time constants (WT: 24 ± 0.9ms, α1HetKO: 27 ± 1.3ms, P=0.034). C: α1HetKOcaused a significant reduction in the average mIPSC peak amplitude when all mIPSCs were analyzed together in a cumulative histogram as well as when averaged among individual neurons (WT: -43 ± 4.1pA, α1HetKO: -32 ± 2.5pA, P=0.032). Data acquired by Dr. Chengwen Zhou.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

β Subunit Expression

We found that α1HetKO reduced the expression of the β2 subunit of the $GABA_A$ receptor, but had no significant effect on the expression of the β3 subunit. Given the fact that the α1 subunit is the most abundant $GABA_A$ receptor subunit and is expressed almost ubiquitously throughout the brain, it was somewhat surprising that we only observed a modest reduction in the expression of functional $GABA_A$ receptors as measured by β subunit expression. These results are consistent with the idea that increased expression of other α subunit isoforms may occur to compensate for the loss of the α1 subunit. Indeed, we showed a significant increase in both the surface and total expression of the α3 subunit, discussed in further detail below.

Because we observed compensatory increases in the expression of the α3 subunit, this raises the intriguing possibility that the α3 subunit may preferentially assemble into receptors with the β3 subunit rather than with β2. This is consistent with observations that brain regions almost exclusively expressing α3—such as the reticular nucleus of the thalamus—express the β3 subunit and not the β2 subunit (Eyre 2012). Further studies will be required to determine if α3 does in fact preferentially assemble with β3.

Alterations in α Subunit Expression

Two important findings regarding $GABA_A$ receptor α subunit expression in cortical neurons were revealed by these studies. First, we found that α1HetKO increased the surface : total expression ratio of the α1 subunit by eliciting significantly smaller

reductions in surface expression as compared to the reductions observed in total α1 subunit expression. Secondly, α1HetKO increased both the total and surface expression of the α3 subunit. Previous reports using Western blot and immunohistochemistry reached conflicting conclusions regarding the effect of α1HetKO on the total expression of the α3 subunit. However, using semiquantitative Western blotting with linear detection of protein, we have demonstrated that α1HetKO does in fact increase total α3 subunit expression in the cortex. Likewise, we have shown here that α1HetKO also significantly increased the expression of the α3 subunit on the cell surface which would be expected to alter GABAergic physiology.

We explored the mechanism by which these two modes of compensation could have occurred by first measuring the levels of WT α1 and α3 subunit mRNAs in cortices from WT and α1HetKO mice. These studies revealed that compensation does not occur at the level of transcription—there was no significant change in α3 subunit mRNA expression and we did not observe increased expression of α1 mRNA from the remaining WT allele. These data indicate a post-transcriptional mode of upregulation consistent with previous reports in homozygous α1 knockout mice that that neurons upregulate the $GABA_A$ receptor subunits they normally express rather than expanding their subunit repertoire (Kralic 2006).

Next we investigated the possibility that α1HetKO reduced the baseline rate of endocytosis of $GABA_A$ receptors which could lead to both an increase in surface α3 expression as well as the increased surface : total ratio of α1 expression. When treated with an inhibitor of dynamin-dependent endocytosis, WT neurons exhibited a time dependent increase in mIPSC peak amplitude while α1HetKO neurons did not display

any significant response. These findings could indicate that the α1HetKO neurons may compensate for the loss of the α1 subunit by decreasing the rate of baseline $GABA_A$ receptor endocytosis and thus increasing the number of $GABA_A$ receptors on the cell surface. Phosphorylation of various $GABA_A$ receptor subunits is known to regulate the rate of receptor endocytosis and surface expression. Phosphorylation of the β3 subunit reduces endocytosis and increases surface expression by inhibiting the interaction of the clathrin adapter protein AP2 with the receptor, whereas phosphorylation of the β2 subunit via PKC enhances endocytosis and thus would be expected to reduce surface expression (Terunuma 2008; Saliba 2012; Herring 2005). It is possible that α1HetKO alters phosphorylation in such a way that facilitates reduced endocytosis and increased surface expression of $GABA_A$ receptors. However, further studies specifically examining $GABA_A$ receptor phosphorylation in the α1HetKO will be needed to address this issue.

α1HetKO Alters Synaptic GABAergic Physiology

In mIPSCs recorded from α1HetKO neurons, we observed increased rise times, longer current decay constants, and reduced peak current amplitudes. The increase in rise time and the lengthening of the decay constant are consistent with the observed increase in surface expression of the α3 subunit. Recall from Chapter II that the biophysical properties of $GABA_A$ receptors are in large part dictated by their α subunit isoform. It is well established that α3 containing receptors activate more slowly than α1 containing receptors and that α3 containing receptors exhibit slower decay kinetics than those containing α1. Additionally, α3 subunit containing $GABA_A$ receptors exhibit a decreased GABA EC_{50} as compared to those containing the α1 subunit, meaning that α3 subunit containing $GABA_A$ receptors are less sensitive to GABA. Reduced GABA

sensitivity could feasibly cause the decrease in mIPSC peak current amplitude that we observed in the α1HetKO neurons.

Implications for the Effects of α1HetKO in the Development of Seizures

Hyperexcitability in the cortex of both humans and rodents with absence epilepsy has been reported in neurophysiological studies (Badawy 2012; Fedi 2008; Lüttjohann 2011). In these studies we have demonstrated that α1HetKO mice with absence epilepsy displayed alterations in cortical $GABA_A$ receptor expression and composition as well as reduced mIPSC peak current amplitudes and increased mIPSC current decay times in layer VI pyramidal neurons. Recall that layer VI pyramidal neurons are thought to initiate absence seizures (Polack 2007). Reduced GABAergic current amplitudes in these neurons could lead to disinhibition in this layer and increase the likelihood of seizure initiation. Reduced synaptic GABAergic currents have been reported previously in upper cortical layers using other rodent models of absence epilepsy (Sasaki 2006; Tan 2007). However, to our knowledge this is the first case demonstrating synaptic disinhibition in layer VI cortical neurons—those thought to be responsible for the initiation of absence seizures.

Lengthening of the decay times of GABAergic currents has not previously been associated with absence epilepsy. One possibility is that the increase in current decay times partially compensates for the reduced peak current amplitudes in the α1HetKO neurons by normalizing the charge transfer carried by the chloride ions. Conversely, it is also possible that the increase in current decay times could exacerbate seizures by allowing for increased de-inactivation of T-type calcium channels (Mann 2008) or by promoting increased neuronal synchrony. Experiments in which IPSC decay times could

be selectively manipulated will clarify the role of prolonged IPSC decay times in the development of absence seizures.

The changes we observed in the endocytosis and recycling of $GABA_A$ receptors in α1HetKO neurons could also be related to the occurrence of seizures. Previous reports indicated that large pharmacologically induced increases in neuronal activity lead to a decrease in $GABA_A$ receptor endocytosis and thus increased surface expression (Rannals 2011). Thus it is possible that the occurrence of absence seizures in the α1HetKO mice caused the reductions in endocytosis that we observed. However, the fact that the inhibition of dynamin-dependent endocytosis had no effect on mIPSC peak current amplitudes indicates that the α1HetKO neurons had maximized their ability to alter endocytosis. These neurons would be incapable of increasing their GABAergic transmission any further in response to higher frequency activity. This would also increase seizure susceptibility in α1HetKO mice, especially during times of high frequency neuronal activity. In the future, experiments designed to specifically test the relationship between absence seizures and alterations in $GABA_A$ receptor endocytosis will help determine whether the absence seizures cause a reduction in endocytosis or if the inability to further decrease endocytosis in response to high frequency activity facilitates the occurrence of seizures. It is important to note that these two possibilities may not be mutually exclusive: absence seizures could initially cause a reduction in $GABA_A$ receptor endocytosis, and once this effect is maximized, the neurons will no longer be able to adjust to high frequency activity which would lead to increased seizure susceptibility.

Conclusion

In order to understand the pathophysiology behind generalized epilepsy, it is imperative to delineate the molecular and physiological alterations associated with the disorder. Although most idiopathic generalized epilepsies are thought to be multigenic which renders animal modeling fairly difficult, the identification of monogenic forms of generalized epilepsy has permitted the generation of animal models based on human disease associated mutations—a powerful tool for advancing our understanding of epilepsy. One such model, the α1HetKO mouse, has been established as a model of generalized absence epilepsy which is construct, face, and predictively valid (Arain 2012). The model is based on a human genetic mutation, recapitulates the phenotype associated with the mutation in a mouse, and responds to the same therapeutic interventions used to treat human patients with absence epilepsy.

In these studies, we discovered that α1HetKO altered both the total and surface expression and subunit composition of $GABA_A$ receptors in cortical neurons. Through our investigation of endocytosis and recycling of these receptors, we identified a molecular mechanism that likely contributes to the development of these changes. Unsurprisingly, the changes in receptor expression and makeup were accompanied by changes in synaptic GABAergic physiology in the α1HetKO neurons. By characterizing the molecular mechanisms modulating inhibitory transmission in a model of epilepsy, we have begun to elucidate the pathophysiology that may contribute to the paroxysmal development of generalized seizures.

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