Two human epilepsy mutations cause developmentally dependent changes in seizure phenotype and $GABA_A$ receptor expression in genetically modified mice

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To my country Pakistan, that I love and want to serve!

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CHAPTER I

Introduction to epilepsy and $GABA_A$ receptors

Introduction

The literal meaning of the Greek word seizure is *"to take hold"*[1]. Although the understanding of epilepsy has come a long way; the first description of an epileptic seizure appears in a text from 2000 BC written in Akkadian language[2]. Despite the observed association with brain injury, as early as 400 BC, epilepsy is still believed to occur in individuals possessed by 'evil spirits' in many cultures.

Today, The International League Against Epilepsy (ILAE) defines a seizure as a sudden and transient occurrence of abnormally excessive or synchronous neuronal activity in the brain, possibly accompanied by altered state of consciousness and other neurological and behavioral manifestations. The occurrence of two or more unprovoked seizures is defined as epilepsy[3]. Epilepsy is a common disease and its worldwide prevalence is 0.5-1%[4]. Furthermore the cumulative lifetime risks of epilepsy and unprovoked seizures are 3.1% and 4.1% respectively[5]. Epilepsy causes serious physical, psychological, social and economic consequences[6].

Classification of seizures

Seizures are broadly classified as generalized or focal (table 1). Generalized seizures are believed to originate at some location within bilaterally distributed networks, while focal seizures originate in networks limited to one cerebral hemisphere[7]. Furthermore, based on electroencephalographic (EEG) recordings and behavioral changes, seizures are described as clinical (that have identifiable behavioral manifestations along with EEG changes) or subclinical (that only have EEG changes)[8]. Among the clinical seizures, the generalized seizures can further be classified based on the associated behavioral change[7] (table 1-1).

Table 1-1. Classification of seizures (adapted from Berg et.al. 2010)			
Gross classification	**Classification**	**Subclassification**	**Behavioral Manifestation**
Generalized seizure	Tonic-clonic		Tonic phase consists of stiffening of body while clonic phase consists of jerking of upper and/or lower limbs
	Absence	Typical	Non-convulsive abrupt cessation of motor activity along with loss of consciousness.
		Atypical	Relatively longer lasting cessation of motor activity as compared to a typical absence seizure. They occur with loss, or partial loss, of consciousness and have a more insidious start and end.
	Myoclonic		Sudden shock-like jerk, usually of the upper body
	Atonic		Sudden loss of posture
Focal seizures			Depends on the region of the brain involved. For example, motor seizures and hippocampal seizures
Unknown	Epileptic spasms		Unprovoked epileptic movements not classified in any of the above mentioned categories. These events are re-classified on the basis of their EEG patterns

Classification of epilepsy

Epilepsy has been classified historically as idiopathic, symptomatic and cryptogenic. It has been proposed to replace these classifications with the following terms (*adapted from Berg et.al. 2010*)[9]:

Genetic: The term "genetic" encompasses all the epilepsy syndromes that are known or presumed to be associated with genetic defect(s) and have seizures as their core symptoms. Genetic epilepsies are estimated to represent 20-30% of all epilepsy cases[10].This classification will further be discussed in the next section.

Structural/metabolic: The terms "structural or metabolic" include distinct structural or metabolic conditions or diseases that have been demonstrated to be associated with a substantially increased risk of developing epilepsy. Examples of such structural or metabolic conditions and diseases are any type of brain insult, including traumatic brain injury, infections, brain tumors and stroke etc.

Unknown: This includes conditions in which the underlying cause is *"as yet unknown"*. Epilepsy syndromes belonging to this category are meant to be viewed neutrally until the underlying genetic or structural/metabolic cause is identified; following which, that group is reassigned to its appropriate classification.

Genetic epilepsy

The term, "genetic" is proposed to replace the previously described term, "idiopathic"[11]. A number of genes that influence the risk for distinct forms of epilepsy have been identified, although this search is complicated by several factors, including reduced penetrance of mutations, genetic heterogeneity of epileptic syndromes, variable expressivity of single gene mutations and gene-environment interactions[12]. Genetic epilepsies include[13]:

1: *Benign neonatal familial convulsions*: Dominantly inherited disorder that presents in second and third day of life with clonic and apneic (respiratory arrest) seizures with no specific EEG criteria. 14% of these patients continue to have seizures later in life.

2: *Benign neonatal convulsions*: These patients present with clonic and apneic seizures at fifth day of life but there is no recurrence of seizures later in life. EEG often shows characteristic sharp theta waves (4-8Hz).

3: *Benign myoclonic epilepsy in infancy*: These patients have brief bursts of myoclonic seizures during the first and second year of life. EEG shows generalized spike waves occurring in brief bursts during early stages of sleep.

4: *Childhood absence epilepsy*: It occurs in 6-8 year old children and is characterized by frequent absence seizures that also show a 3 Hz spike wave discharge on EEG. These seizures usually remit with age.

5: *Juvenile absence epilepsy*: Juvenile absence epilepsy is similar to childhood absence epilepsy except that onset of symptoms occurs around puberty, the frequency of seizures is lower and it is more commonly associated with GTCS than childhood absence epilepsy.

6: *Juvenile myoclonic epilepsy*: These patients present with bilateral, single or repetitive, arrhythmic or irregular myoclonic jerks. GTCS also occur frequently in these patients while absence seizures may also occur. The onset of symptoms is around puberty. The EEG often shows irregular spike-waves and polyspike-waves.

7: *Epilepsy with Generalized Tonic Clonic Seizures (GTCS) on awakening*: The onset of this disease is usually in the second decade of life and presents with GTCS, predominantly after awakening.

Animal models of epilepsy

Animals models for seizures and epilepsy have played a fundamental role in our understanding of physiological and behavioral changes associated with human epilepsies, along with their treatments[14]. However the validity of animal models has been challenged for three main reasons[14]: 1) Often mechanistic conclusions about epilepsy are drawnbased on studies performed on animal models with normal, but not epileptic, brains. 2) Behavioral manifestations associated with each model can vary from subtle to extremely complex and not replicate human behavior. 3) The complexity of cellular mechanisms of seizure origination and pathways of seizure propagation can differ based on species studied. For example, seizure propagating networks may differ in primates and rodents. What are the criteria of a good animal model of epilepsy? It is recommended that before an animal can be considered a model for human seizures or epilepsy it should satisfy the most of the following six general criteria[14-16]:

1) Age of onset: If the human condition is characterized by a unique age of onset, then the proposed animal model should scale to or reflect a similar age to humans. For example, childhood absence epilepsy usually has earlier onset compared to juvenile myoclonic epilepsy[17].

2) Etiology: The underlying cause of seizure or epilepsy, i.e. genetic or acquired, should be similar to that of human condition that the model is replicating.

3) Seizure phenotype and EEG abnormalities: The animal model should exhibit similar electrophysiological patterns as seen in human. For example in human absence seizures have characteristic 3-4Hz spike and wave pattern on EEG. The abnormality noted on the animal EEG should be similar in morphology to that observed in humans.

4) Pathological insult: If the human condition is characterized by a specific pathological change, such as unilateral hippocampal sclerosis seen in some patients with temporal lobe epilepsy, the animal should also carry a similar pathological change.

5) Response to typical treatment: Although the pharmacological treatment has not been developed for every human condition, the more 'classical' drugs used to treat humans (for example ethosuximide is used to treat absence seizures) should also be effective in the animal model.

6) Behavioral characteristics: Seizure induced behavioral manifestations observed in humans (for example behavioral arrest during absence seizures, or automatisms observed in some complex partial seizure in patients with temporal lobe epilepsy) should, in some way, be reflected in the animal model.

Currently, the majority of the animal models of epilepsy replicate only some of the above mentioned criteria. A brief summary of some of the commonly used models of genetic and acquired epilepsies is summarized in the table 1-2 and 1-3 respectively.

Table 1-2: Examples of commonly used animal models of genetic epilepsy

Species	Line/mutation	Model of	Reference
Rat	WAG/Rij (Wistar Albino Glaxo rats from Rijswijk)	Absence seizures	Coenen et.al.[18]
Rat	GAERS (Genetic absence epilepsy rats from Strasbourg)	Absence seizures	Vergnes et.al.[19]
Rat	GEPRs (Genetically epilepsy-prone rats	Tonic-clonic seizures	Epps et.al.[20]
Mouse	Stargazer	Absence seizures	Letts et.al.[21]
Mouse	Lethargic	Focal motor and absence seizures	Burgess et.al.[22]
Mouse	Tottering	Focal motor and absence seizures	Noebels et.al.[23]
Mouse	ARX (Aristaless-related homebox)	Infantile spasms (West syndrome)	Marsh et.al.[24]

Table 1-3: Examples of commonly used animal models of acquired epilepsy

Approach	Method/ material used	Epilepsy/ seizure types	References
Chemical	Pentylenetetrazole	Tonic-clonic seizures	Zhang et.al.[25]
Chemical	Kainic Acid	Temporal lobe epilepsy	Vincent et.al.[26]
Chemical	Pilocarpine	Temporal lobe epilepsy	Curia et.al.[27]
Chemical	Flurothyl	Myoclonic jerks and tonic-clonic seizures	Velisek et.al.[28]
Chemical	Bicuculline	Myoclonic jerks and tonic-clonic seizures	Velisek et.al.[28]
Chemical	Picrotoxin	Myoclonic jerks and tonic-clonic seizures	Veliskova et.al.[29]
Kindling	Electrical kindling	Focal seizures depending on the site of depth electrode	Epps et.al.[20]
Kindling	Maximum electric shock	Generalized seizures	Epps et.al.[20]
Kindling	Corneal electric kindling	Clonic seizure	Rowley et.al.[30]
Hypoxia	Exposure to hypoxic or anoxic gas mixture	Tonic-clonic seizures	Jensen et.al.[31]
Temperature	Acute hyper-thermia	Tonic-clonic seizures	Bender et.al.[32]

Mechanism of seizure generation

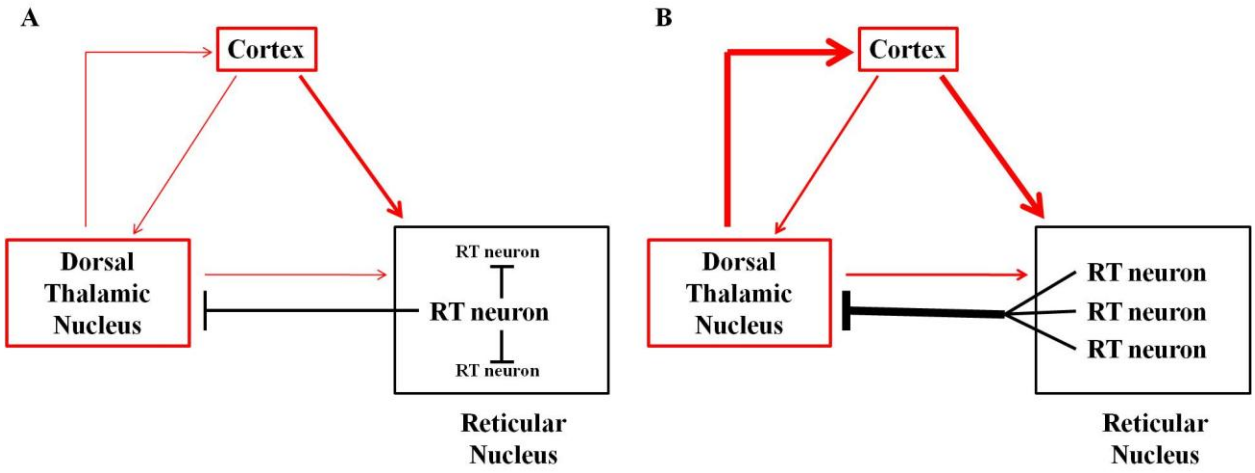
Synchronized firing among groups of neurons within a network is required for normal brain function. For example, synchronized thalamocortical oscillatory activity generates sleep spindles[33], but under pathological conditions, these networks can be used to generate aberrant oscillatory network activity associated with disease states such as schizophrenia and epilepsy[34]. Some groups have also put forward the view that spike-wave discharges, a characteristic of absence seizures, are a "perverted" form of sleep spindles[35]. So in order to better understand the mechanism of seizure generation, it is important to first understand the initiation and continuation of physiologic rhythms of neuron firing.

The initiation and generation of sleep-related thalamocortical spindle oscillations have been studied extensively *in vitro* and *in vivo*[33]. Here, I will briefly describe how the modulation of firing due to reciprocal connections among cortical neurons, reticular thalamic (RT) neurons and thalamocortical neurons (TC) of dorsal thalamic nucleus results in spindle-like oscillatory activity(description adapted from Beenhakker et.al.[33]) (figure 1-1 A). The GABAergic RT neurons project to dorsal thalamic nucleus to inhibit TC neurons that express $GABA_A$ and $GABA_B$ receptors. The TC neurons in turn send excitatory glutamatergic input to both cortical neurons and RT neurons. The cortical neurons send excitatory input to both TC and RT neurons, although the cortical input to RT neurons is much stronger. When RT neurons fire a burst of action potentials they release GABA onto TC neurons and inhibits them. This decreases the excitatory input to the cortical neurons which eventually decreases the activation on RT and TC neurons. The inhibition of TC neurons hyperpolarizes them and de-inactivates low-threshold T-type Ca^{2+} channels, enabling the TC neurons to fire post-inhibitory rebound burst of action potentials. This sends excitatory input to both RT and cortical neurons. The RT neurons again send inhibitory GABAergic input to hyperpolarize the TC neurons, creating this physiologic spindle rhythm of excitation and inhibition. It must also be kept in mind that the activation of RT GABAergic neurons not only silences the TC neurons but also results in *intra-RT* neuron inhibition via the activation of $GABA_A$ receptors, present on RT neurons. How does RT send any inhibitory signal to TC neurons? It is thought that a centrally located RT

neuron fires a strong burst of action potentials releasing GABA onto neighboring RT neurons and also the TC neurons. This "limited activation" of RT neurons restricts the number of neurons that contribute to any one cycle and reduces the feed forward disinhibition of TC neurons.

Dysregulation of the above explained circuitry has been implicated in seizure generation (figure 1-1 B). It has been hypothesized that blockade of $GABA_A$ receptors (by genetic or pharmacological factors) eliminates the *intra-RT* neuron inhibition resulting in a greater number of RT neurons firing and a stronger inhibition of TC neurons by activating the $GABA_B$ receptors. The resulting de-inactivation of low-threshold T-type Ca^{2+} channels, present on TC neurons, is even greater which results in an increased activation signal to the cortical neurons. This causes a highly robust and synchronized activity of cortical neurons which is reflected in high amplitude spike-wave discharges seen on EEG, a characteristic of absence seizures.

Figure 1-1: Simplified schematic of thalamocortical circuitry involved in generation of (A) sleep spindles and (B) absence seizures



The above described circuitry and the effects of its dysregulation show how important it is to maintain the physiology of neurotransmitters and their receptors. Therefore it is very essential to study the modulation of the physiology of neurotransmitter regulated ion channels (in other words study the "channelopathies") because any aberration in this can possibly be involved in the generation of seizures and epilepsy.

Some genetic epilepsies are "channelopathies"

Genetic epilepsies account for almost 20-30% of all epilepsies[10]. Recently a number of genes that influence the risk for distinct forms of epilepsy have been identified, although this search is complicated by several factors, including reduced penetrance of mutations, genetic heterogeneity of epileptic syndromes, variable expressivity of single gene mutations and gene-environment interactions[36]. It is now thought that the mutations in transmembrane ligand- and voltage-gated ion channel genes are associated with a significant number of these epilepsy syndromes[37]. Furthermore, rare cases of monogenic mutations in transmembrane ion channels have been identified in several large pedigrees and sporadic cases of epilepsy[36, 38, 39]. Over the last 10 years rare forms of familial epilepsy, where the mode of inheritance is clearly Mendelian, have been reported to be associated with mutations in $GABA_A$ receptors[40]. It is important to note that patients suffering from these rare forms of familial epilepsies also present with similar symptoms as those suffering from polygenic epilepsies. Therefore, these rare familial epilepsies, with associated mutations in $GABA_A$ receptors, are phenotypically identical to the more common polygenic forms of epilepsy and serve as important models for them. A better understanding of the GABA system can help us further our knowledge about the pathology of seizures

Introduction to GABA

γ-aminobutyric acid (GABA) is an amino acid neurotransmitter that is synthesized by decarboxylation of glutamate by the enzyme glutamic acid decarboxylase. It was discovered in 1950 by Eugene Roberts and Jorge Awapara[41]. It is the major inhibitory neurotransmitter in mammalian central nervous system[42]. In the brain, 17-20% of all neurons make GABA and are hence called GABAergic neurons[43]. GABA binds to two separate classes of receptors, namely $GABA_A$ and $GABA_B$ receptors[41]. $GABA_A$ receptors are ionotropic receptors that consist of heteropentameric protein complexes consisting of five subunits, which are arranged pseudo-symmetrically around a central chloride selective channel[44]. Although they are excitatory in nature early in development, they become the major inhibitory neurotransmitter receptors in mammalian brain later in development[45]. This phenomenon is explained in more detail in later sections. $GABA_B$ receptors, however, are G protein coupled receptors that constitute of heterodimer of two subunits, R1 and R2[46]. Although their role is not completely understood it is known that $GABA_B$ receptors are present on both pre and post-synaptic membranes. It has been reported that on the presynaptic membrane they inhibit the release of other neurotransmitters through a decrease in membrane Ca^{2+} conductance (by modulating the activity of P/Q-, N- and possibly L- type Ca^{2+} channels[46]) and an increase in membrane potassium conductance (by rectifying the activity of GIRK or Kir3 potassium channels[47, 48]).

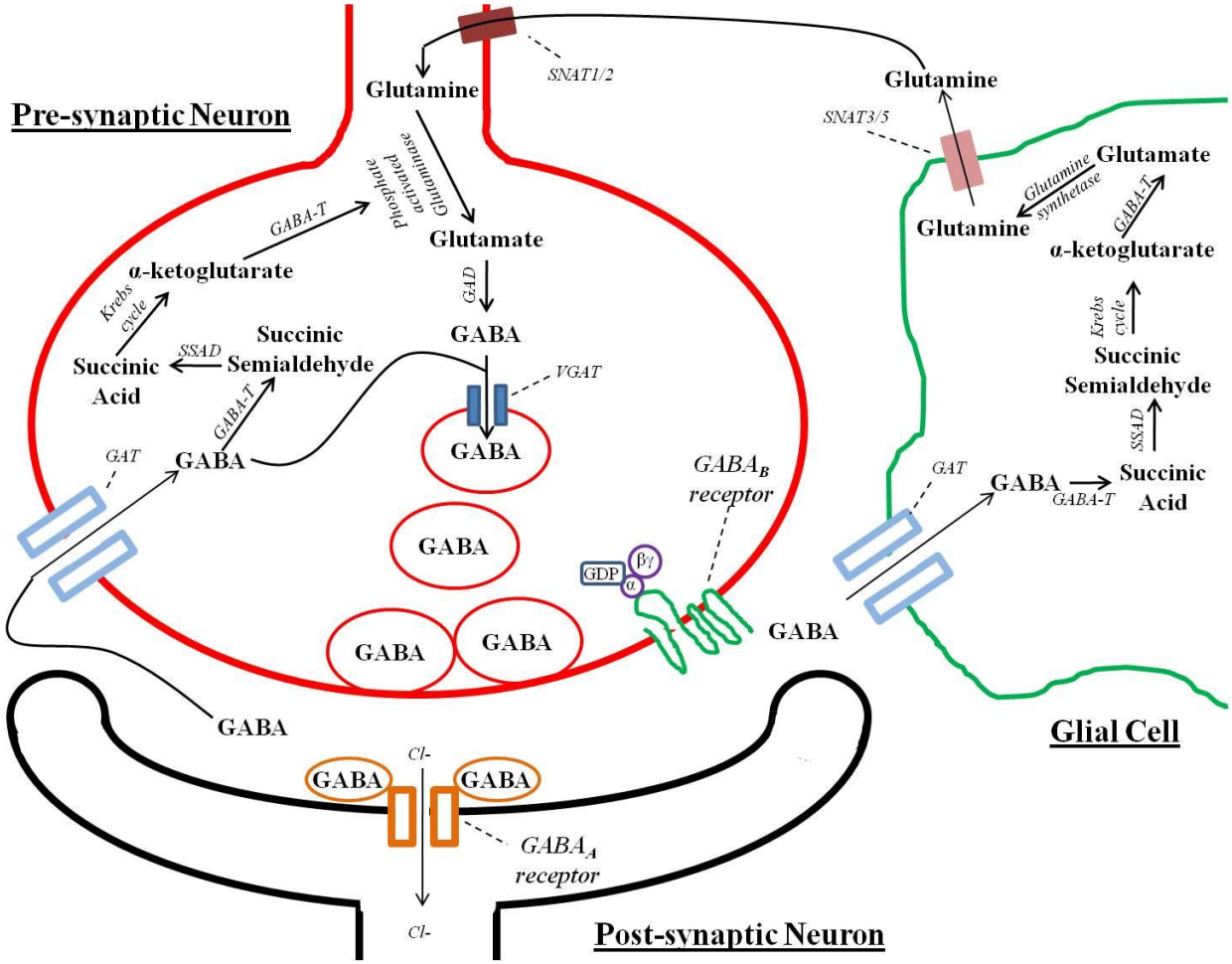
GABA synthesis, storage, release and inactivation

The principal precursor of GABA is glucose. The Krebs cycle forms α-ketoglutarate, which is then converted to the amino acid glutamate by the enzyme GABA-transaminase (GABA-T)[49]. In GABAergic neurons (GABA synthesizing neurons), glutamate is further converted to GABA by the enzyme glutamic acid decarboxylase (GAD)[50]. GAD appears to be present exclusively in GABAergic neurons and hence serves as a good immunohistochemical marker to identify them[51].

GABA is then packaged into vesicles by vesicular GABA transporter (VGAT)[52]. It is then released into synaptic cleft upon depolarization of GABAergic neurons[50]. The release of GABA is regulated by $GABA_B$ autoreceptors located of GABAergic axon terminals[50] by the inhibition of Ca^{2+} and potassium conductance, as explained earlier.

Exocytosed GABA is removed from the synaptic cleft both by diffusion and active reuptake. Reuptake, via GABA transporters (GAT) present on GABAergic and surrounding glial cells, is the primary mode of inactivation of GABA signaling[50, 53]. When GABA is taken up into surrounding glial cells, the enzyme GABA-T combines it with α-ketoglutarate to form succinic semialdehyde that is further converted to succinic acid by succinic semialdehyde dehydrogenase (SSAD) and eventually returned to the Krebs cycle that produces glutamate[49]. Since GAD is not present in glial cells, glutamate is converted to glutamine by glutamine synthetase[54]. Glutamine is then pumped into the extrasynaptic space, by sodium-coupled neutral amino acid transports (SNAT3/5) from which it is taken up into neurons by SNAT1/2[55]. In the neuron, glutamine is converted back to glutamate by the enzyme phosphate activated glutaminase[56]. This 'renewed' glutamate can then be converted to GABA by the action of GAD, hence completing the cycle which has classically been described as the "GABA shunt"[49, 57]. On the other hand, when GABA is imported by GAT present on GABAergic neurons themselves, it can be packaged into vesicles again. GABA can also be metabolized to succinic semialdehyde by GABA-T present in the GABAergic neurons, which is further converted to succinic acid by SSAD and returned to the Krebs cycle[49]. This entire process is summarized in figure 1-2.

Figure 1-2: Schematic depiction of GABA synthesis, storage, release and recycling (adapted from Mackenzie et.al. 2004, Bak et.al. 2006, Seigel et.al. 1998 and Squire et.al. 2008)

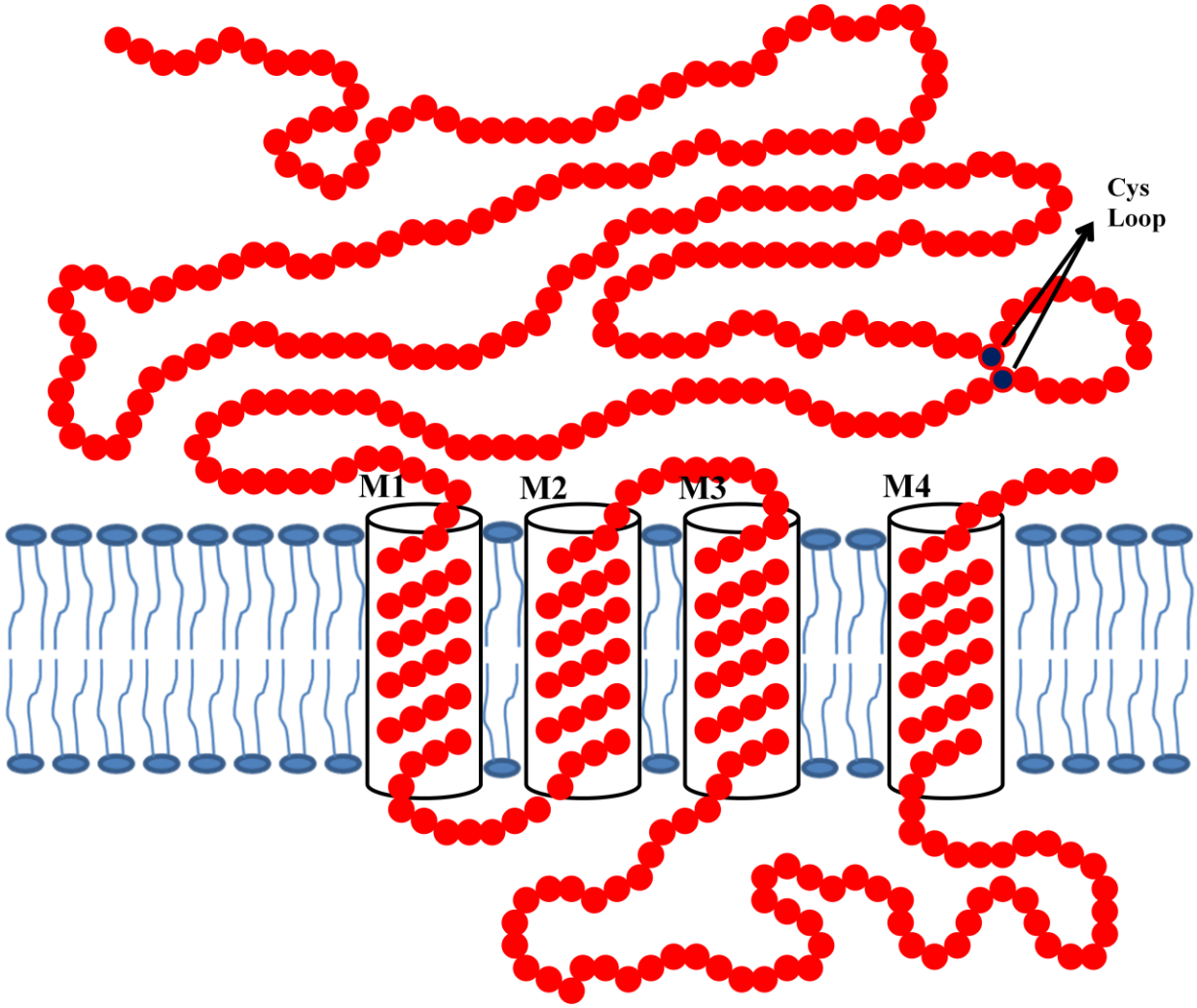


$GABA_A$ receptors

$GABA_A$ receptors are the most abundant and important inhibitory neurotransmitter receptors in the CNS[58]. They are located throughout the mammalian nervous system and are involved in almost all of brain physiological functions[59]. $GABA_A$ receptors are chloride ion channels that can be opened by GABA and modulated by a variety of different drugs such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants[58, 60-62]. In addition to their use as anticonvulsants, $GABA_A$ receptor targeting drugs are used in the treatment of hyperalgesia, anxiety, depression and other neuropsychiatric disorders[63, 64].

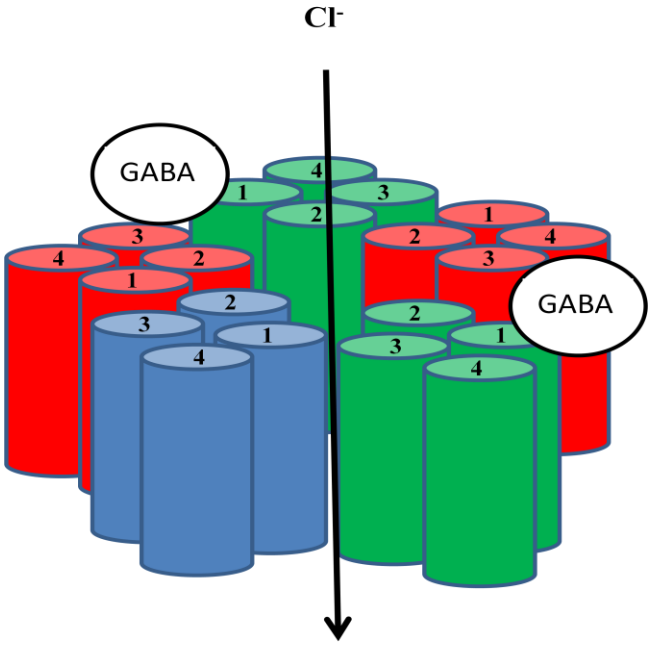
$GABA_A$ receptors are members of the cys-loop family of ligand-gated ion channels that also includes nicotinic, cholinergic, serotonin 5-HT3 and glycine receptors[65, 66]. $GABA_A$ receptors are pentameric assemblies of 19 different subunit subtypes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ, and ρ1–ρ3)[59, 62, 65, 67, 68]. Additional diversity of receptor structure is generated by alternative splicing of some of these subunit mRNAs[69]. Based on these factors, it is theoretically possible to assemble more than 10,000 pentameric combinations[67]. However, the majority of $GABA_A$ receptors are composed of two α subunits, two β subunits, and a γ or δ subunit[70]. All of the subunits share a common ancestral structure that includes a large extracellular N-terminal domain, four transmembrane domains (TM1-4) and an extended cytoplasmic loop region between TM3 and TM4 that mediates interactions with trafficking and signaling factors[71] (figure1-3).

Figure 1-3: Schematic diagram of $GABA_A$ receptor subunits (adapted from Macdonald et.al. 2010[72]).



The central pore of $GABA_A$ receptors is lined by M2 transmembrane domains of each subunit (figure 1-4)[42]. The arrangement of subunits around the channel pore is γβαβα counterclockwise, when viewed from the synaptic cleft[44, 73]. Interestingly, $GABA_A$ receptors consisting of two α, two β and one δ subunits are present on exclusively in extrasynaptic membrane[74, 75], while the $GABA_A$ receptors consisting of two α, two β one γ subunits are present on both synaptic and extrasynaptic membrane[76]. Furthermore, it has been shown that postsynaptic clustering of $GABA_A$ receptors requires the γ2 subunit[77, 78].

Figure 1-4: Schematic diagram of $GABA_A$ receptor (adapted from Macdonald et.al. 2010[72]). 2 α subunits (red complexes), 2 β subunits (green complexes) and δ/γ subunits (blue complex) surround a central Cl^- ion channel.



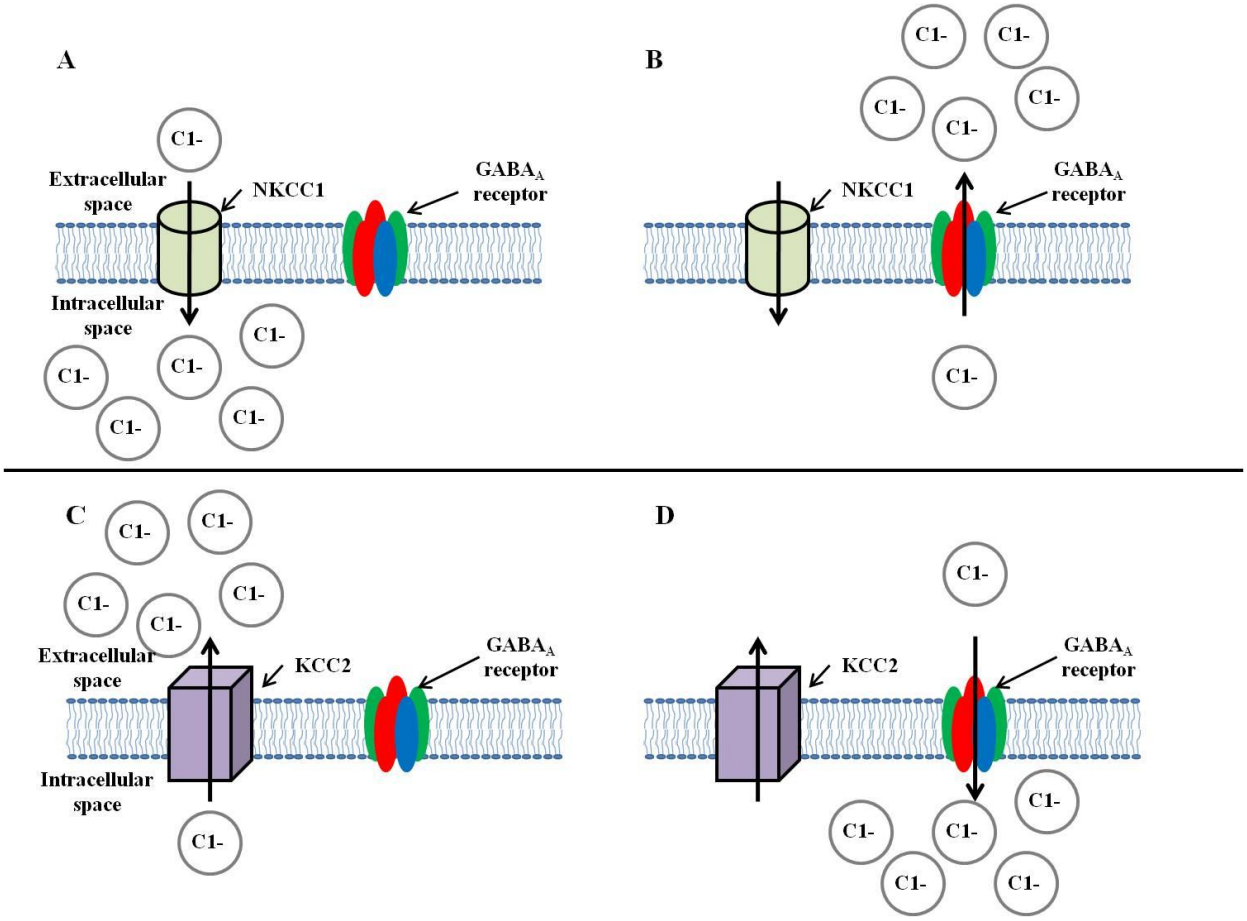
Full activation of $GABA_A$ receptor requires the binding of two GABA molecules at βα subunit interfaces[79, 80] (figure 1-4). Upon activation, chloride (Cl^-) and bicarbonate ions flow down their concentration gradient into the postsynaptic neurons causing hyperpolarization (in mature brains [81, 82]) or out of the postsynaptic neurons causing depolarization (in immature brains[83]).

$GABA_A$ receptors play different roles in immature and mature brains

Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) expression is high in neurons during early development and lasts until the first week of postnatal life in rodents[84, 85]. NKCC1 imports Cl^- ions into immature neurons creating a higher concentration of Cl^- ions in the cytoplasm than is found in mature neurons (figure 1-5 A). At this point activation of $GABA_A$ receptor results in an efflux of Cl^- ions, resulting in depolarization of the neuron[83] (figure 1-5 B). Hence during early development GABA act as an excitatory neurotransmitter. As the brain ages the expression of NKCC1 declines and the expression of the K^+-Cl^-

cotransporter (KCC2) increases[86]. KCC2 extrudes Cl^- ions from neurons, decreasing their intracellular concentration (figure 1-5 C). The activation of $GABA_A$ receptors in these mature neurons allows the Cl^- ions to flow down their concentration gradient back into the neuron, resulting in its hyperpolarization (figure 1-5 D). Hence after the early period of development GABA act as inhibitory neurotransmitter.

Figure 1-5: Activation of $GABA_A$ receptors results in depolarization of neurons during early development (A and B) but hyperpolarization later in development (C and D). A) Early in development, resting state NKCC1 transports Cl- ions into the neurons increasing Cl- ion concentration in the cytoplasm and hyperpolarizing the neuron. B) Activation of $GABA_A$ receptors in these young neurons results extrusion of Cl- ion from the cytoplasm and depolarization of the neuron. C) In adult brain KCC2 extrudes the CL- ions out of the neuron, D) but activation of $GABA_A$ receptors results in allowing the Cl- ions to flow back into the neurons and hyperpolarizes them.



$GABA_A$ receptor synthesis assembly and trafficking

A summary of $GABA_A$ receptor assembly and transport is illustrated in figure 1-6. $GABA_A$ receptor assembly occurs in the endoplasmic reticulum (ER) and involves the chaperon proteins, calnexin and binding immunoglobulin protein (BiP)[87]. Furthermore, it has been shown, using forced expression in heterologous cells, that the receptors composed of α, β and γ or α and β or αβδ, and other subunits are capable of oligomerizing and can be transported to cell surface, while other combinations of subunits are retained in the ER and subsequently degraded[87, 88]. When α, β and γ subunits are expressed in heterologous cells, receptors containing all three subunits preferentially assemble as compared to α and β subunits alone[89].

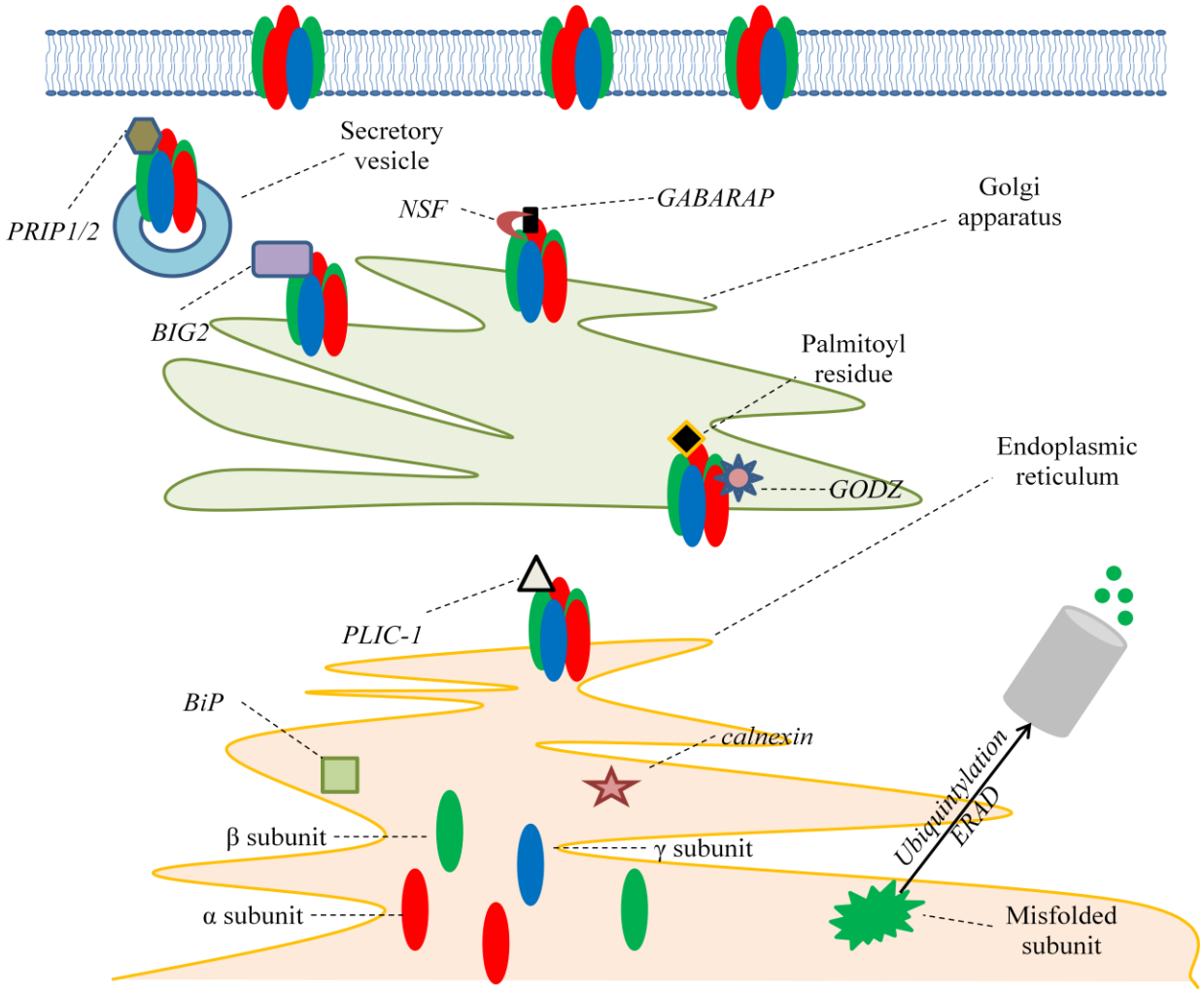
The first step in the assembly of complexes that are eventually translocated to the cell surface, begins in the ER and involves the initial formation of α and β subunit heterodimers, which is principally controlled by the N-terminal/ luminal domain of these subunits[90]. This process requires chaperone proteins calnexin and BiP[87]. Interestingly, *in vitro* studies show that treatment with GABA itself appears to increase the surface expression of $GABA_A$ receptors[91]. If the $GABA_A$ receptor is improperly organized or the constituting subunits are improperly folded, the $GABA_A$ receptor is quickly subjected to ubiquination and subsequent ER associated degradation (ERAD) [90, 92]. Furthermore, blockade of neuronal activity also enhances the processes of degradation[93, 94].

After $GABA_A$ receptors are assembled, inhibition of their polyubiquitination by *protein linking integrin-associated protein with cytoskeleton-1* (PLIC-1)[95] (a protein that interacts with $GABA_A$ receptor subunits α1-3, α6 and β1-3[96]) increases their stability. The $GABA_A$ receptors are then transported out of the ER and reach the Golgi apparatus where γ2 subunit is palmitoylated at its cytoplasmic cysteine-rich domain by *Golgi-specific DHHC zinc finger protein* (GODZ)[97, 98]. If this process is interrupted by reducing the expression of GODZ, for example by GODZ specific shRNA vectors, it causes selective loss of $GABA_A$ receptors at synapses[99].

Next *brefeldin A-inhibited GDP/GTP exchange factor 2* (BIG2) binds to the intracellular loop of $GABA_A$ receptor β subunit at the site that overlaps with binding site of PLIC-1[100] and catalyzes activation of the class I ADP-ribosylation factors (ARF), which allows the budding of vesicles from the Golgi apparatus[71]. It is also suggested that another population of BIG2, associated with recycling endosomes, might play a role in receptor recycling[101].

Two factors, namely *GABA receptor associated protein* (GABARAP) (which interacts with the intracellular loop of γ subunits[102]), and N-ethylmaleimide-sensitive factor (NSF) (which binds to both GABARAP and intracellular loop of β subunits[103]), enhance surface expression of $GABA_A$ receptor clusters upon activation of the glutamate receptor NMDA[104]. In addition to GABARAP and NSF, this process might also involve Ca^{2+} *calmodulin-dependant kinase II* (CaMKII), *glutamate receptor interacting protein* (GRIP)[71] and *phospholipase C-related inactive protein* (PRIP1/2)[105].

Figure 1-6: $GABA_A$ receptor assembly and trafficking (adapted from Luscher et.al. 2011 and Tretter et.al. 2008)



$GABA_A$ receptor stability at synapse

Proteins embedded in lipid bilayer of a neuron can have a relatively high mobility unless it is associated with anchoring proteins[96]. In cultured neurons, assembled $GABA_A$ receptors are likely delivered to the plasma membrane at an extrasynaptic site, from which they later migrates to their final destination[106, 107]. The proteins discussed below might play a role as scaffold to allow the distribution of $GABA_A$ receptors between synaptic and extrasynaptic sites (figure 1-7).

Gephyrin

Gephyrin, a 93-kDa protein, was originally discovered as a copurified protein associated with glycine receptors expressed in rat spinal cord[108]. It has also been identified at GABAergic synapses[109]. Gephyrin is most probably associated with the intracellular loop between third and fourth transmembrane domain of $GABA_A$ receptor α subunits[110]. It is also hypothesized that interaction between $GABA_A$ receptor γ2 subunit and gephyrin exists and plays a role in synaptic anchoring of $GABA_A$ receptor clusters[96, 111]. *In vitro* studies using neuronal cultures from gephyrin knockout mice, show significant loss of $GABA_A$ receptor clusters[109]. Similarly, gephyrin clusters are significantly reduced in γ2 subunit knockout mice[77].

In vitro studies have also demonstrated a direct interaction between α1 and gephyrin[112] and α3 and gephyrin[113], that requires collybistin to form a stable ternary complex[114]. The N terminal portion of gephyrin (known as G-gephyrin) associates with N terminal portion of other gephyrin monomers to form a trimeric structure[115] while the C terminal portion (known as the E domain) of gephyrin associates with other gephyrin monomers to form a dimer[116]; eventually leading to the formation of hexagonal lattice[117] (figure 1-7 B). It is hypothesized that $GABA_A$ receptor α1, α2 and α3 subunits and glycine receptor β subunit all bind to the same site on E domain of gephyrin[118].

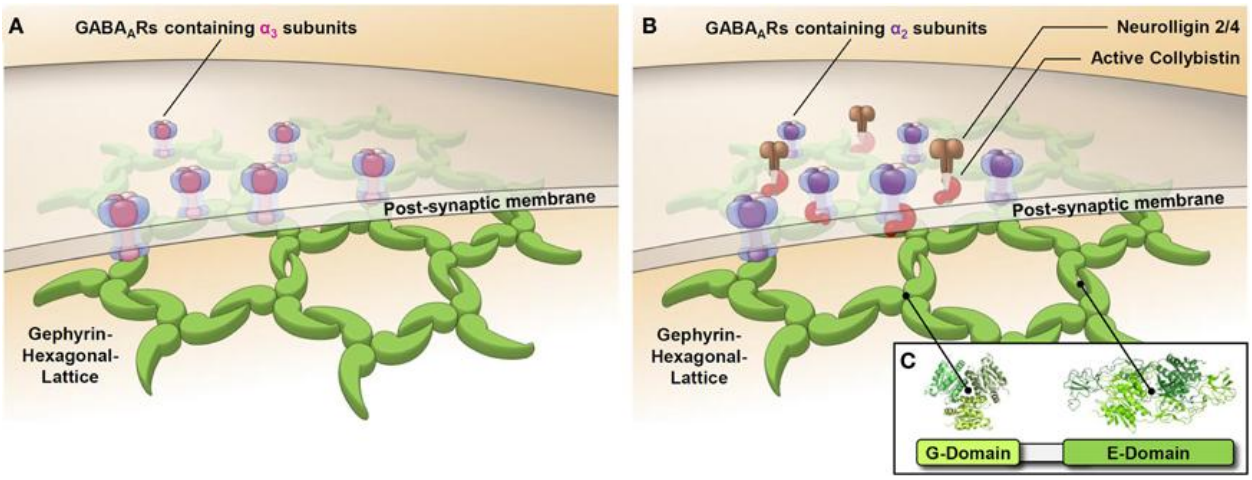
Collybistin

Collybistin is a cytoplasmic protein that binds to gephyrin and facilitates gephyrin clustering and transport to cell membrane[119]. It is also suggested that the cell adhesion molecules, neuroligins (NL-2/4), activate collybistin[111]. Collybistin knock-out mice show a loss of $GABA_A$ receptor clusters in hippocampus and basolateral amygdala[120]. Moreover, an *in vitro* study showed that co-transfection of collybistin and gephyrin resulted in the formation of numerous and larger synaptic clusters (which the writers described as "superclusters"[121]).

Radixin

Radixin is required for the clustering of $GABA_A$ receptors containing α5 subunits on membrane in the extrasynaptic space[122]. Phosphorylation of radixin causes a structural change which allows the binding of f-actin, a prerequisite for clustering of $GABA_A$ receptors containing α5 subunit[122].

Figure 1-7 A and B: $GABA_A$ receptor clustering and stability at synapses (Tretter et.al. 2012, reproduced with permission of Dr. Hermann Schindelin)



$GABA_A$ receptor endocytosis, recycling and degradation

Receptor endocytosis is known to regulate the cell surface expression of neurotransmitter receptors[123]. 17-25% of $GABA_A$ receptors undergo constitutive endocytosis[124]. Internalization of $GABA_A$ receptors is mediated by clathrin-dependent endocytosis[125]. This interaction is made possible by the *adaptor protein 2* (AP-2 complex) that binds to $GABA_A$ receptor and clathrin. First the μ and σ subunits of AP-2 interact with the intracellular loops of $GABA_A$ receptor β and γ subunits[123, 125-127]. The phosphorylation of the AP-2 interaction site on β1 and β3 subunits by *protein kinase A* (PKA) and *protein kinase C* (PKC) prevents their interaction with AP-2 and thus reduces $GABA_A$ receptor internalization[128 129]. On the other hand,

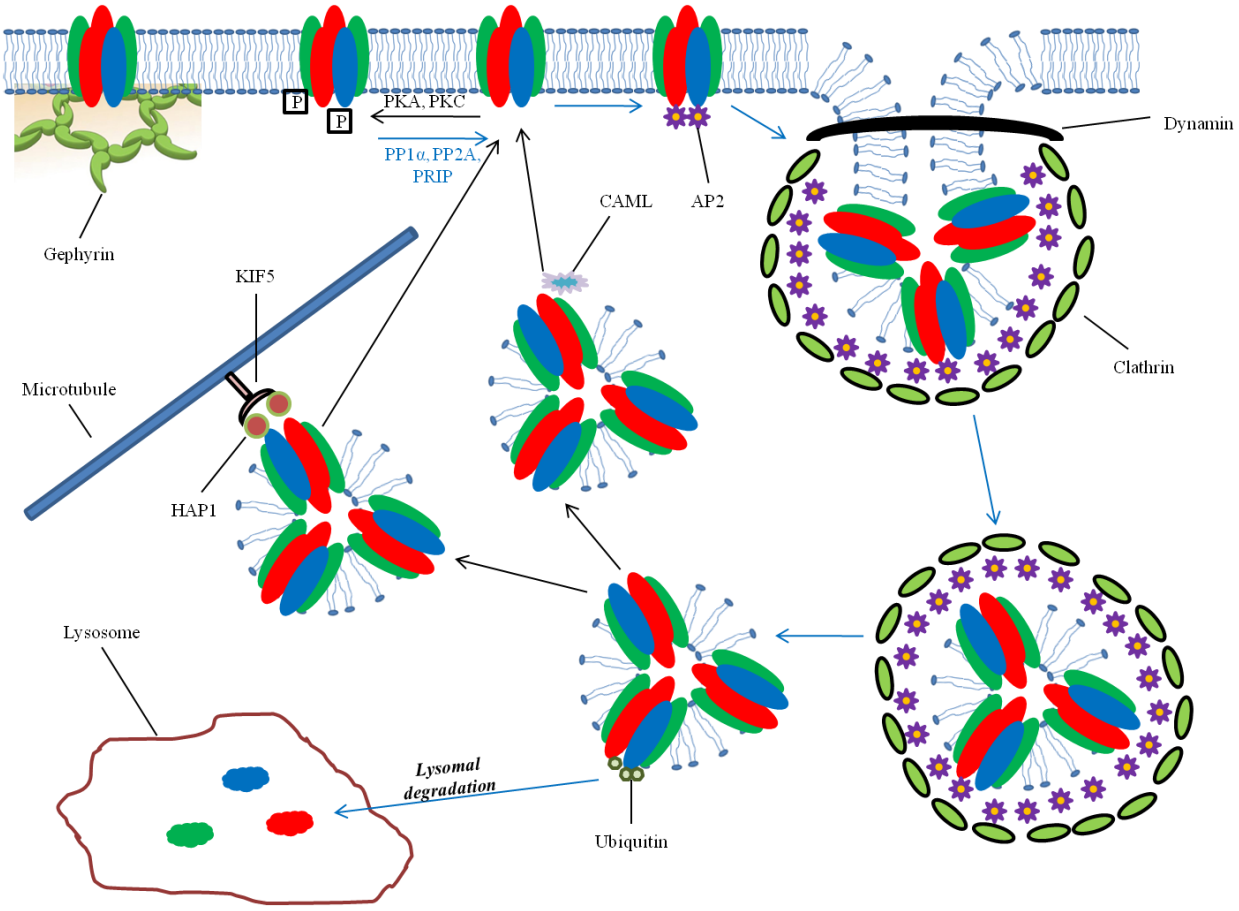
differential activation of protein phosphatases (PP1α and PP2A) by *phospholipase C-related catalytically inactive proteins 1* (PRIP1)[130, 131] results in dephosphorylation of AP-2 interacting sites on $GABA_A$ receptor β and γ subunits and facilitates receptor internalization.

After AP2 binds to $GABA_A$ receptors, clathrin is recruited to the plasma membrane. The polymerization of clathrin causes the membrane to bend into a pit[127, 132]. Next dynamin, (a protein in the GTPase family), assembles around the neck of the clathrin coated pits and assists in pinching vesicles from the plasma membrane into the cytoplasm[133].

Upon internalization, vesicles are either subjected to rapid recycling to the cell surface or targeted for lysosomal degradation. The *Huntingtin associated protein 1* (HAP1) inhibits degradation of internalized vesicles and hence facilitates recycling[124, 134]. HAP1 also acts as an adapter protein that links the internalized $GABA_A$ receptors to *kinesin family motor protein 5* (KIF5), which provides the machinery that controls the transport of $GABA_A$ receptors along the microtubules in the dendrites allowing the recycling of $GABA_A$ receptors[135]. Interestingly the disruption of the $GABA_A$ receptor/ HAP1/ KIF5 complex and it's dissociation from microtubules has been reported in a mouse model of Huntington disease[136]. In a separate study, it was shown *Calcium-modulating cyclophilin ligand* (CAML) interacts with γ2 subunit of $GABA_A$ receptor and is selectively involved in the recycling of endocytosed $GABA_A$ receptors[137].

It is estimated that of all the internalized $GABA_A$ receptors almost 30% are rapidly recycled back to the cell surface[96]. The internalized $GABA_A$ receptors that do not get recycled to the cell membrane undergo slow lysosomal degradation[138]. This degradation pathway is facilitated by ubiquitination of lysine residues between amino acid 317-328 in the intracellular domain of the γ2 subunit[139]. The process of $GABA_A$ receptor internalization, recycling and degradation is summarized in figure 1-8.

Figure 1-8: $GABA_A$ receptor endocytosis, recycling and degradation (adapted from Luscher et.al. 2011, Tretter et.al. 2008, Tretter et.al. 2012 and McMahon et.al. 2011).



$GABA_A$ receptor temporal and spatial expression

The temporal and spatial expression of different $GABA_A$ receptor subunits is tightly regulated in the brain but the expression of $GABA_A$ receptor subunits in human brain has not been studied in detail[140]. It is generally believed that brain development in rats at postnatal day (P) 8-10 is equivalent to a newborn human brain, P7-21 is the infantile stage, P21-32 is the juvenile stage, 32-37 is the onset of puberty and adult begins at two months[141]. Therefore studies on rodent brains have been very useful in understanding

the transcript and protein expression of $GABA_A$ receptors. A summary of findings from studies that looked at mouse and rat brains are discussed below.

$GABA_A$ receptor α1 subunit

Expression of the α1 subunit is low early in development and restricted to a few areas including brainstem and basal forebrain[142] (figure 1-9). The levels of the α1 subunit dramatically increases in the first postnatal week and the mRNA and protein levels of α1 subunits begin to be expressed widely throughout all brain regions with age[143], except for the reticular nucleus of thalamus, CA3 region of hippocampus, nucleus accumbens and striatum where expression remains very low[143-146] (figure 1-10).

Figure 1-9: Relative expression of $GABA_A$ receptor subunits across development (adapted from Galanopoulou 2008)[141].

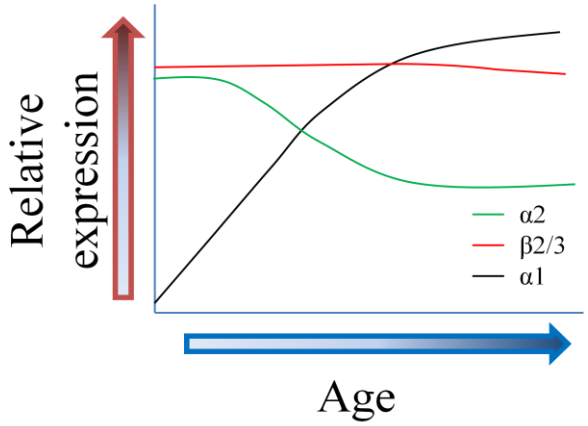
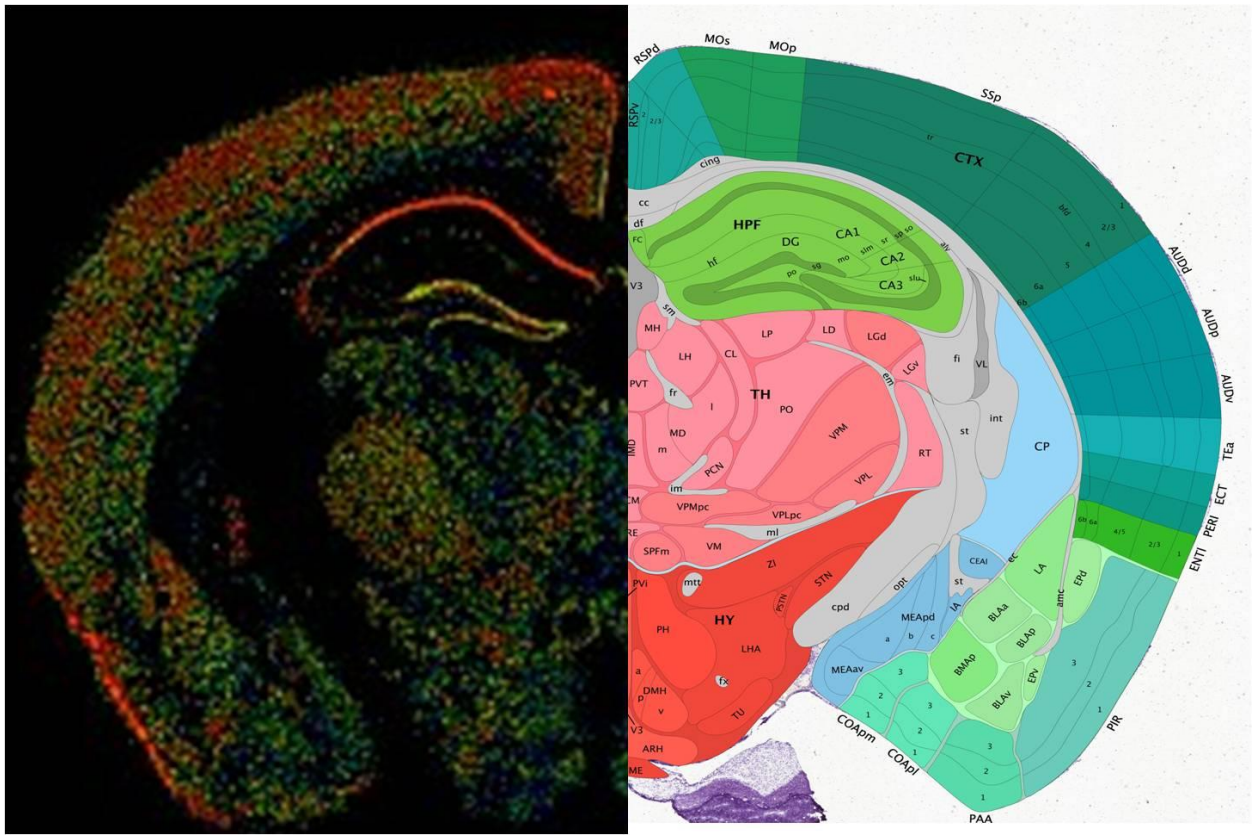


Figure 1-10: Expression of $GABA_A$ receptor α1 subunit mRNA in adult mouse brain (reproduced from ©2012 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas).



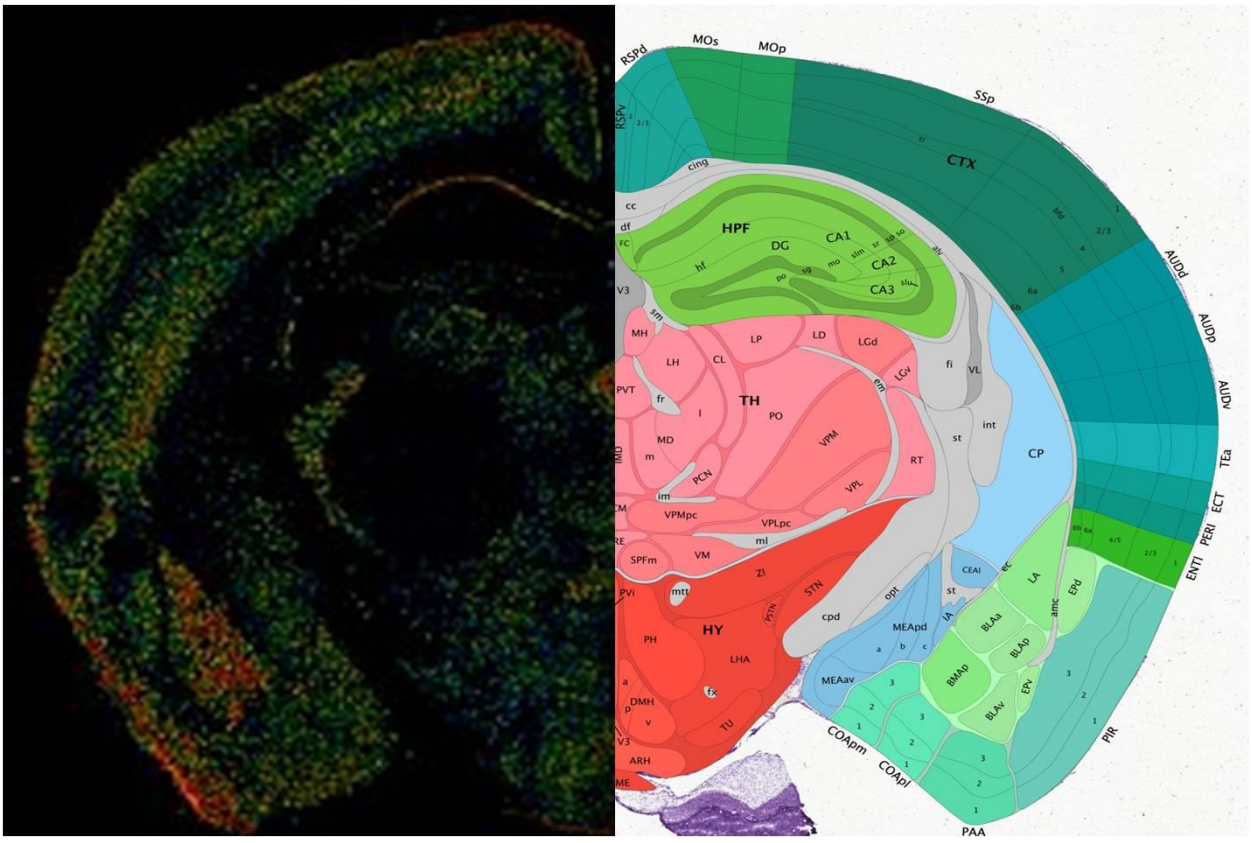
$GABA_A$ receptor α2 subunit

The expression of the α2 subunit is high early in development but begins to decline progressively with age[142] (figure 1-9). In adult mouse brain, the α2 subunit is highly expressed in hippocampus, amygdala, striatum, nucleus accumbens and hypothalamus[143-146]. In the thalamus, the α2 subunit is expressed only in paraventricular nucleus and reticular nucleus; it is also weakly expressed in anterodorsal nucleus, laterodorsal nucleus and rhomboid nucleus[143-146]. In the cerebellum and substantia nigra, it is completely absent or very weakly expressed[143-146].

GABA$_A$ receptor α3 subunit

Expression of the α3 subunit is high early in development but declines with age[147]. In adult brain, α3 subunit expression is higher in the inner layers of the cortex. In mouse hippocampus, the α3 subunit is only expressed in CA3, and not in the dentate gyrus or CA1[146]. In the thalamus, the α3 subunit is expressed highly in the reticular nucleus (a region of thalamus where α1 subunit is not expressed) and the midline nuclei[146] (figure 1-11). It is also strongly expressed in the Purkinjee cell layer of the cerebellum[146].

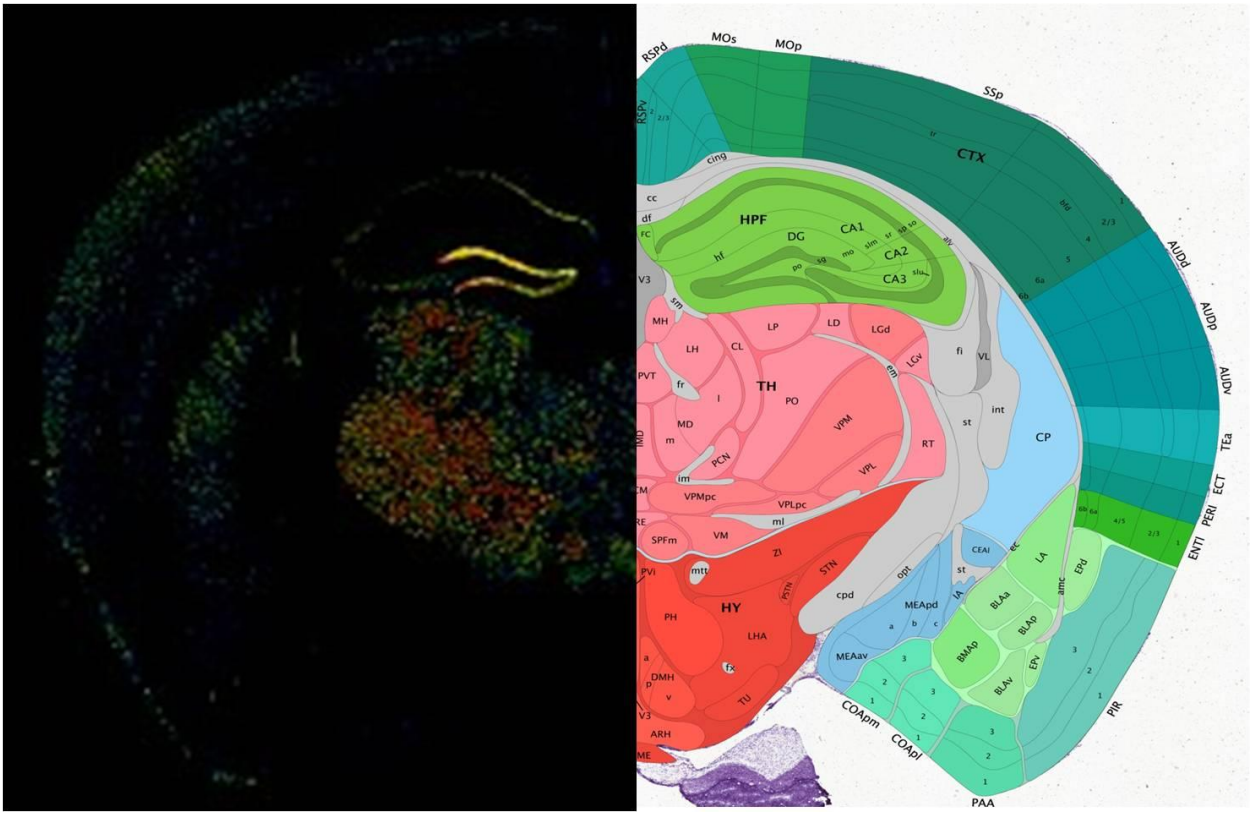
Figure 1-11: Expression of GABA$_A$ receptor α3 subunit mRNA in adult mouse brain (reproduced from ©2012 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas).



$GABA_A$ receptor α4 subunit

Expression of the α4 subunit is high in the superficial layers of cortex[143, 144]. In the thalamus, the α4 subunit is highly expressed in all regions except the reticular nucleus[146]. It is also highly expressed in the striatum, and nucleus accumbens and the dentate gyrus[143]. It is almost completely absent in the hypothalamus[143, 144, 146] (figure 1-12).

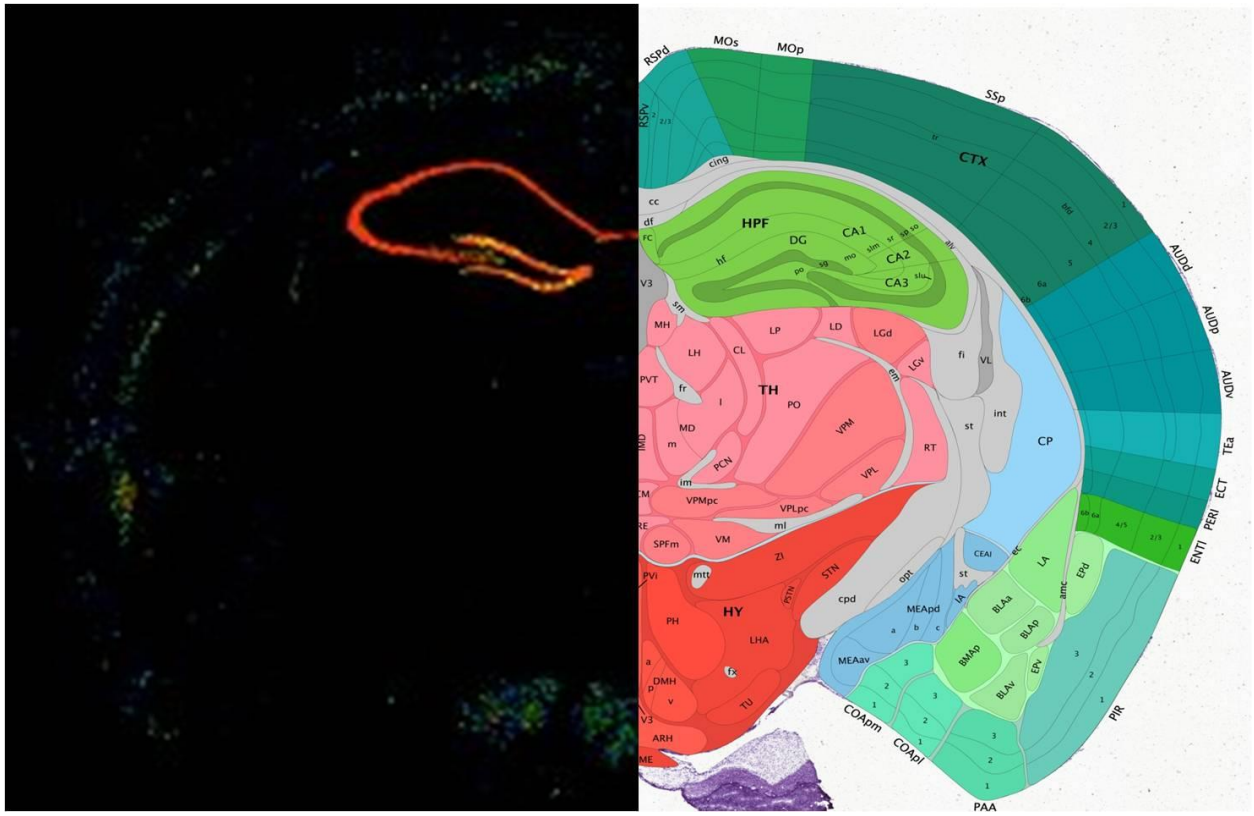
Figure 1-12: Expression of $GABA_A$ receptor α4 subunit mRNA in adult mouse brain (reproduced from ©2012 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas).



$GABA_A$ receptor α5 subunit

The expression of α5 subunit is considerably less prominent than the previously discussed α subunits[145]. It is expressed mainly in the deeper layers of cortex and the hypothalamus[143-146]. In the hippocampus it is expressed in the CA3 and dentate gyrus[146] (figure 1-13).

Figure 1-13: Expression of $GABA_A$ receptor α5 subunit mRNA in adult mouse brain (reproduced from ©2012 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas).



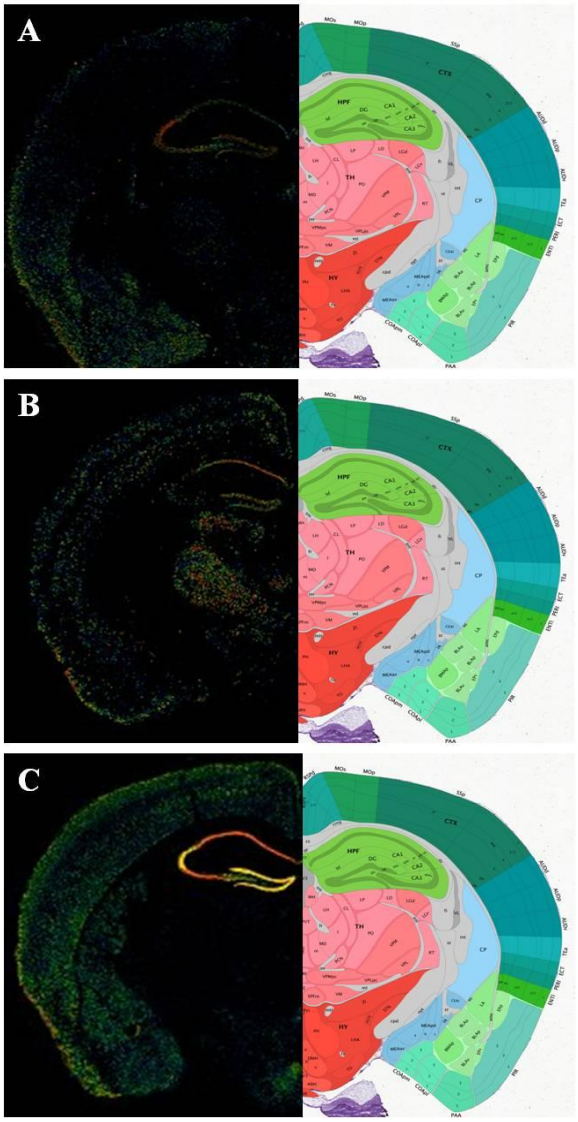
$GABA_A$ receptor α6 subunit

The α6 subunit is only found the granule cell layer of the cerebellum[143, 146] and the cochlear nucleus[148].

$GABA_A$ receptor β subunits

All 3 β subunit subtypes are expressed highly in all regions of cortex (figure 1-14). β1 and β3 subunits are expressed more strongly than the β2 subunit[145, 149 146]. In the striatum and hippocampus, the β3 subunit is more highly expressed as compared to other β subunits[145, 146, 149]. The β2 subunit expression is high in most thalamic nuclei except for the reticular nucleus; the β1 and β3 subunits are considerably less abundant throughout the remainder of the thalamus[149].

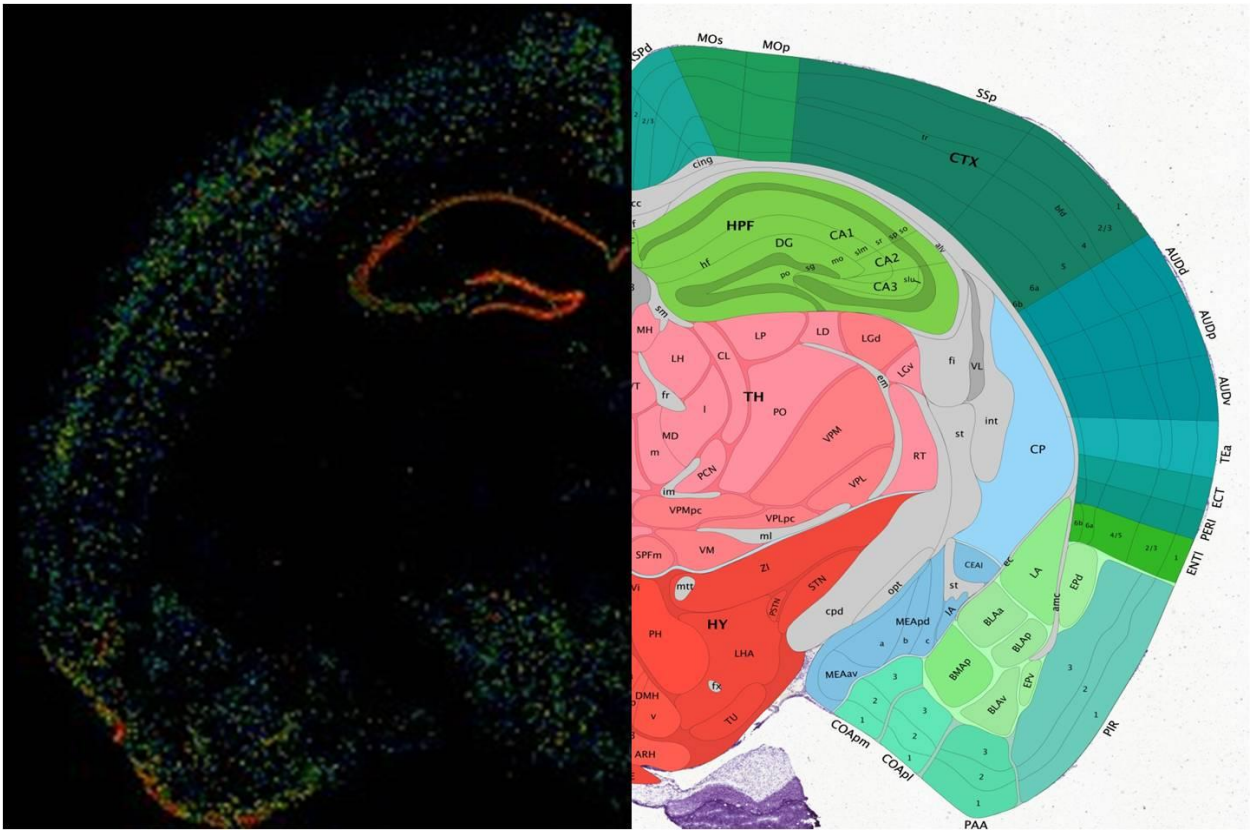
Figure 1-14: Expression of $GABA_A$ receptor β1 (A), β2 (B) and β3 (C) subunit mRNA in adult mouse brain (reproduced from ©2012 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas).



$GABA_A$ receptor γ subunits

Among the γ subunits, the γ2 subunit appears to be the most widely distributed in cortex, hippocampus, amygdala, globus pallidus and hypothalamus[145, 146, 149] (figure 1-15). All three γ subunits are weakly expressed in thalamus[149].

Figure 1-15: Expression of $GABA_A$ receptor γ2 subunit mRNA in adult mouse brain (reproduced from ©2012 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas).



$GABA_A$ receptor δ subunits

The δ subunits are expressed in the cortex, striatum and nucleus accumbens. It is also expressed highly throughout the thalamus except for the reticular nucleus[146]. The distribution of δ subunits in hippocampus is almost exclusively restricted to the dentate gyrus[146]. In the cerebellum it is only expressed in the granule cell layer[146, 150].

Mutations in $GABA_A$ receptor subunits associated with genetic epilepsies

Although most genetic epilepsies are associated with complex inheritance[38], there are many families in which genetic epilepsy syndromes are associated with monogenic inheritance. The mutations in transmembrane ligand- and voltage-gated ion channel genes are associated with a significant number of these monogenic epilepsies[37]. Some of the key human mutations in the $GABA_A$ receptor subunits associated with different forms of genetic epilepsies are summarized in tables I-III A and B and discussed in detail separately.

Table 1-4 A: Summary of mutations in $GABA_A$ receptor subunits and their association with epilepsy

Subunit	Mutation	Seizure/ Epilepsy	References
α1 subunit	A322D	Juvenile myoclonic epilepsy	Cossette et.al.[151]
α1 subunit	S326fs328X	Childhood absence epilepsy	Maljevic et.al.[152]
α1 subunit	K353delins18X	Idiopathic generalized epilepsy	Lachance-Touchette et.al.[153]
α1 subunit	D219N	Febrile seizure and idiopathic generalized epilepsy	Lachance-Touchette et.al.[153]
α6 subunit	R46W	Childhood absence epilepsy and atonic epilepsy	Dibbens et.al.[154]
β3 subunit	P11S	Childhood absence epilepsy	Tanaka et.al.[155]
β3 subunit	S15F	Childhood absence epilepsy	Tanaka et.al.[155]
β3 subunit	G32R	Childhood absence epilepsy	Tanaka et.al.[155]
δ subunit	E177A	Generalized epilepsy with febrile epilepsy plus	Dibbens et.al.[154]
δ subunit	R220C	Generalized epilepsy with febrile epilepsy plus	Dibbens et.al.[154]
δ subunit	R220H	Generalized epilepsy with febrile epilepsy plus	Dibbens et.al.[154]

Table 1-4 B: Summary of mutations in $GABA_A$ receptor subunits and their association with epilepsy

Subunit	Mutation	Seizure/ Epilepsy	References
γ2 subunit	K328M	Generalized epilepsy with febrile epilepsy plus	Baulac et.al.[156]
γ2 subunit	R82Q	Childhood absence epilepsy and febrile seizures	Wallace et.al.[157]
γ2 subunit	R177G	Febrile seizures	Audenaert et.al.[158]
γ2 subunit	P83S	Idiopathic generalized epilepsy and febrile seizures	Lachance-Touchette et.al.[153]
γ2 subunit	Q390X	Idiopathic generalized epilepsy and febrile seizures	Harkin et.al.[159]
γ2 subunit	W429X	Generalized epilepsy with febrile epilepsy plus	Sun et.al.[160]
γ2 subunit	S443delC	Idiopathic generalized epilepsy and febrile seizures	Tian et.al.[161]
γ2 subunit	IVS6+2T→G	Childhood absence epilepsy and febrile seizures	Kananura et.al.[162]

Mutations in the $GABA_A$ receptor α1 subunit associated with genetic epilepsies (figure 1-16)

A322D Missense Mutation

The A322D missense mutation was identified in a large French-Canadian family suffering with an autosomal dominant form of juvenile myoclonic epilepsy (JME)[151]. This mutation results in the replacement of alanine with a charged aspartate in the third transmembrane domain of the $GABA_A$ receptor α1 subunit[65]. Further *in vitro* studies showed that this mutation destabilizes the insertion of third transmembrane domain of α1 subunit in the lipid bilayer and hence causes its gross misfolding[163]. *In vitro* studies also showed that mutant α1 subunits have significantly reduced total and surface expression[164]. This reduced expression has been associated with degradation due to endoplasmic reticulum associated degradation (ERAD)[165] and lysosomal degradation[166]. It was further shown that residual assembled $GABA_A$ receptor containing the mutant α1 subunit reduced the surface expression of wild type subunits possibly by oligomerizing with and trapping wild type subunits in the endoplasmic reticulum; hence causing a small but significant dominant negative effect[165].

S326fs328X Frameshift Mutation

This mutation was originally described in one patient (*de novo*, the unaffected parents were negative for the mutation) suffering with childhood absence epilepsy (CAE)[152]. It causes a single base pair deletion (975delC) which eventually results in a frameshift mutation (S326fs328X)[152]. This mutation is situated in the third transmembrane domain of α1 subunit. *In vitro* studies showed that it completely abolished GABA-evoked current[167]. *In vitro* studies also showed that mutant mRNA was degraded through nonsense-mediated mRNA decay (NMD) and the small amount of protein produced from the remaining mRNA that escaped NMD was degraded by ERAD[167]. These two mechanisms completely eliminated the α1 subunit expression making S326fs328X a null mutation. These findings also make the $GABA_A$ receptor α1 subunit knock out mouse model a very relevant model to study this form of genetic epilepsy.

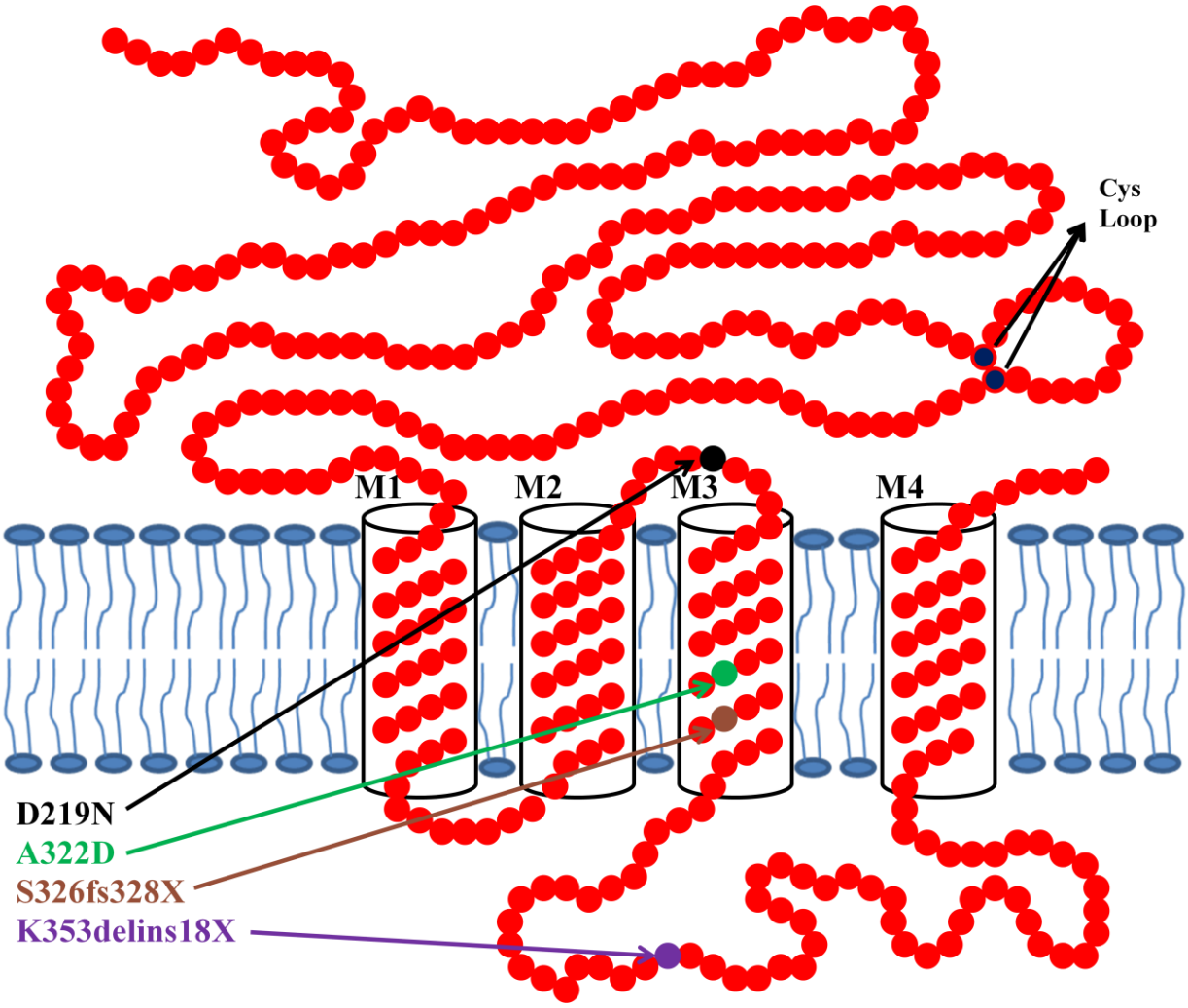
K353delins18X Mutation

Three patients suffering with IGE and one obligated carrier, all belonging to the same French-Canadian family, were reported to carry the K353delins18X mutation in the $GABA_A$ receptor α1 subunit gene[168]. The mutation resulted in the insertion of 25 nucleotides in the intron close to the splice acceptor site of exon 11[168]. This splice mutation is predicted to rearrange the α1 subunit transcript such that the fourth transmembrane is deleted as well as 18 amino acids and a premature stop codon is inserted[168]. *In vitro* studies, conducted on transfected HEK-293 cells using surface biotinylation assay and electrophysiology, show that the mutant α1 subunit is expressed but not transported to the plasma membrane[168].

D219N Missense Mutation

Four out of five individuals belonging to the same French-Canadian family, suffering with either febrile seizures or IGE, were reported to carry a missense mutation (D219N) that resulted in the replacement of the negatively charged amino acid aspartate with a non-charged polar amino acid asparagine[168]. *In vitro* studies showed that the mutant $GABA_A$ receptor α subunits were able to assemble as part of $GABA_A$ receptor and transported to cell membrane, although there was a 50% reduction in the ratio of surface to total expression of mutant D129N α1 subunit as compared to controls[168].

Figure 1-16: $GABA_A$ receptor α1 subunit mutations associated with genetic epilepsies (adapted from Macdonald et al 2010 and Lachance-Touchette et al 2011)



Mutations in $GABA_A$ receptor α6 subunits associated with genetic epilepsies

R46W Missense Mutation

A novel mutation in $GABA_A$ receptor α6 subunit, described in a patient suffering from CAE and atonic seizures, results in substitution of arginine at position 46 with tryptophan(R46W)[169]. This mutation causes impaired functioning of the receptor, as determined by electrophysiology experiments, and decreased surface expression of $GABA_A$ receptor[170].

Mutations in $GABA_A$ receptor β subunits associated with genetic epilepsies

Three missense *de novo* mutations in $GABA_A$ receptor β3 subunit gene (P11S, S15F and G32R) have been identified in four cases of CAE[171]. P11S and S15F are located on exon 1a and are part of the β3 subunit signal peptide, while G32R is located at amino acid 10 from the N terminus in mature protein and located on exon 2[171]. Initial *in vitro* studies showed that each of these three mutations were associated with hyperglycosylation and decreased peak amplitude of GABA-evoked whole-cell currents[171]. Further *in vitro* studies done on the G32R mutation showed three changes: 1) co-expression of β3 (G32R) subunit with α1 or α3and γ2L subunits resulted in an increased surface expression of β3 subunits and reduced surface expression of γ2L subunits; 2) G32R β3 subunits were more likely to be glycosylated at Asn-33 than in controls and finally 3) $GABA_A$ receptors assembled with α1, β3(G32R) and γ2L subunits had reduced macroscopic current density relative to controls[172]. Interestingly, another study showed an association of the P11S mutation and autism[173]. It should also be noted that studies conducted on β3 subunit knockout mouse model have shown that homozygous β3 knockout mice have a high mortality, stunted growth, cleft palate and numerous neurological symptoms including hyperactivity and seizures[174].

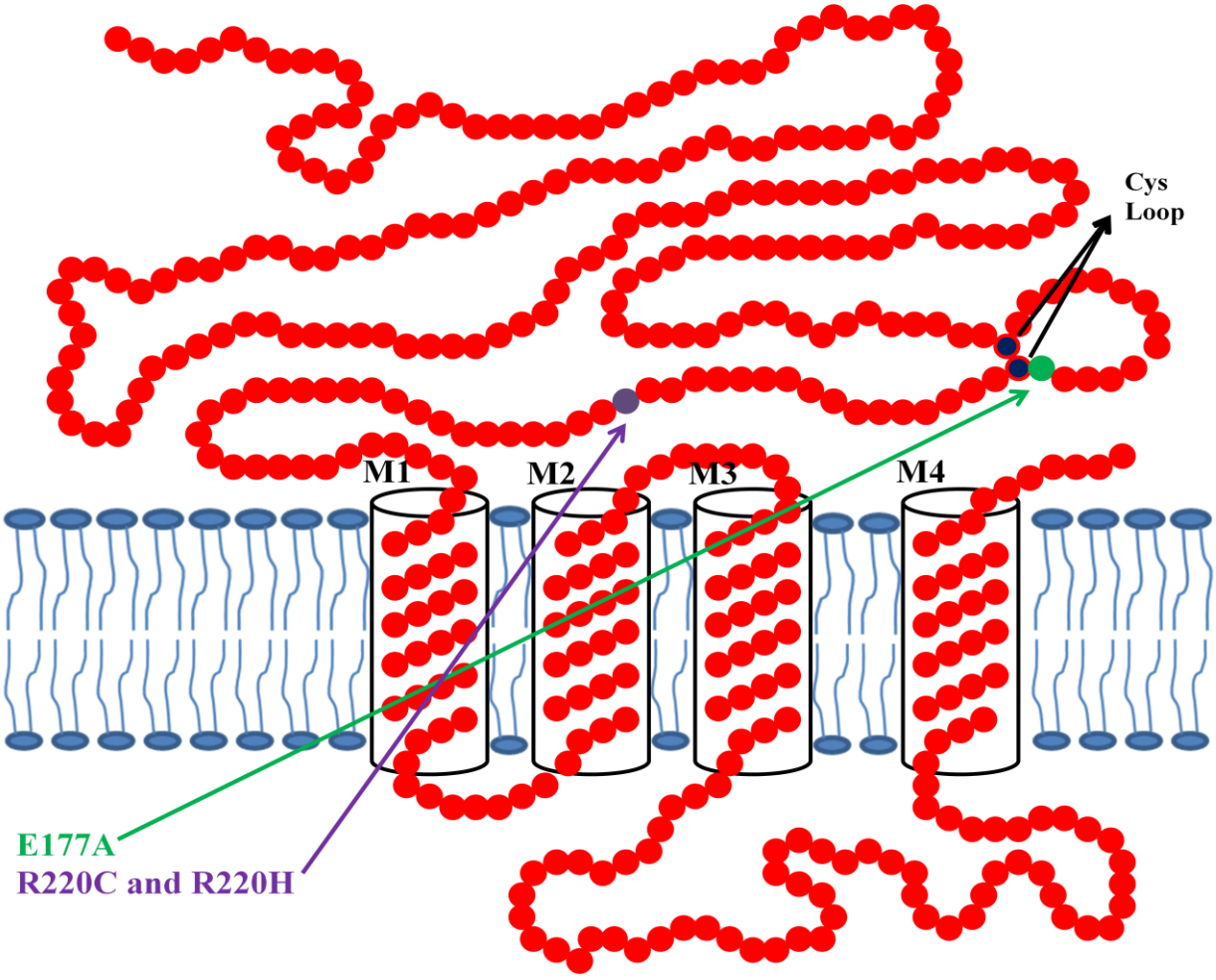
$GABA_A$ receptor δ subunits associated with genetic epilepsies (figure 1-17)

Two missense mutations in $GABA_A$ receptor δ subunit gene, E177A and R220C, have been identified in two separate small families suffering with generalized epilepsy with febrile epilepsy plus (GEFS+)[175]. Both of these substituted amino acid residue in the extracellular domain of the amino terminal of $GABA_A$ receptor δ subunit, however it should be noted that E177A is located adjacent to one of the two invariant cysteines that form the disulfide bond[175] (figure1-17).

It was also shown that although the EC_{50} of GABA was not shifted by heterozygous and homozygous expression of E177A but a significantly reduced maximal current in saturating concentrations of GABA occurs[175]. Although no functional effect of R220C was reported, heterozygous expression of another variant, R220H, found in GEFS+ and febrile seizure (FS) patients and controls, also showed significantly

decreased peak current; indicating that R220H could be a modifier or susceptibility gene[175]. No change in the total receptor protein has been reported but the surface expression was significantly reduced for both E177A and R220H mutation carrying $GABA_A$ receptor[176]. Although it should be noted that association analysis done as part of another study has also shown that there is no evidence of association of R220H with IGE susceptibility[177].

Figure 1-17: $GABA_A$ receptor δ subunit mutations associated with genetic epilepsies (adapted from Macdonald et al 2010)



Mutations in $GABA_A$ receptor γ2 subunits associated with genetic epilepsies (figure 1-18)

A large number of mutations in the $GABA_A$ receptor γ2 subunit gene have been reported. Some of these mutations will be discussed here based on their classification.

Missense mutations of $GABA_A$ receptor γ subunits

Autosomal dominant missense mutation in $GABA_A$ receptor γ2 subunit (K328M) was identified in a family suffering from GEFS+[178]. The K328M mutation causes the substitution of a residue located in the short extracellular loop between the M2 and M3 transmembrane segment of the γ2 subunit. This mutation causes a reduced amplitude of GABA currents in oocytes[178], but studies in HEK cells show an accelerated deactivation and no change in amplitude[179]. This mutation does not have any effect on $GABA_A$ receptor cell surface expression at normal room temperature[180] but a recent *in vitro* study showed that upon elevation of temperature, by a few degrees, the postsynaptic aggregation of the mutant K328M γ2 subunit was significantly reduced[181]. This finding makes K328M a strong candidate for further studies on GEFS+.

The R82Q, a missense mutation that resides in the extracellular domain of the amino terminal of the $GABA_A$ receptor γ2 subunit gene, has been reported in a large Australian family suffering with CAE and FS[182, 183]. *In vitro* studies have shown that, when expressed with α1 and β2 subunit, this mutation causes a slow $GABA_A$ receptor deactivation and increased rate of desensitization[184]. When mutant γ2 subunit was expressed with α1and β3 subunits, in another *in vitro* experiment, no such change was seen[179]. The R82Q mutation also causes reduced surface expression of $GABA_A$ receptors[185]. R82Q knock-in mice have absence seizures while cortical neuronal cultures of these mutant mice showed reduced surface expression of γ2 subunits[186].

Another missense mutation, R139G in $GABA_A$ receptor γ2 subunit gene, has been identified in a family suffering with autosomal dominant form of FS[158]. *In vitro* studies showed that this substitution of the highly conserved arginine with glycine caused a rapid desensitization but no change in peak current amplitude[158].

In a large French-Canadian family, suffering from FS and genetic epilepsy (GE) over three generations, another missense mutation (P83S) in $GABA_A$ receptor γ2 subunit gene has been identified[168]. *In vitro* electrophysiology studies, however, did not identify any significant functional deficit, but the high degree of penetrance and conservation shows that further studies should be conducted to study this mutation better.

Nonsense mutations of $GABA_A$ receptor γ2 subunits

An Australian family suffering from $GEFS^+$, FS and IGE has been identified in which a single base pair substitution in the γ2 subunit gene caused a premature stop codon at Q390, which is present in the large cytoplasmic loop between the third and fourth transmembrane domains[187]. *In vitro* studies showed that the application of GABA failed to activate current responses, indicating a failure of surface expression of assembled $GABA_A$ receptor[187]. This could be attributed to the retention of the mutant γ2 subunit and the wild type α and β subunits in the endoplasmic reticulum[188]. The mutant γ2 subunit has been shown to have a stable conformation and a slow rate of degradation than wt γ2 subunits[189].

An autosomal dominant nonsense mutation (W429X), present in the large intracellular loop between third and fourth transmembrane domain of γ2 subunit, with 87.5% penetrance was reported in a Chinese family suffering with $GEFS^{+}$[190].

Frameshift mutations of $GABA_A$ receptor γ2 subunits

In a non-consanguineous Italian family a frameshift mutation γ2S(S443delC) was reported in four patients suffering from mild generalized seizures and febrile seizures[191]. *In vitro* studies showed that the mutation caused an extension in the C terminal of γ2 subunits by 26 amino acids. The total expression of the mutant protein was reduced and the residual expressed protein was not trafficked to the cell membrane and was retained in the endoplasmic reticulum[191].

Mutations of $GABA_A$ receptor γ subunits in untranslated region

In a German family suffering with CAE and FS a mutation, IVS6+2T→G, was found that destroyed the 5'-splice site of intron 6 and hence interfered in correct splicing[162]. Further studies on this mutation are required as it can possibly result in the formation of nonfunctional $GABA_A$ receptor γ subunits and play a role in the development of epilepsy phenotype.

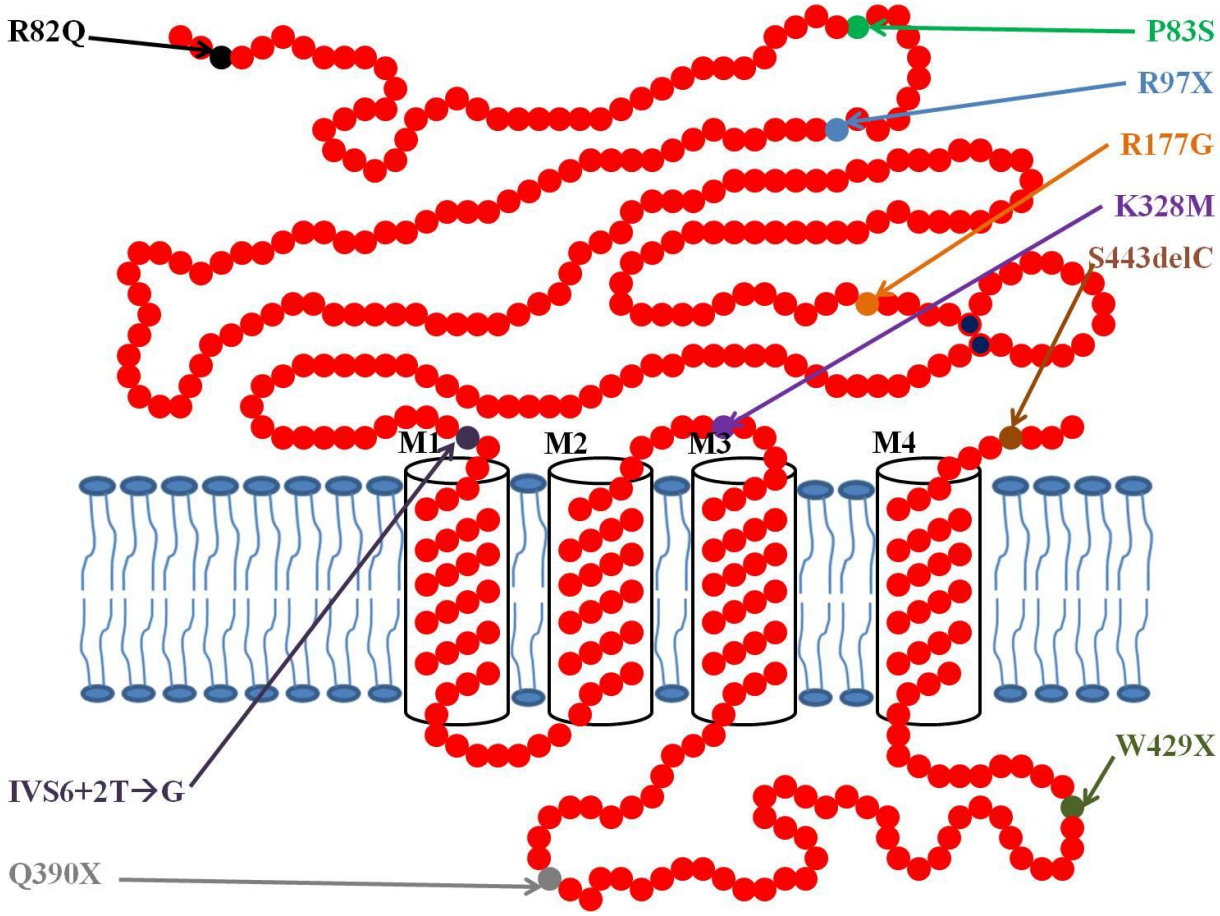


Figure 1-18: $GABA_A$ receptor γ2 subunit mutations associated with genetic epilepsies (adapted from Macdonald et al 2010)

Modulation of $GABA_A$ receptor expression in models of acquired seizures

Gross changes in brain structure of rodents have been reported after pilocarpine-induced seizures, that includes enlarged ventricles, enlarged dentate gyrus, damaged piriform cortex and amygdala, along with cell loss in thalamus and hippocampus[192-194]. $GABA_A$ receptors are the target of many chemoconvulsants including pentylenetetrazol, an antagonist of $GABA_A$ receptors[195]. Studies done on human temporal lobe epilepsy and related animals models of acquired epilepsy have shown modulation of the expression of $GABA_A$ receptor subunits[196]. A brief summary of the more notable changes in the expression $GABA_A$ receptor subunits, in acquired models of epilepsy, are discussed below.

$GABA_A$ receptor α subunits

Pilocarpine induced seizures have been reported to cause a decrease in α1 subunit and an increase in α4 subunit mRNA expression[197]. Electrically-induced status epilepticus appears to increase the level of both α2 and α4 subunits[198]. In both electrically-induced status epilepticus[198] and pilocarpine-induced seizures[199], α5 subunit expression is decreased in the CA1, CA2 and CA3 region of hippocampus.

In the hippocampal neurons of *tremor rats*, a model of spontaneous absence-like seizures and tonic convulsions, mRNA and protein levels of α1 subunit were reported to be upregulated[200]. Further studies showed that mRNA and protein levels of α4 and γ2 subunits were also increased in the hippocampus of these animals[201].

$GABA_A$ receptor β subunits

The mRNA levels of all three β subunits show a general trend towards an increase in electrically induced status epilepticus rats[198]. However in another study it was shown that the β2 and β3 subunits were internalized more than controls in pilocarpine induced status epilepticus[202].

$GABA_A$ receptor δ subunits

In a mouse model of pilocarpine-induced status epilepticus it was shown that the expression of δ subunits was decreased in the molecular layer of dentate gyrus, while it was increased in the interneurons present in CA1 region of the hippocampus[192]. A progressive decline in mRNA expression of δ subunits is also observed in electrically induced status epilepticus in rats[198]. In kainic acid-induced seizure model, δ subunit expression is reduced in dentate gyrus and CA1 region of hippocampus[203].

$GABA_A$ receptor γ2 subunits

Studies using insitu-hybridization show that the levels of γ2 subunit mRNA increased slightly in electrically induced status epilepticus rats[198]. In pilocarpine-induced status epilepticus mice, γ2 subunit expression increased throughout hippocampus, especially in the dentate gyrus[192, 193].

Conclusions

Epilepsy, known as the "sacred disease" in ancient times, has been the target of many anticonvulsant treatments that ranges from barbaric (for example bleeding and branding in the Middle Ages) to serendipitous (for example development of valproic acid in recent times)[204]. Despite centuries of research epilepsy remains a poorly understood disorder[205]. However the use of animals models has played a fundamental role in increasing our understanding of seizures and epilepsy[14] and developing new treatments for them[206].

Genetic epilepsies account for about 30% of all epilepsy cases[10] and their complex inheritance is a significant hurdle in developing a better understanding of their etiologies. Research on these epileptic syndromes has identified that the mutations in ligand- and voltage-gated ion channel genes are associated with genetic epilepsy[37]. Furthermore, the fact that some monogenic mutations in ion channels show clear

Mendelian pattern of inheritance, stresses the importance of why they should receive more attention. The development of animal models of genetic epilepsy provides an invaluable source of information for these syndromes.

Over the last 10 years many mutations in $GABA_A$ receptors have been shown to be associated with epilepsy[40]. The discovery of the A322D missense mutation, identified in a large French-Canadian family suffering with an autosomal dominant form of juvenile myoclonic epilepsy (JME)[151], is a good example. Therefore further studies conducted on animal models of $GABA_A$ receptor mutations can help us increase our understanding of epilepsy and provide insights for the development of more effective treatments.

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Chapter II

Decreased viability and absence-like epilepsy in mice lacking or deficient in the $GABA_A$ receptor α1 subunit

Introduction

It is estimated that 50 million people suffer from epilepsy world wide[1]. Genetic generalized epilepsy (GGE) syndromes constitute 10-20% of all epilepsies[2]. These epilepsy syndromes that confer generalized seizures (for example absence, myoclonic and generalized tonic clonic seizures), do not result from any known acquired lesion[3], and are often clustered in families as are observed in twin and family studies[4].

Although GGEs are thought to result from a genetic etiology, Mendelian inheritance is not often observed[3]. For example, although autosomal dominant EFHC1 mutations have been shown to cause juvenile myoclonic epilepsy (JME) in some families, some cases of JME result from *de novo* EFHC1 mutations while majority are believed to result from polygenic inheritance[5, 6].

As discussed in chapter I, $GABA_A$ receptors are the primary mediators of fast inhibitory synaptic transmission in the central nervous system and mutations in these receptors have been implicated in many cases of genetic epilepsies[7]. Two mutations, S326fs328X and A322D, in $GABA_A$ receptor α1 subunit gene have been shown to be associated with childhood absence epilepsy[8] and juvenile myoclonic epilepsy[9] respectively. *In vitro* studies have shown that A322D mutation causes a 88% reduction in $GABA_A$ receptor α1 subunit expression[10], while S325fs328X causes complete elimination of $GABA_A$ receptor α1 subunit expression[11].

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Despite the evidence that heterozygous loss-of-function mutation in $GABA_A$ receptor α1 subunit caused GGE in humans[8], heterozygous or even homozygous $GABA_A$ receptor α1 subunit knockout (Gabra1 KO) mice (maintained in a mixed genetic background) were not reported to have any visually apparent seizures or changes in viability[12]. These surprising *in vivo* finding could have resulted if, 1) α1 subunit haploinsufficiency alone does not cause epilepsy and a dominant negative effect is necessary, 2) Gabra1 KO mice do not fully replicate the human disease, 3) modifier genes present in the mixed background alter the phenotype, or 4) Gabra1 KO mice have nonconvulsive seizures that require synchronized video/EEG monitoring to be diagnosed.

Here we investigated whether heterozygous loss of α1 subunit reduced viability or caused seizures in mice maintained in the C57BL/6 and DBA/2J congenic backgrounds.

Methods and materials

Generation and maintenance of Gabra1 KO mice in congenic strains

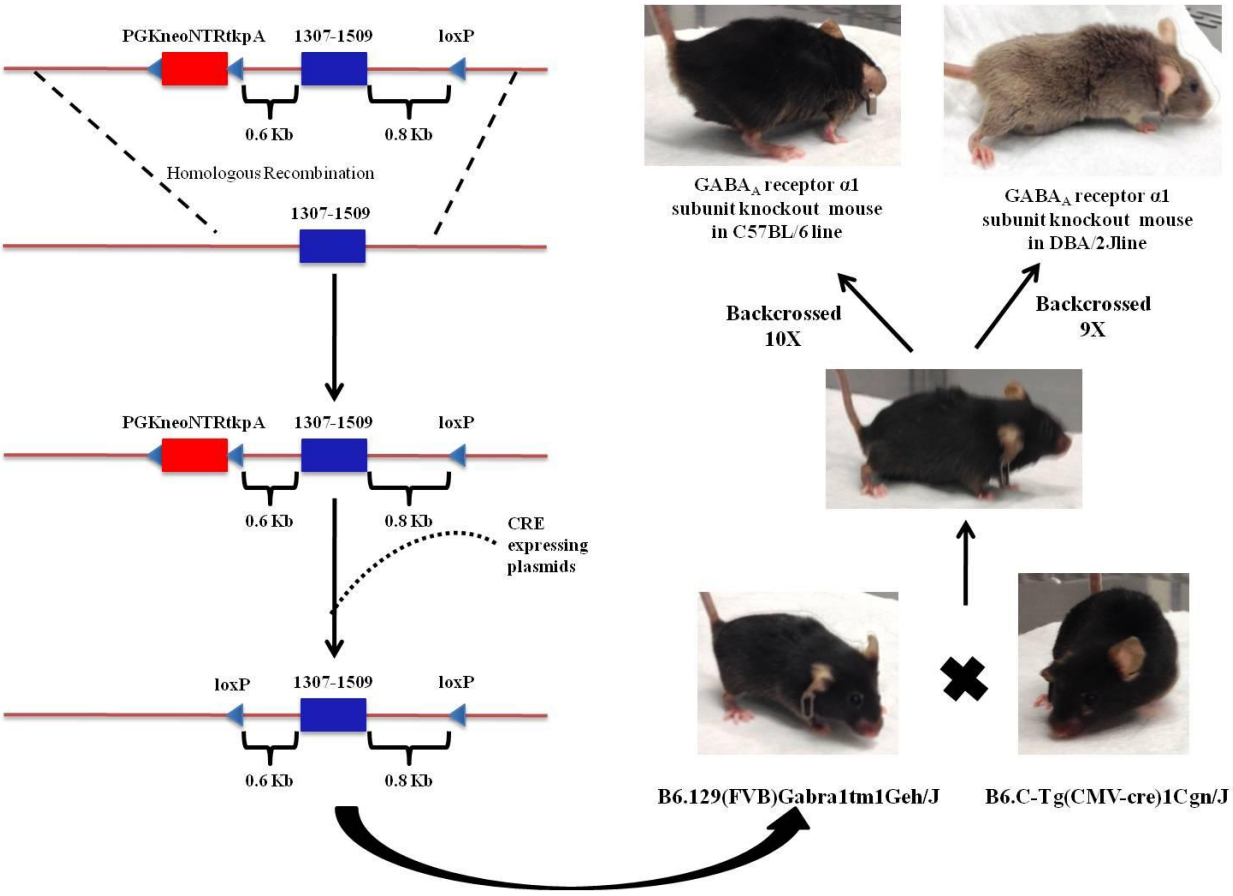
We obtained the Gabra1 subunit mutant mice, that possessed *loxP* sites flanking the DNA encoding the second transmembrane domain of α1 subunit, from Jackson Laboratories (B6.129(FVB)Gabra1tm1Geh/J; stock 004318). These mice were first generated by Vicini et.al.[12]. A short description of the mutant mouse production is as follows.

Vicini et.al. created a BAC clone containing a targeting vector (derived from Strain 129-derived mouse genomic DNA) that consisted of nucleotides 1307-1509, which are predicted to encode for amino acids starting putative second transmembrane domain and ending in the intercellular loop between transmembrane 3 and 4 of mouse Gabra1 subunit[12]. It should be noted that deletion of nucleotides 1307-1509, that are predicted to encode amino acids starting in the putative second transmembrane domain and ending in the intercellular loop between transmembrane 3 and 4, is also expected to create a frame-shift

mutation and prevent translation of downstream exons, possibly creating a truncated protein[12]. The vector also contained the selectable marker gene PGKneoNTRtkpA flanked by loxP sites into a site ~0.6 kb upstream of the target exon and another loxP site 0.8 kb downstream of nucleotides 1307-1509[12]. This entire construct was injected into mouse embryonic stem cells (ESC). The PGKneoNTRtkpA selectable marker cassette allowed identification of correctly targeted ESC clones. The correctly targeted ESCs were transfected with CRE expressing plasmids to delete the selectable marker cassette. Four ESC clones that had the target sequence flanked by loxP sites were injected into C57BL/6 blastocysts. These blastocysts were implanted in a pseudo-pregnant female mouse. Among the resulting offspring two chimeric animals were chosen to establish the germ line transmission. A summary of the creation of congenic Gabra1 KO mouse line is summarized in Figure 2-1 (adapted from Vicini et.al. 2001)

Although previous studies of Gabra1 KO mouse used interbred strain, we obtained this mouse after it had been backcrossed to C57BL/6 congenic line for 6 generations. We then mated these mice with B6.C-Tg(CMV-cre)1Cgn/J (stock 006054) (that expresses cre in all tissues) and the resulting litter contained heterozygous Gabra1 KO mice. We then continued backcrossing the heterozygous Gabra1 KO mice into C57BL/6 strain (backcrossed at least 10 times) and also into a separate congenic strain, DBA/2J (backcrossed at least 9 times).

Figure 2-1: A summary of the creation of Gabra1 KO mouse is summarized below (adapted from Vicini et.al. 2001)



Mouse colony maintenance and mating strategy for experimental mice

All procedures were performed in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Mice were housed in a temperature and humidity controlled environment, with a 12 hour light/dark schedule. Water and food were provided ad libitum. We mated either C57BL/6 or DBA/2J heterozygous Gabra1 KO breeding pairs to produce wild type (WT), heterozygous (het) and homozygous (hom) knockout mice in their respective congenic strain.

Gabra1 KO mice genotyping

Mouse tail snips were collected between postnatal day (P) 17 and P21or at the date of death. DNA was extracted and amplified using a commercially available PCR protocol (red Extract-N-AMP,Sigma). Forward and reverse primers used to identify the wild type allele were CAGCAGACCTGTGCTTGTTC and TTCTGCATGTGGGACAAAGA, respectively. Forward and reverse primers used to identify the mutant allele were CTAGGGTAGACTAGGGAGTGG and CTGCATGTGGGACAAAAGA, respectively.

The PCR conditions are as follows:

Initial denaturation 95°C for 5 minutes

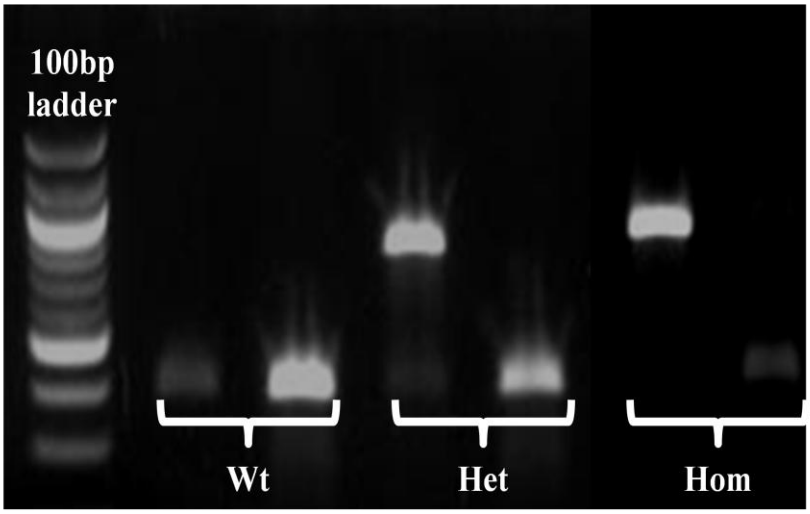
PCR cycle denaturation 95°C for 30 seconds

PCR cycle annealing 54°C for 30 seconds

PCR cycle extending 72°C for 90 seconds

Final extension 72°C for 10 minutes

Figure 2-2: Genotyping for $GABA_A$ receptor α1 subunit knockout allele



Analyzing viability of Gabra1 KO mice

The number of pups produced in each litter was documented along their sex, genotype, date of birth and age at death. We calculated the Mendellian ratios by genotyping the mice at the time of weaning or day of death. We measured the body mass of the mice every one to three days from P7 to P30, after which the animals were used for different experiments including video/EEG recording and immunohistochemistry.

Using P25 WT, heterozygous and homozygous Gabra1 KO mice, we performed pathologic analyses, looking at gross morphology and histopathology of all major organs in collaboration with an expert veterinary pathologist, Kelli L. Boyd (Associate Professor, Department of Pathology, Microbiology and Immunology; Vanderbilt University).

Surgical implantation of prefabricated skull head mounts and video/EEG monitoring

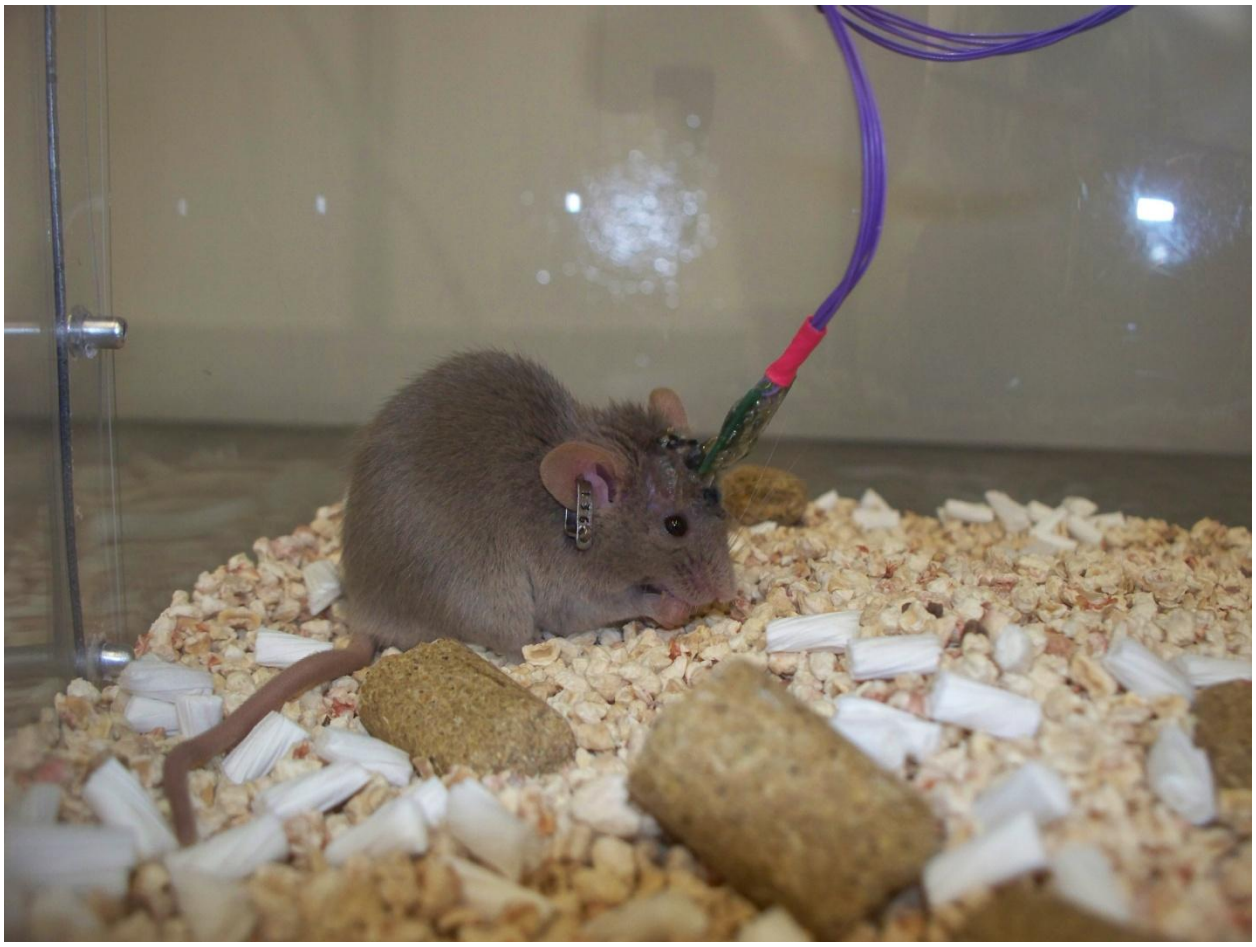
Surgical implantation of prefabricated mouse headmounts (Pinnacle Technologies, Lawrence, KS, USA), that allows two bipolar EEG channels and one subcutaneous nuchal EMG channel monitoring was done as described earlier[13], at least 48 hours prior to the scheduled EEG recording. Animals were anesthetized with continuous flow of isoflurane. A saggital incision was made on shaved and sterilized scalp to expose the skull. The headmount was placed using central sulcus and bregma as landmarks and held in place with four stainless steel screws (Pinnacle Technologies), inserted through burr holes. These screws also served as electrodes that allowed us to obtain intracranial EEG signals. The two EMG wires were placed along the vertebral muscles to monitor gross body movement. Headmount was further secured using dental acrylic and suturing loose skin around the headmount. Mice were given the analgesic ketoprofen 10mg/kg intraperitoneally before the start of surgery and were monitored daily for signs of infection or distress after surgery.

In separate set surgeries the implantation of headmounts was modified to obtain referential EEG recordings from each hemisphere separately. A coronal incision was made on shaved and sterilized scalp

to expose the skull. The headmount was placed along the bregma using the central sulcus as the midpoint. The referential electrode was placed on the skull instead of intracranial.

At the time of the recording, a 100X mouse preamplifier (Pinnacle Technologies) was attached to the headmount to amplify and filter the EEG waveforms. EEG signals then passed through the low-torque mouse commutator/swivel (Pinnacle Technologies) to the 8206 DCAS for final stage conditioning and filtering (Pinnacle Technologies). Acquisition of Video/EEG was done using the software Sirenia®**. D**ata were analyzed using 3 different computer programs: 1) Sirenia® Seizure, 2) EDFbrowser and 3) NicoletOne EEG Reader V5.71.4.2530.

Figure 2-3: Picture of a post-operative mouse showing the implanted headmount connected to a preamplifier.



The EEG was analyzed by blinded reviewer to identify spike-wave discharges (SWDs) using criteria established for the analysis of rat models of absence epilepsy[14]. Briefly SWDs were defined as trains of rhythmic biphasic spikes, with a voltage at least twofold higher than baseline and that were associated with after-going slow waves. The reviewer quantified the incidence, duration and spike frequency of SWDs in uniform 5-min samples each hour. To determine if SWDs were associated with behavioral arrest, manifestations of absence seizures, we determined whether the longer SWDs (>2 s) were associated with attenuation of the EMG signal and behavioral changes on video. Because mouse movements produce slow (1–4 Hz) EMG waveforms, we were also able to objectively quantify the effects of SWDs on movement by measuring the relative EMG spectral power (1–4 Hz delta power, Carefusion, San Diego, CA, U.S.A.) in 2 second segments before, during, and after the SWDs. Finally, we determined the effects of ethosuximide, a drug used to treat absence seizures in humans, on the incidence SWDs. Mice were given 200 μl intraperitoneal (i.p.) saline, and a two hour baseline EEG was obtained. The mice then received either ethosuximide (200 mg/kg, i.p.) or another dose of 200 μl saline (placebo), and the incidence of SWDs after the drug/placebo treatment was compared with that during the baseline.

Statistical Analyses

Statistical analyses were performed using the R 2.12.2 Statistical Package for Windows (R Foundation for Statistical Computing, Vienna, Austria). We first determined the effects of Gabra1 KO separately in male and female mice; if there was no sex-dependent effect, we grouped male and female mice together. Parametric data (body mass, SWD incidence) are presented as the mean ± standard error of the mean (SEM) and are compared using the Student's two-tailed t-test or analysis of variance with Tukey posttest, as appropriate. Nonparametric data (Mendelian ratios, mortality) were analyzed by chi-square.

Results

The Gabra1 KO mutation causes a decrease in viability

Two previous studies have reported that heterozygous or even homozygous loss of α1 subunit of Gabra1 receptors is not lethal in mice[15, 16]. In both of these studies, WT and mutant Gabra1 KO mice used were generated on a mixed genetic background, i.e. a mix of C57BL/6, 129Sv/SvJ, and FVB/N[17] and a mix of ~50% C57BL/6–50% 129SvEv genetic background[15, 16].

Recent research has underlined the importance of the genetic background of the animal model used to study complex human diseases. The genetic basis of disease in DBA, GAERS, EL and other epileptic rodent strains is usually multifactorial, owing to a combination of additive and epistatic genetic variables, along with environmental and even random stochastic effects[17]. Although "non-genetic factors" can sometimes be controlled by experimental procedures or overcome by increasing sample size, it is the genetic complexity itself that presents the greatest obstacle[17]. For example, an inbred mouse strain, PL/J, has been reported to be susceptible to handling and rhythmic tossing-induced seizures. However, when PL/J is crossed with the seizure resistant C57BL/6 strain , the genetic inheritance of seizure susceptibility of PL/J is non-Mendelian[18]. Interestingly when PL/J strain was crossed to DBA/2J, severity and frequency of seizures were higher in the progeny[18].

Therefore, using mutant heterozygous Gabra1 KO animals maintained in a congenic genetic strain allows us to study the effects of any mutation without the results being confounded by other genetic variables. That is why we decided to conduct all of our experiment on the progeny of the mice fully backcrossed to either the C57BL/6 strain (backcrossed at least 10 times) or the DBA/2J strain (backcrossed at least 9 times).

Mendelian ratio shows a lack of prenatal mortality

First, we determined the effect of Gabra1 KO mutation on viability. A total of 199 C57BL/6 and 115 DBA/2J mice obtained from het Gabra1 KO and het Gabra1 KO intercross were analyzed. Mendelian

ratios were calculated as a measure of prenatal mortality. In the absence of any significant prenatal mortality, the ratio of the genotype among the offspring of het Gabra1 KO and het Gabra1 KO intercross is expected to be approximately 25% wt, 50% het and 25% hom. In both C57BL/6 and DBA/2J congenic lines no significant deviation from the expected Mendelian ratio was observed (results are summarized in table 2-1), indicating a lack of significant prenatal mortality.

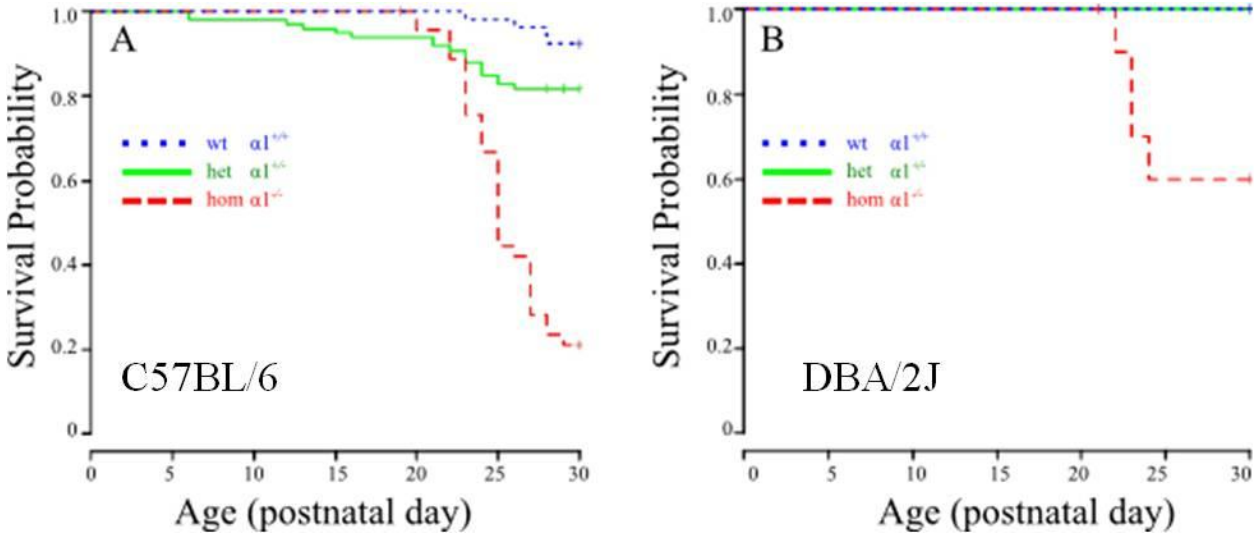
Table 2-1: Mendelian ratios of F1 progeny of heterozygous Gabra1 KO mice.

	Mendelian Ratio			
	Wild Type	Heterozygous	Homozygous	*p value*
C57BL/6	27%	50%	24%	*0.780*
DBA/2J	34%	47%	19%	*0.273*

Homozygous Gabra1 KO mice have high mortality after P19

Offspring of heterozygous Gabra1 KO and heterozygous Gabra1 KO intercross were followed until postnatal day 30 (P30) for mortality and gain in mass. As shown in the Kaplan Meier plots in figure 2-4, a robust decrease in survival probability of homozygous (hom) Gabra1 KO mice was observed, which reached significance after P19 as shown by chi-square test (*p value* < 0.001), in both congenic strains. It should also be noted that the mortality of hom Gabra1 KO mice was significantly higher in C57BL/6 compare to DBA /2J congenic strains (*p value* $= 0.028$). There was no significant difference in mortality between WT and het mice in either the C57BL/6 or DBA/2J strains.

Figure 2-4: Kaplan Myer plots showing decreased survival probability in hom Gabra1 KO mice of both C57BL/6 (figure 2-4A) and DBA/2J (figure 2-4B) congenic strains.



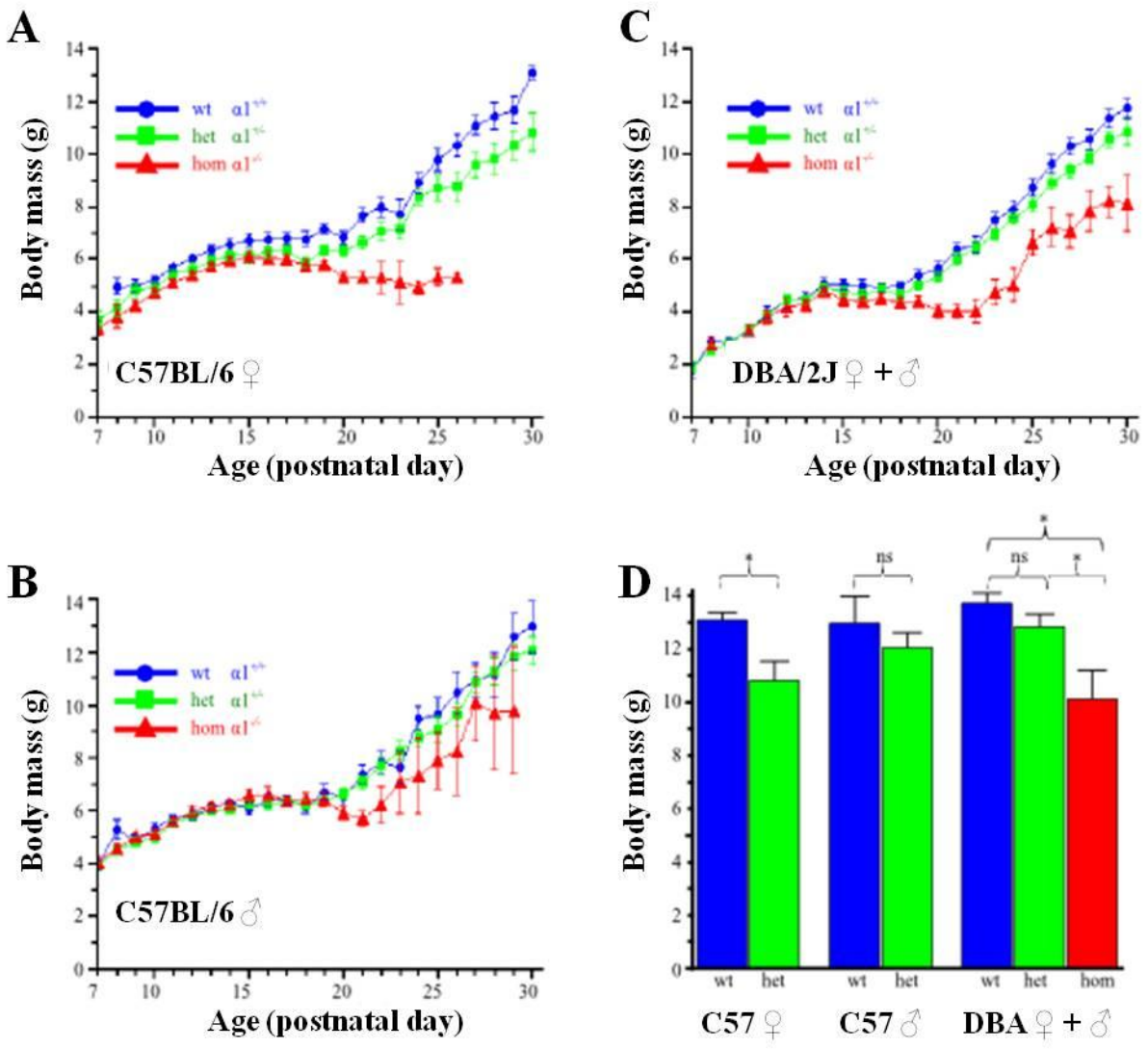
Gain in body mass is affected by genotype and sex

We measured the body mass of these mice every 1 to 3 days from P7 to P30 to determine the effect of the Gabra1 KO mutation on daily gain in body mass (figure 2-5). At P30 statistical analysis revealed a sex dependent effect in the C57BL/6 congenic strain where female het Gabra1 KO mice have significantly less body mass compared to WT ($p = 0.0074$). No such difference was observed in het Gabra1 KO males of C57BL/6 or either males or females in DBA/2J. Although, the significance of the difference of body mass at P30 hom Gabra1 KO mice of either sexes in both C57BL/6 and DBA/2J congenic strains could not be calculated due to the highly mortality earlier than P30, the rate of gain in body mass was severely less compared to WT after P19.

Therefore it can be concluded that in the C57BL/6 congenic strain homozygous Gabra1 KO mice had significantly increased mortality compared to heterozygous mice which had increased mortality compared

to wild type mice. Among the DBA/2J congenic strain, homozygous Gabra1 KO mice had increased mortality compared to wild type and heterozygous mice.

Figure 2-5: Body mass of both C57BL/6 and DBA/2J mice is affected by Gabra1 KO mutation. Body mass of (A) female C57BL/6, (B) male C57BL/6 and (C) both male and female DBA/2J is shown as the mice age. (D) Body mass at P30 is shown for both congenic lines.



Reduced viability of hom Gabra1 KO mice is not a result of gross pathological abnormalities

We determined whether or not the reduced viability resulted from gross anatomical changes. We performed pathologic analysis on all major organs in collaboration with an expert veterinary pathologist, Kelli L. Boyd (Vanderbilt University). No gross morphologic changes in the brain and all of the organs were seen except for the thymus. A significant lymphoid depletion was observed in the thymus, which could be the response to stress the mutant mice had due to repeated and prolonged seizures.

Therefore any change in viability or epileptiform activity cannot be associated with any other gross pathological insults apart from the modulation of neurotransmitter physiology in the central nervous system.

Gabra1 KO mutation causes absence seizures

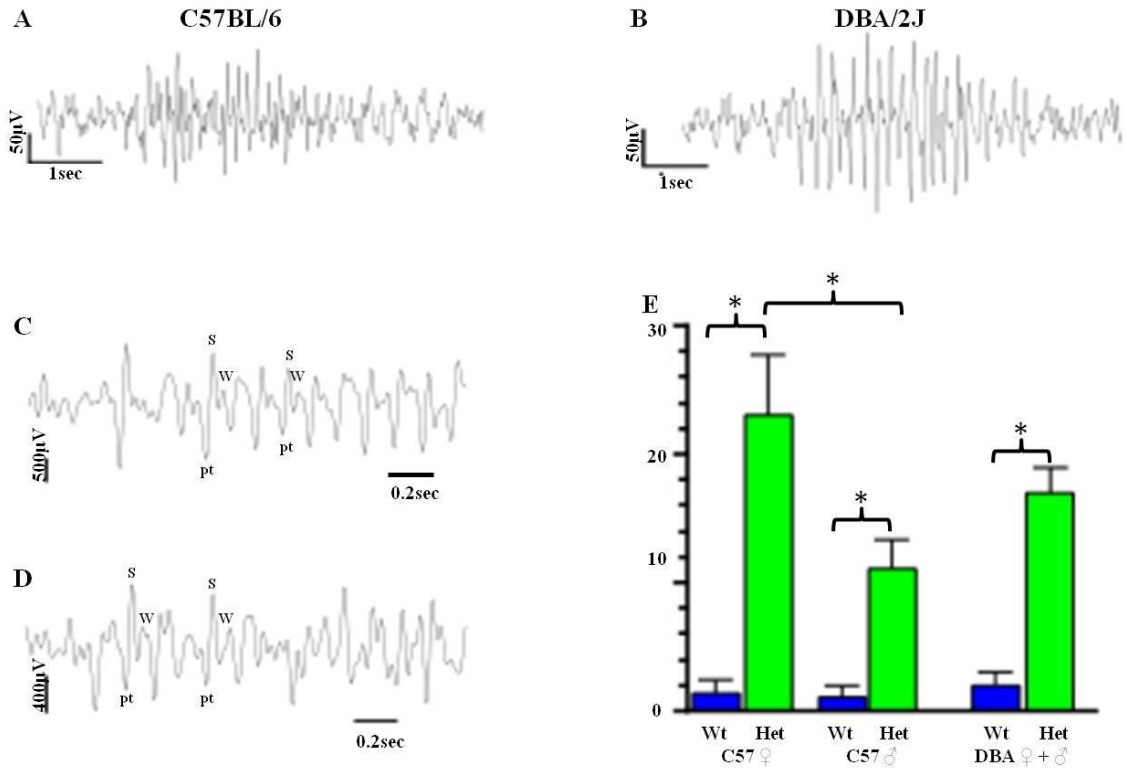
Previous studies have shown that loss of Gabra1 subunit in mice kept in a mixed genetic background does not result in any spontaneous visually apparent convulsive seizures[15, 19]. The possible reasons for this result could include: (A) het loss of Gabra1 subunit alone is not sufficient to cause seizures, (B) a murine model do not fully replicate the human phenotype, (C) modifier genes present in the mixed background alter the phenotype or (D) the mutant mice may be having nonconvulsive seizures that are not visually apparent. Therefore, we conducted synchronized video/EEG recording on congenic Gabra1 KO mice to determine if they exhibited any electrographic or behavioral seizures using 3 different computer programs: 1) Sirenia® Seizure, 2) EDFbrowser and 3) NicoletOne EEG Reader V5.71.4.2530.

Synchronized video/EEG shows Gabra1 KO mice have SWDs

We observed frequently occurring abnormal discharges in the EEG of the mutant mice (figure 2-6 A and B). Viewing the discharges on an expanded time scale showed that they consisted of a repetitive pattern consisting of spikes (s), positive transients (pt) and waves (w) (figure 2-6 C and D). This pattern

resembled the EEG pattern consisting of spike wave discharge (SWD), previously reported for a rat[20] and mouse model of absence epilepsy[21]. Therefore we decided to investigate further if the Gabra1 KO mice were having absence seizures.

Figure 2-6: Spontaneous abnormal EEG discharges were observed in Gabra1KO mice. Both (A) C57BL/6 and (B) DBA/2J Gabra1 KO mice have spontaneous SWDs. Expanded time scales show characteristic spikes (s), positive transients (pt) and waves (w) in both (C) C57BL/6 and (D) DBA/2J Gabra1 KO mice. The incidence of SWDs is shown for both congenic strains (E).



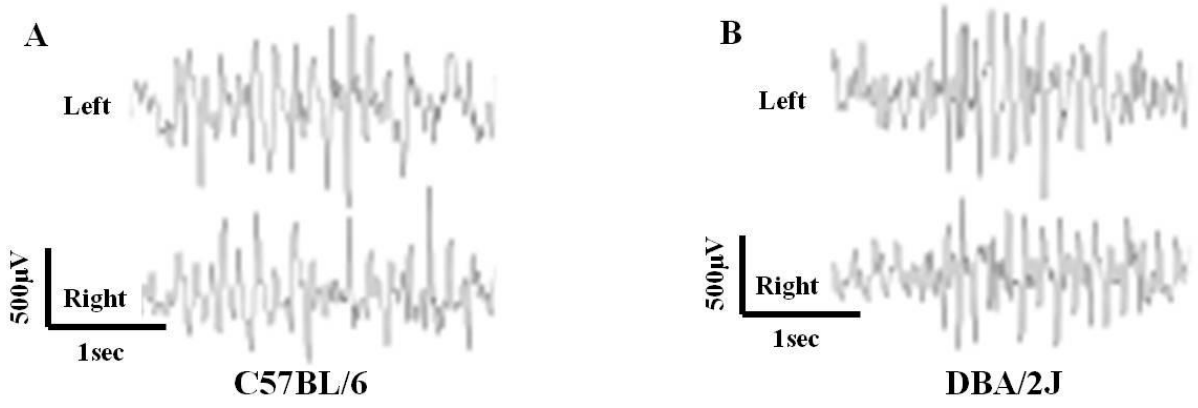
Quantification of SWDs showed that heterozygous loss of Gabra1 subunit resulted in an increase in the incidence of SWDs in both C57BL/6 (15 ± 3 SWD/hr, N = 21, p = 0.001) and DBA/2J mice (19 ± 2 SWD/hr; N = 19, p<0.001) (figure 2-6E). The infrequent SWDs observed in WT mice of both C57BL/6 and DBA/2J congenic strains were consistent with previous reports[22]. No sex dependent effect was observed in DBA/2J with both male and female het Gabra1 KO mice having similar incidence of SWD

(17 ± 3 SWD/hr). However, in the C57BL/6 strain, het female Gabra1 KO mice had a significantly higher incidence of SWD (23 ± 5 SWD/hr) than males (11 ± 2 SWD/hr; p = 0.024) (figure 2-6F). No statistical difference in the duration of SWD was observed within each congenic line (C57BL/6J 1.78 ± 0.12 sec; p = 0.06 and DBA/2J 2.47 ± 0.12 sec; *p = 0.123*).

Spike wave discharges in Gabra1 KO mice have bi-hemispheric origin

Absence seizures are a type of generalized seizure[23]. Therefore to further investigate if the SWDs observed in mutant mice were generalized in origin we devised an altered surgical approach that allowed us to acquire referential EEG recordings from both hemispheres independently. Recording from Gabra1 mice of both C57BL/6 and DBA/2J congenic line shows similar amplitude, frequency and duration of synchronous SWD in both EEG electrodes indicating that these SWDs are bihemispheric in origin (figure 2-7).

Figure 2-7: Bihemispheric EEG recording showing similar duration, amplitude and frequency in left and right hemispheres of het Gabra1 KO of C57BL/6J (A) and DBA/2J (B) congenic strains.

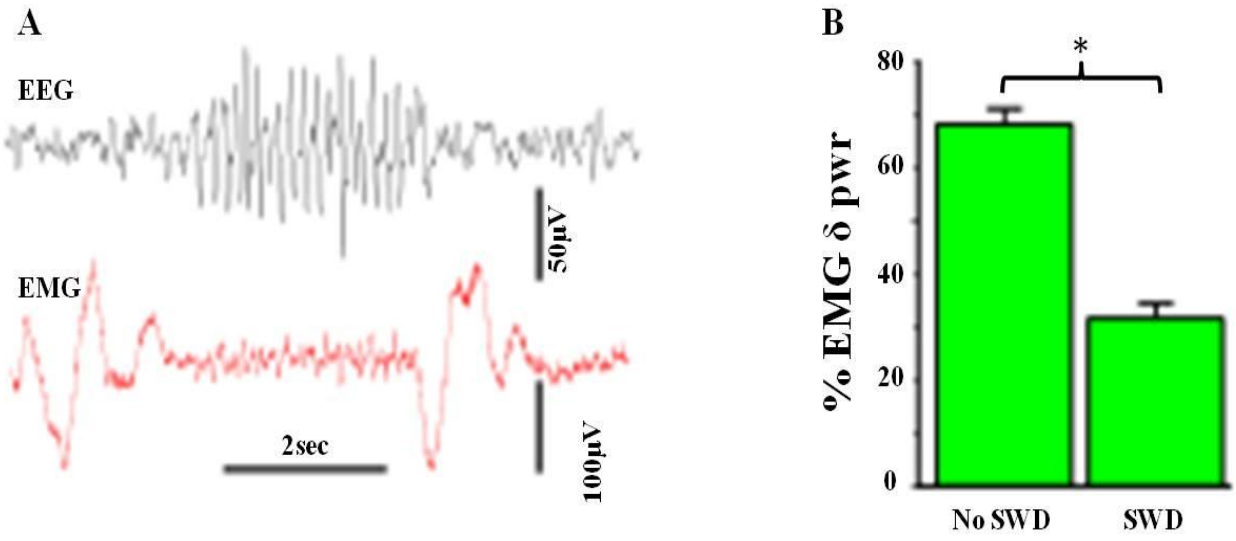


Spike wave discharges are associated with behavioral arrest

A typical absence seizure is a non-convulsive epileptic seizure, characterized by brief impairment of consciousness accompanied by a characteristic spike wave discharge[24]. For the duration of the seizure the patient remains unresponsive to any stimuli and, upon recovery, has no recollection of the event. As described in chapter 1, the behavioral arrest seen in absence seizure is so subtle that diagnosis is difficult with visual inspection alone; that is why it can remain undiagnosed for a long time even in human patients.

We therefore looked for evidence of similar behavioral arrest associated with spike wave discharges on EEG recordings. Visual inspection of the electrographic recording showed that SWDs were sometimes associated with behavioral arrest on EMG (figure 2-8A). Movement usually produces slow EMG delta waves. We quantified the effect of the SWDs on these slow, movement-associated EMG waves by measuring the EMG relative delta spectral power (1-4Hz) of 83 sequential SWD from 14 mice. We found a significant reduction of mean EMG amplitude during SWDs relative to times immediate before or after the SWDs (*p value* < *0.001*, figure 2-8B), indicating a behavioral arrest during SWDs.

Figure 2-8: Pattern of EMG discharge before, during and after the SWD on EEG indicates a behavioral arrest. (A) A sample of EMG and EEG discharges indicating behavioral arrest. (B) Mean normalized EMG spectral power (1-4 Hz).



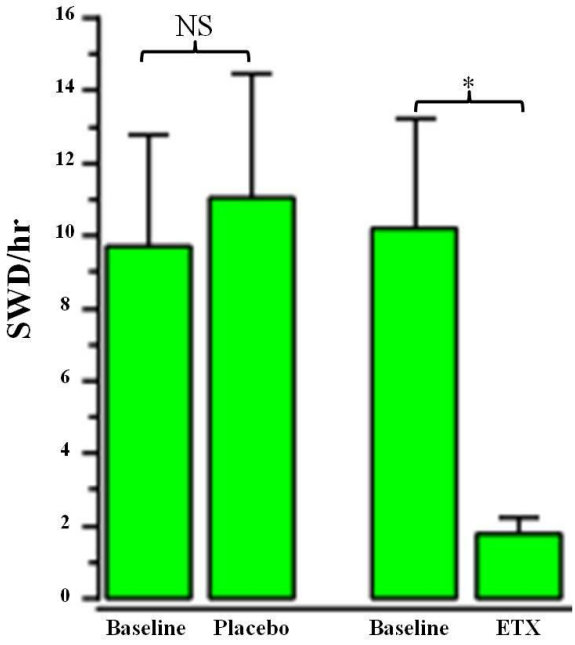
Anti-absence seizure drug reduces the incidence of spike wave discharges

Ethosuximide, valproic acid and lamotrigine are used to treat childhood absence epilepsy. Recently, a randomized, double-blinded study published in New England Journal of Medicine provided evidence demonstrating that ethosuximide and valproic acid are more effective than lamotrigine and that ethosuximide is better tolerated than valproic acid[25-28]. Therefore, ethosuximide is considered the first line treatment for absence epilepsy[25-28]. Ethosuximide has also been used successfully in reducing the incidence of SWDs in other mouse models of absence epilepsy[29-31].

Therefore, we decided to test the efficacy of acute ethosuximide treatment in reducing the incidence of SWDs. After recording a 2 hr baseline video/EEG, DBA/2J Gabra1 KO het mice were given either placebo (saline) or an equal volume of 200mg/kg of ethosuximide and monitored for another two hours to

determine the effect on SWD. We found that ethosuximide reduced the incidence of SWDs by 83% (*p value < 0.05*) (figure 2-9).

Figure 2-9: Ethosuximide (ETX), but not placebo, reduced SWD incidence by 83% compared to baseline



Discussion

Mutations in $GABA_A$ receptor genes, resulting in alteration of their expression and function, have been described as a possible mechanism for the development of epilepsy[32, 33]. Even though two mutations, S325fs328X and A322D, identified in the α1 subunit of $GABA_A$ receptor cause reduced expression and altered electrophysiological properties in *in-vitro* studies[34, 35], heterozygous and *even homozygous* Gabra1 KO mice were previously reported to lack visually apparent seizures[36]. Some of the possible reasons for these results could have included:

1) *α1 subunit haploinsufficiency alone does not cause epilepsy*. Epilepsy, especially CAE, clearly aggregates in families. However, the genetics is often complex. Even in CAE, the majority of epilepsy patients do not have any affected first degree relative[37]. Even in cases when a Mendelian epilepsy mutation is identified in families, the penetrance is rarely complete[37]. Moreover, a single epilepsy mutation can produce different epilepsy phenotypes in different individuals. For example, K289M mutation in the $GABA_A$ receptor γ2 subunit was identified in members of a large French family suffering with febrile seizures plus (GEFS+), but only 50% of mutation carriers also had tonic clonic seizures[38]. Therefore it is possible that the heterozygous loss of Gabra1 subunit alone is not sufficient to induce an epileptiform phenotype and modifier genes are required.

2) *Gabra1 KO mice do not fully replicate the human disease*. It is commonly believed that mice are a good model to mimic human diseases. However, some studies have shown the simple animal model systems are not sufficient to mimic complex human diseases[39, 40]. It is important to note that mice differ from humans in numerous ways including rate of metabolism, overall development and brain size etc. One possible reason why previous studies conducted on the Gabra1 KO mouse model did not reveal any spontaneous visually apparent seizures could be that mice are not a good model to study the role of α1 subunit of $GABA_A$ receptors.

3) *Modifier genes present in the mixed background alter the phenotype*. The importance of using congenic mouse strains has been emphasized because phenotypes caused by specific genetic modification can be strongly influenced by genes unlinked to the target locus[41]. It has been shown that the congenic strain diversity of mice also plays an important role in determining the seizure threshold[42]. C57BL/6 mice have been reported to be one the most seizure-resistant, while DBA/2J mice are considered to be the most seizure sensitive strains[17]. In the previously reported studies Gabra1 KO mice used were the progeny of strains intercrossed immediately after the Gabra1 KO mutant line was derived. The lack of a fully backcrossed congenic strain could be another reason why no epilepsy phenotype was observed.

4) *The Gabra1 KO mice have subtle nonconvulsive seizures that require video/EEG monitoring for diagnosis.* Absence seizures are brief nonconvulsive generalized seizures that have a sudden onset and termination and the patients suffering from them usually have transient impairment of consciousness and episodes of staring unresponsively[43, 44]. The diagnosis of an absence seizure in human patients requires an electroencephalogram (EEG) recording showing synchronous symmetrical spike-wave, approximately 3 Hz[45]. Even though EEG has a number of limitations, and a normal EEG does not rule out the possibility of epilepsy[46], it is not possible to rule out epilepsy without EEG testing. Although previous studies have reported a lack of visually apparent seizures, the conclusion that no seizures are occurring in Gabra1 KO mice cannot be drawn without EEG confirmation.

Gabra1 KO mice have absence epilepsy

Our data shows that Gabra1 KO mutation is a good model to study absence epilepsy. We showed that heterozygous loss of Gabra1 subunit results in SWDs in both C57BL/6 and DBA/2J congenic strain. These SWDs are bihemispheric in origin and consist of typical spike-wave patterns, previously seen in other models of absence epilepsy. Furthermore by quantifying the frequency of delta activity in EMG channels, associated with movement of mouse, we showed a significant decrease in the incidence of movement during SWDs. We have also recorded examples of visible movement arrest associated with SWDs using synchronized Video/EEG. Finally we showed that acute treatment with ethosuximide, a drug typically used for treatment of absence seizures in humans, resulted in significantly decreasing the frequency of SWDs, as compared to placebo (saline). These results present a convincing argument that heterozygous loss of Gabra1 is sufficient to cause absence seizures in a mouse model.

Because the absence seizure phenotype is so subtle, we would not have been able to draw this conclusion without using synchronized video/EEG and just studying the Gabra1 KO mice with visual inspection. Synchronized video/EEG not only allowed us to study the involuntary behavioral arrest during SWDs but also to show how similar our findings are to other known models of absence epilepsy.

Congenic background and sex can be significant variables in the expression of a phenotype

Knowing the importance of the influence of genetic modifiers[41] and how the congenic strain diversity of mice also play an important role in determining the seizure threshold[42], we decided to conduct our studies in fully backcrossed congenic mouse strains. We showed that SWDs occurred equally in het Gabra1 KO mice of both sexes in DBA/2J congenic strain, but in C57BL/6 the female het Gabra1 KO mice had significantly more SWDs compared to males. This sex dependent discrepancy is consistent with previous findings that showed that human absence epilepsy is more prevalent in females than males[47] and that the expression of $GABA_A$ receptor is sex dependant[48]. In fact women with preexisting epilepsy experience cyclical occurrence of seizure exacerbations during particular phases of menstrual cycle (commonly referred to as catamenial epilepsy)[49]. Seizure exacerbation in these women is believed to be due to the withdrawal of the progesterone-derived $GABA_A$ receptor modulating neurosteroid, allopregnanolone[49, 50]. Therefore, the increased incidence of SWDs in C57BL/6 female het Gabra1 KO mice makes them a possible model to study catamenial epilepsy.

Due to general causes like accidents, suicides, sudden unexpected death in epilepsy (SUDEP), mortality in epilepsy patients is 2-3 times higher than in age and sex matched general population[51, 52]. Epilepsy patients are also significantly more likely to have medical or psychiatric comorbidities along with associated negative impacts on quality of life[53]. Consistent with these facts, we showed that, contrary to previous reports, hom Gabra1 KO mice had significantly reduced viability in both C57BL/6 and DBA/2J congenic strains. Even though the Mendelian ratio of WT, het and hom Gabra1 KO mice after birth was not significantly different than expected, there was a significantly sharp decline in survival at approximately P19 in hom Gabra1 KO mice of both C57BL/6 and DBA/2J congenic strains. But interestingly, hom Gabra1 KO mice of C57BL/6 congenic strains had significantly less chances of survival than DBA/2J. The decrease in viability of the hom Gabra1 KO mice of C57BL/6 and DBA/2J congenic strain makes them a better model to study GGE, because even in human the risk of premature mortality in epilepsy patients is higher compared to general population[54], but no such decrease in viability

has been reported in other traditional models of absence epilepsy, for example GAERS and WAG/Rij rats[55, 56]. The gain in mass after birth was significantly lower in the hom Gabra1 KO mice of both C57BL/6 and DBA/2J congenic strains. Interestingly only female C57BL/6 het Gabra1 KO mice had significantly less body mass compared to WT at P30, while no such difference was reported in male C57BL/6 het Gabra1 KO and either sexes of DBA/2J.

In vitro studies have shown that activation of $GABA_A$ receptors induces a hyperpolarizing response that reduces cellular excitability, whereas loss of $GABA_A$ receptor function results in highly synchronized bursts of action potentials[57, 58]; a defining feature of seizures. In humans also it has been hypothesized that epilepsy may result from a decrease in GABA-mediated inhibition[58]. We have demonstrated that haploinsufficiency of Gabra1 subunit results in epilepsy phenotype. Our results can thereby guide future investigations to determine the precise mechanisms by which α1 subunit loss (in particular) and disinhibition (in general) produces generalized seizures.

Acknowledgement statement

We thank Dr. Kelli L. Boyd (Vanderbilt University) for her expertise with the pathology studies. We are also grateful to Dr. Jennifer Kearney, Dr. Gregg Stanwood and Ms. Nicole Hawkins (Vanderbilt University) for their helpful comments regarding this study. This research was supported by National Institute of Health Grants NS055979 and NS064286.

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Chapter III

Gabra1 KO and A322D KI mutations cause a persistent absence epilepsy and also an adult-onset myoclonic-like epilepsy

Introduction

Generalized genetic epilepsy (GGE) is typically inherited as a complex trait[1]. Within GGE, juvenile myoclonic epilepsy (JME) represents about 5-30% of all epilepsies[2-4]. Eighty percent of the reported cases of JME are sporadic and only a small proportion of patients have identified genetic mutations or predisposition factors[5]. Two chromosomal loci, on chromosome 6p and 15p, have been identified for JME[6].

Several genes including EFHC1[7, 8], calcium-channel β_4 subunit[9], BRD2 and TAP1[10, 11] have been associated with JME. However the first mutation shown to segregate with a Mendelian phenotype was a missense mutation, A322D, in the $GABA_A$ receptor α 1 (Gabra1) subunit gene[12]. This mutation was discovered in a large French-Canadian family suffering with an autosomal dominant form of JME[13]. It had 100% penetrance and was found in all of the individuals who presented with clinical and electroencephalogram (EEG) features found in classic cases of JME, and was not found in any of the unaffected family members[13]. *In vitro* studies showed that the Gabra1 subunit A322D mutation caused misfolding of the α1 (A322D) subunit, which caused its degradation and thereby resulted in a substantial reduction in its expression[14,15]. When overexpressed *in vitro*, the residual non-degraded A322D mutant Gabra1 subunit conferred a small but significant dominant negative effect that reduced the expression of the wild type Gabra1 subunit more than would be expected to result from haploinsufficiency alone[16].

JME presents with bilateral, single or repetitive, arrhythmic and irregular myoclonic jerks with retained consciousness[17]. Ninety percent of JME patients present with generalized tonic clonic seizures (GTCS) and 20-30% patients also have absence seizures[12, 18]. The onset of these symptoms is around puberty and the sex distribution is equal[2, 19]. The electroencephalogram (EEG) of these patients often show 4-6 Hz

irregular spike-waves and polyspike-waves[12, 20, 21]. The majority of JME patients report seizure onset (typically GTCS) between the ages of 12-18 years[22]. Approximately 15% of childhood absence epilepsy patients develop JME later in life[23, 24].

Few animal models have been described to model myoclonic epilepsy. In a BRD2 haploinsufficiency model, rare spontaneous seizures and interictal discharges have been reported[25]. *Efhc1*-deficient mice have been reported to have frequent spontaneous myoclonus and enhanced seizure susceptibility to chemoconvulsant stimulation[26]. Myoclonus in these *Efhc1*-deficient mice is characterized by brief electromyographic bursts, lasting ~ 200 ms, associated with visible brief jerks[26].

In our recent study, we showed that mice lacking or deficient in Gabra1 subunit have reduced viability and absence-like seizures[27]. Since Gabra1 A322D KI mutation also causes a reduction in the expression of Gabra1 subunits *in vitro*, we decided to investigate if this mutation also results in reduced viability and absence-like seizures *in vivo*. In addition, we set out to investigate if the Gabra1 A322D KI mutation also caused JME-like phenotype. Because *in vitro* experiments showed that in addition to causing loss of function, $GABA_A$ receptor α1 subunit mutation (A322D) caused a small, but significant, dominant negative effect on $GABA_A$ receptor expression[28], we hypothesized that the Gabra1 A322D KI mice would have a different phenotype than the Gabra1 KO mice. Therefore, we designed these experiments to directly compare the presence of JME-like phenotype in Gabra1 KO and A322D KI mice.

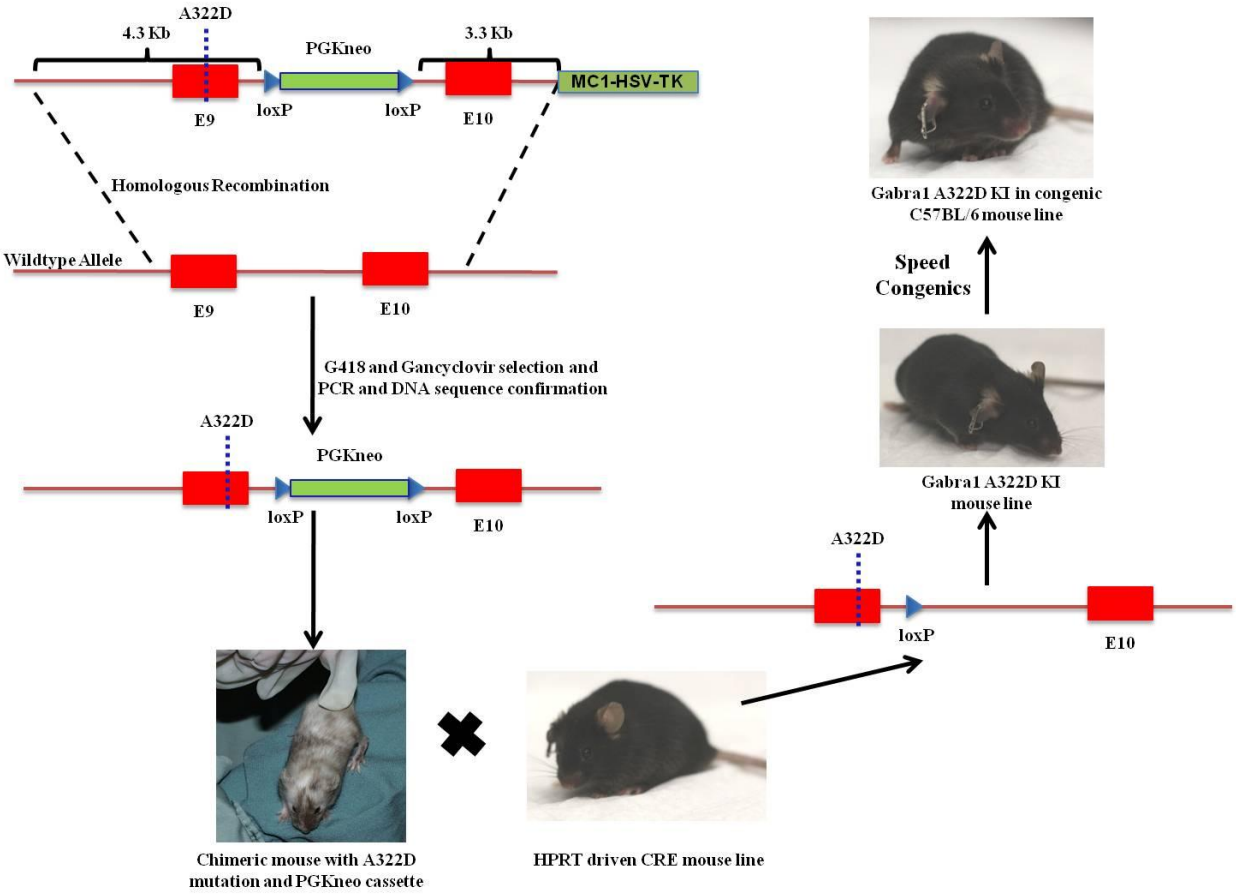
Methods and Materials

Generation and maintenance of Gabra1 A322D KI mice in congenic strains

We collaborated with the Gene Targeting and Transgenic Facility at The University of Connecticut Health Center to design the Gabra1 A322D KI mouse. Briefly, a bacterial artificial chromosome (BAC) construct of Gabra1 subunit exon 9 (containing the A322D missense mutation, created by the replacement of GCC codon with GAC), loxP flanked PGKneo, exon 10, and MC1-HSV-TK sequence was created. The BAC construct was transfected into embryonic stem cells (ESC). Correct homologous recombination

was confirmed using selection with G148 and Gancyclovir. Further verification was done using PCR and DNA sequencing. These targeted ESC were then injected into growing blastocysts and implanted into pseudo-pregnant females. The resulting offspring that showed chimerism were used to test for germ line transmission in future generations. These Gabra1 A322D KI mutant mice were then crossed with hypoxanthine guanine phospho rybosyle transferase (HPRT) (a housekeeping enzyme, responsible for recycling purines, expressed in every cell of the body[29]) driven CRE mouse line to remove the loxP flanked PGKneo cassette. The resulting A322D KI mouse line was verified using PCR and DNA sequencing. Using speed congenic services provided by DART mouse Gabra1 A322D KI mice were backcrossed into C57BL/6 congenic line at an accelerated pace. After 6 speed congenic guided backcrosses we were able to obtain a 99.98% congenic background and continue with our experiments. This entire process is summarized in figure 3-1.

Figure 3-1: A summary of generation and maintenance of Gabra1 A322D KI mouse



Gabra1 A322D KI mice genotyping

For genotyping of Gabra1 A322D KI mice, tail snips were collected between postnatal day (P) 17 and P21 or at the date of death. DNA was extracted and amplified using red Extract-N-AMP tissue PCR kit (Sigma). Forward and reverse primers used to identify the wild type (WT) and mutant alleles were CGTGAGCCACACAGATAACC and ACCCTTTGATGGGTTACAGC, respectively. The presence of Lox P site, in the amplified DNA segment of Gabra1 A322D KI mice, resulted in a longer DNA segment as compared to the WT mice, which lacked the Lox P site (figure 3-2).

The PCR conditions are as follows:

Initial denaturation 95°C for 5 minutes

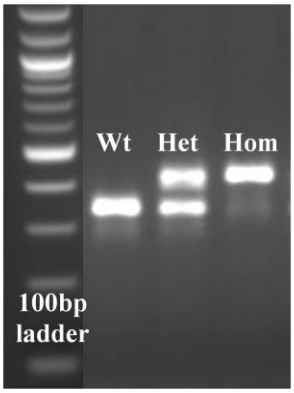
PCR cycle denaturation 95°C for 30 seconds

PCR cycle annealing 59°C for 30 seconds

PCR cycle extending 72°C for 90 seconds

Final extension 72°C for 10 minutes

Figure 3-2: Genotyping for Gabra1 A322D KI mice. The presence of Lox P site in mutant mice DNA increased size of DNA segment being amplified.

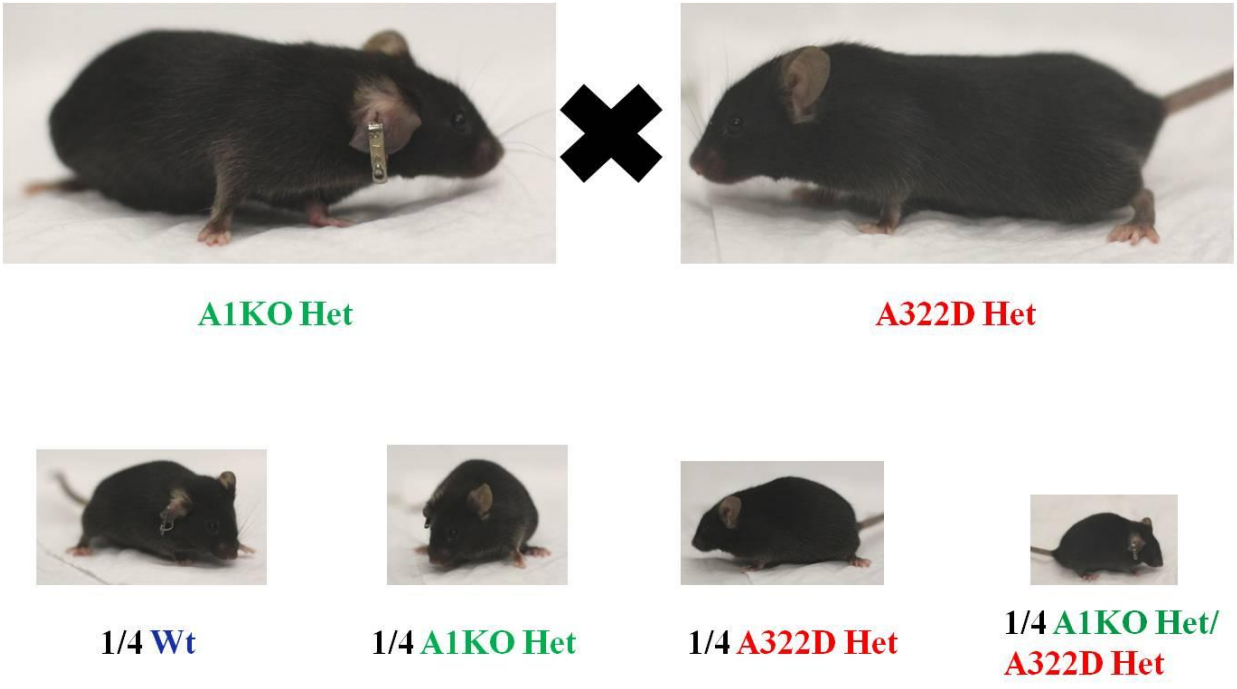


Mouse colony maintenance and mating strategy for experimental mice

All procedures were performed in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Mice were housed in a temperature and humidity controlled environment, with a 12 hour light/dark schedule. Water and food was provided ad libitum.

One goal of our study was to determine whether or not the phenotype of the Gabra1 A322D KI mice differed from the Gabra1 KO mice (generation of Gabra1 KO mice has been described in chapter 2). Because the phenotype may be related to parents as well as the other pups in the litter and thus may confound our interpretation, we wanted to breed WT, Gabra1 KO and A322D KI pups in the same litter. Therefore, we mated Gabra1 KO mice with Gabra1 A322D KI mice to produce WT, heterozygous α1 knockout, heterozygous A322D KI and α1 knockout/A322D KI double mutant mice in equal Mendelian proportions (figure 3-3). Mice generated by this mating strategy were also used for western blot and immunohistochemistry chemistry experiments (described in chapter 4).

Figure 3-3: Mating strategy to obtain WT, Gabra1 KO and A322D KI mice from same litter.



Analyzing viability of Gabra1 KO and A322D KI mice

The number of pups produced in each litter was documented along with their sex, genotype, date of birth and age at death. We measured the body mass of mice every one to three days from P7 to P30, after which the animals were used for different experiments including EEG recording and western blots.

Surgical implantation of prefabricated skull head mounts and video/EEG monitoring

The procedure for the implantation of prefabricated skull head mounts and video/EEG monitoring has been explained in chapter 2. The recorded video/EEGs were analyzed by a blinded reviewer to identify spike-wave discharges (SWDs) using criteria established for the analysis of rat models of absence epilepsy[30]. Other forms of EEG abnormalities, including polyspike discharges (PSDs), were also noted and quantified. The characteristics of these PSDs will be described later in the results section of this chapter.

Determining susceptibility to pentylenetetrazole (PTZ) induced seizures

Prefabricated mouse headmounts (Pinnacle Technologies) were surgically implanted in two groups of animals, aged postnatal day (P) 33-37 (referred to as P30s from here on) and P120-130 (referred to as P120s from here on) as described in chapter 2. After a 48 hour post-op recovery, mice were injected with repeated low doses of PTZ and studied for epilepsy-like behavior using synchronized video/EEG. Although PTZ is typically used to evoke GTCS, Wong et.al. described a modified protocol with repeated low doses of PTZ that produced myoclonic jerks[31]. Briefly, mice were first injected with 25 mg/kg PTZ intraperitoneally and observed for 45 minutes followed by four repetitive doses of 10 mg/kg PTZ 10 minutes apart. If a tonic clonic seizure occurred during the course of the experiment, further doses of PTZ were not administered to prevent further tonic clonic seizures or status epilepticus and consequent death.

Statistical Analyses

Statistical analyses were performed using the R 2.12.2 Statistical Package for Windows (R Foundation for Statistical Computing, Vienna, Austria). We first determined the effects of Gabra1 KO and A322D KI mutations separately in male and female mice; if there no sex-dependent effect was noted we grouped data from the male and female mice. Parametric data (body mass, seizure incidence) are presented as the mean ± standard error of the mean (SEM) and compared using the Student's two-tailed t-test or analysis of variance (ANOVA) with Tukey posttest, as appropriate. The two factor ANOVA test was used to determine the effects and interaction of the age (P30s vs P120s) and genotype factors on the phenotype. Post-hoc pairwise comparison of means was conducted to determine the significance of the effect of each genotype. A multi-factorial test of categorical variables was used to analyze the effect of PTZ on GTCS seizure threshold. Nonparametric data (for e.g. Mendelian ratios, mortality) were analyzed by chi-square.

Results

Heterozygous Gabra1 KO and A322D KI mutations do not decrease viability

As explained in chapter II and reported in our recently published study, we have shown that in C57BL/6 congenic background homozygous loss of Gabra1 subunit causes a significant decrease in viability[32]. Here we directly compared the effects of Gabra1 KO and A322D KI mutations on viability. To better compare the characteristics of WT, Gabra1 KO and A332D KI mutations in mice of the same litter we used the mating strategy (explained in the methods sections and figure 3-3) of crossing het Gabra1 KO with het Gabra1 A322D KI mice. This mating strategy also produced Gabra1 KO/ A322D KI double mutant, which has a sudden decrease in viability past P19 (figure 3-4). Given the results for C57BL/6 homozygous Gabra1 KO mice[32], this high mortality of the Gabra1 KO/ A322D KI double mutant was expected. Since the findings from Gabra1 KO/ A322D KI double mutant mice were not the subject of our interest, they were not pursued and will not be discussed further in this thesis.

Mendelian ratio shows a lack of prenatal mortality

We obtained 115 offspring from het Gabra1 KO and A322D KI mice cross. In the absence of any significant biased prenatal mortality, the ratio of the genotype among these offspring is expected to be approximately 25% WT, 25% het Gabra1 KO, 25% Gabra1 A322D KI and 25% Gabra1 KO/A322D KI double mutant. No significant deviation from the expected Mendelian ratio was observed (results summarized in table 3-1), indicating a lack of significant prenatal mortality.

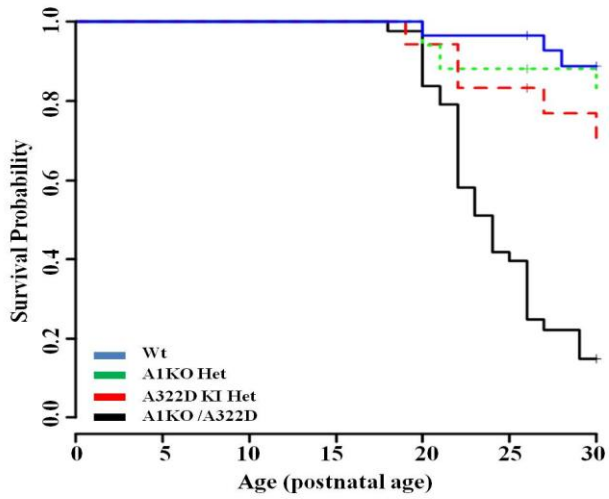
Table 3-1: Mendelian ratios of F1 progeny of heterozygous Gabra1 KO and A322D KI mice. Chi-square test showed no significant difference between the actual and expected Mendelian ratio.

Mendelian Ratio				
WT	Gabra1 KO	Gabra1 A322D KI	Gabra1 KO/A322D KI	*p value*
27%	16%	17%	39%	*0.07*

Neither Gabra1 KO nor A322D KI mutations alter the survival probability of mice.

Offspring of het Gabra1 KO and A322D KI cross were followed until P30 for mortality and gain in mass. Kaplan Meier curve (figure 3-4) showed no significant change in viability in either WT, het Gabra1 KO or A322D KI mice, although a robust decrease in survival probability was observed in Gabra1 KO/A322D KI double mutant past P19, which was similar to that seen in hom Gabra1 KO mice[27] (figure 2-4, chapter 2).

Figure 3-4: Neither Gabra1 KO nor A322D KI mutations significantly alter survival probability. Kaplan-Meier curve showed the survival probability as the animals aged. 29 WT, 17 Gabra1 KO, 18 A322D KI and 43 Gabra1 KO/A322D KI double mutant mice were monitored for survival from birth until P30.



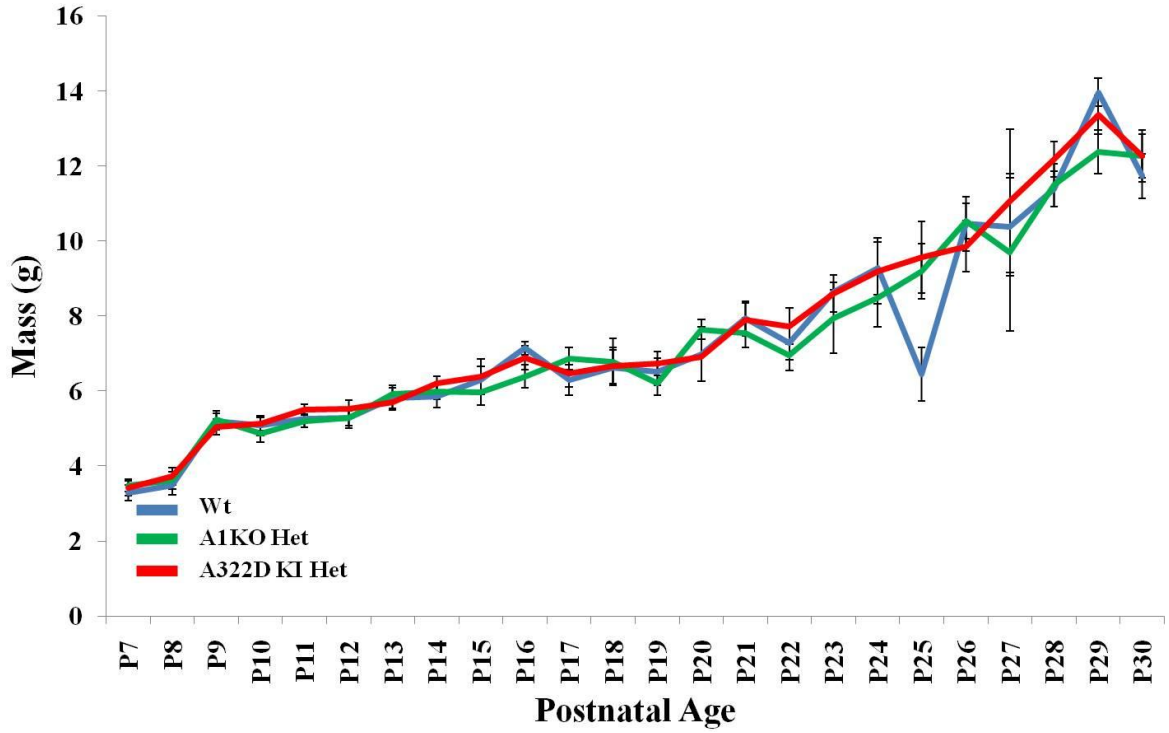
Neither Gabra1 KO nor A322D KI mutations alter the body mass of mice

We measured the body mass of these mice every 1 to 3 days from P7 to P30 to determine the effect of the Gabra1 KO and A322D KI mutations on daily gain in body mass (figure 3-5). In both male and female mice, neither the Gabra1 KO nor A322D KI mutations changed the body mass during development. At P30s, there was no significant difference in body mass of WT, Gabra1 KO or A322D KI mice (*p value = 0.49*). Therefore, it can be concluded that heterozygous loss of Gabra1 subunit or A322D KI mutation does not cause a significant change in viability.

In chapter 2, we described that the C57BL/6 female heterozygous Gabra1 KO mice had significantly reduced body mass compared to males and no such sex dependent effect was observed in the DBA/2J mice. However with the breeding strategy used for these experiment (figure 3-3), no sex dependent effect

was observed between male and female Gabra1 KO (*p value = 0.898*) or A322D KI (*p value = 0.223*) mice. This suggests that the composition of genotypes within the litter modifies the gain in body mass of WT and/or mutant mice.

Figure 3-5: Neither Gabra1 KO nor A322D KI mutations significantly alter body mass. Body mass of 29 WT, 17 Gabra1 KO and 18 A322D KI mice recorded every one to three days from P7 until P30 or death, is shown. At P30s, the average body masses were WT (11.7 ± 0.6g), Gabra1 KO (11.8 ± 0.7g) and A322D KI (12.8 ± 0.5g). *p value = 0.49*



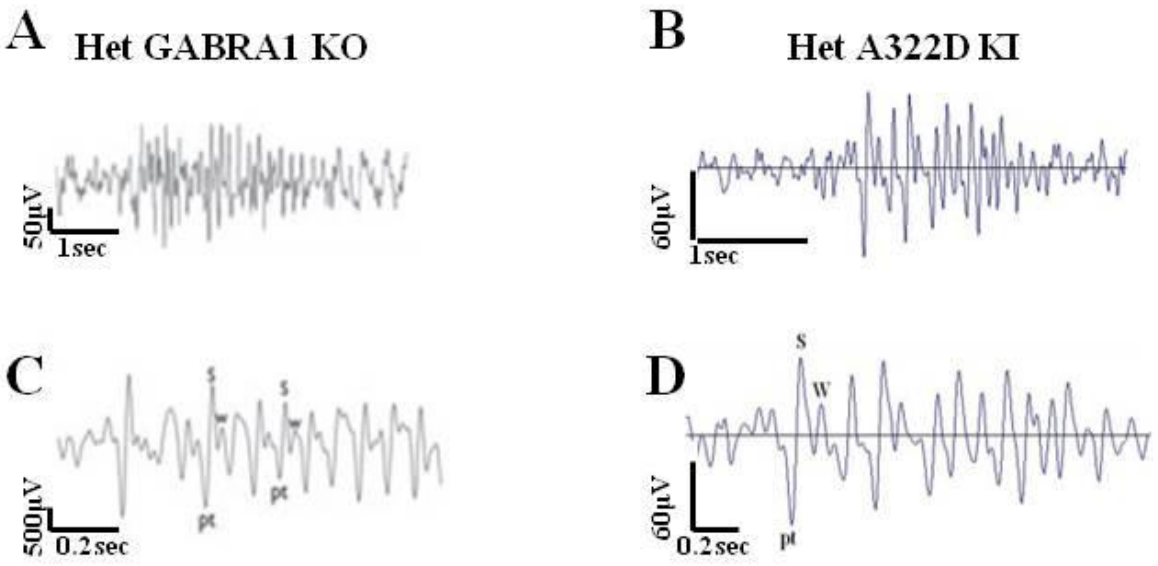
Gabra1 KO and A322D KI mutations cause absence seizures

In our recently published study, and in our findings explained in chapter II, we demonstrated that het Gabra1 KO mice have absence seizures at P30s[27]. *In vitro* studies have shown that the A322D mutation in the Gabra1 receptor causes a significant decrease in total and surface expression of α1 subunits, and that the Gabra1 A322D KI mutation causes a small but significant dominant negative effect[33]. Therefore we hypothesized that het Gabra1 A322D KI mice have a different seizure phenotype than het Gabra1 KO mice.

Synchronized video/EEG shows both Gabra1 KO and A322D KI mice have SWDs at P30s

We conducted synchronized video/ EEG recording on WT, Gabra1 KO and A322D KI mice and analyzed the recording using computer programs: Sirenia® Seizure and EDFbrowser where appropriate. We observed frequently occurring abnormal discharges in EEG of the Gabra1 A322D KI mice that were very similar to the SWDs seen Gabra1 KO mice (figure 3-6 A and B). Analysis of waveforms on an expanded time scale showed that these discharges consist of a repetitive pattern of spikes (s), positive transients (pt) and waves (w) (figure 3-6 C and D). Synchronized video/EEG showed behavioral arrest associated with these SWDs in both Gabra1 KO and A322D KI mice. These findings are consistent with our previously published report on Gabra1 KO mice[27] and other rodent models of absence epilepsy[34, 35].

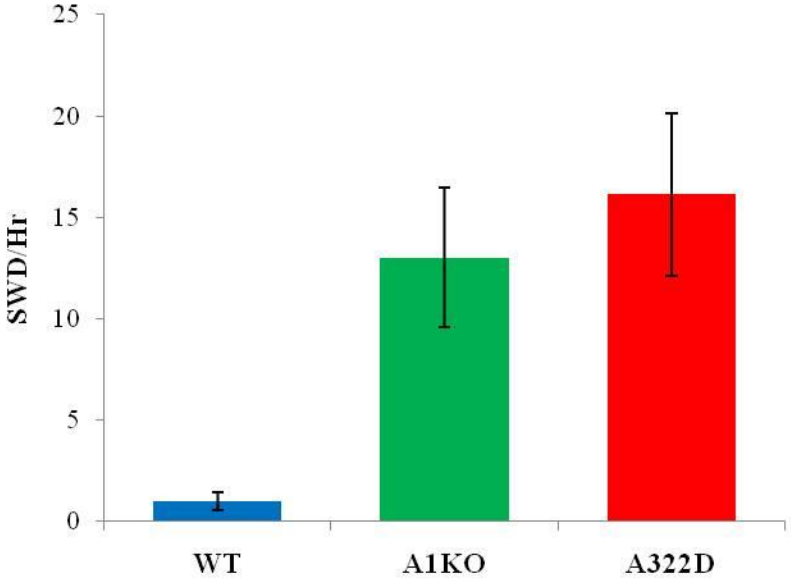
Figure 3-6: Both het Gabra1 KO and A322D KI mice have spontaneous SWDs. Examples of typical discharges associated with absence seizure seen in (A) Gabra1 KO and (B) A322D KI P30s mice that are consistent with previous reports. Expanded time scale shows distinct spike and wave pattern in both (C) Gabra1 KO and (D) A322D KI mice.



Both Gabra1 KO and A322D KI mice have frequent SWDs at P30s

We quantified the frequency of SWDs in WT, Gabra1 KO and A322D KI mice at both P30s as described in the methodology section. ANOVA testing showed a statistical difference in the incidence of SWDs in Gabra1 KO, A322D KI and WT mice. This result is consistent with our previous report, where we showed that the incidence of SWDs was significantly greater in Gabra1 KO mice compared to WT[27].

Figure 3-7: Both het Gabra1 KO and A322D KI mice have significantly more SWDs than WT. The incidence of SWDs in 11 WT (0.99 ± 0.4), 6 Gabra1 KO (13 ± 3.4) and 13 A322D KI (16.1 ± 4) mice is shown. ANOVA testing was significant (*p value = 0.004*).



Incidence of SWDs does not change in Gabra1 KO and A322D KI mice with age

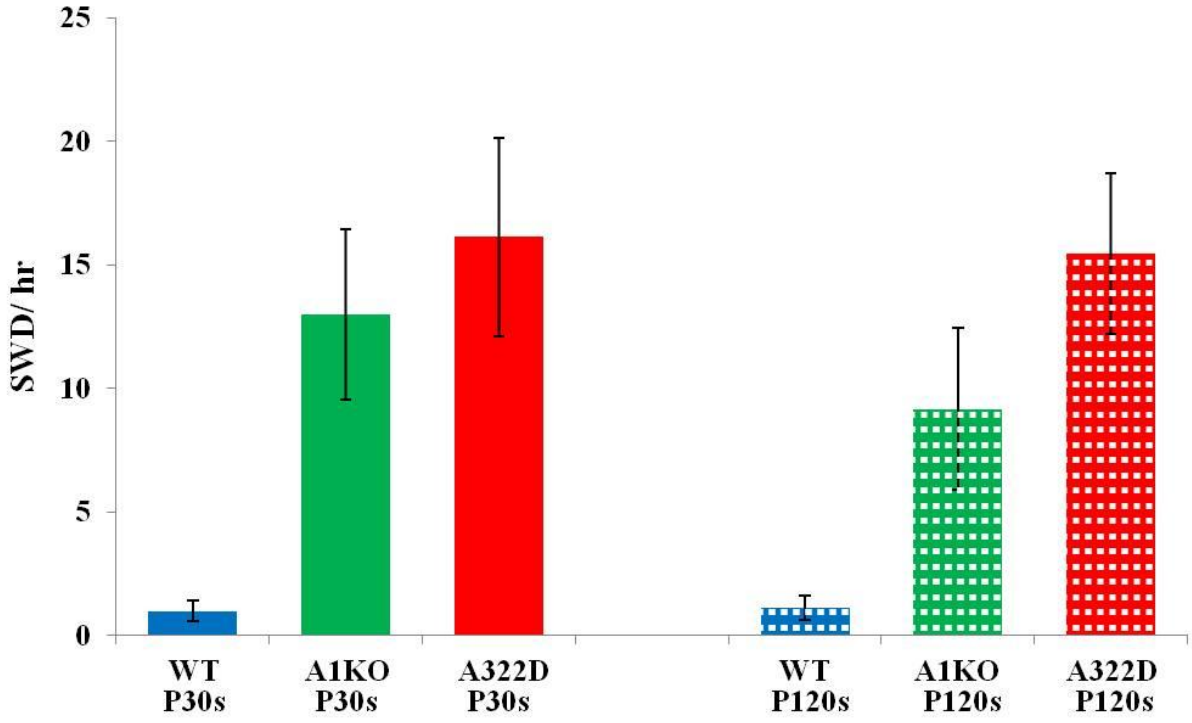
Studies have shown that childhood absence epilepsy (CAE) usually occurs before puberty and then the seizures remit or convert to more resistant forms of epilepsy[36]. The reported rate of remission in CAE patients ranges from 56-84%[24, 37-40], although 12-32% of CAE patients continue to have absence seizures into adolescence and adulthood[41]. Similarly in some of the well established rodent models of absence epilepsy (e.g. WAG/Rij and GAERS rat strains), absence seizures have been reported to persist into adulthood[42]. We therefore decided to investigate if the frequency of SWDs in Gabra1 KO and A322D KI mice changes with age.

We recorded synchronized video/EEG from WT, Gabra1 KO and A322D KI mice at P30s and again at P120s and compared the incidence of SWDs. Multi factor ANOVA test revealed a significant effect of

genotype (*p value < 0.001*), but not of age (*p value = 0.727*), on the incidence of SWDs. Furthermore, no significant interaction of genotype and age was observed (*p value = 0.797*). We then applied a post-hoc pair wise comparison of means to determine the significance of each genotype on the incidence of SWDs. Both Gabra1 KO and A322D KI mice differed significantly from WT (*p value < 0.005*), although there was no significant difference between Gabra1 KO and A322D KI mice (*p value = 0.090*).

At both P30s and P120s, the frequency of SWDs remained high in Gabra1 KO and A322D KI mice. Analysis using two-way ANOVA showed a strong effect of genotype on SWD incidence, but no effect of age or interaction of age and genotype was observed (figure 3-8). Post-hoc testing indicated that there was no difference in the SWD incidence between the Gabra1 KO and A322D KI mutant mice.

Figure 3-8: Genotype, but not age, affected SWD incidence. The incidence of SWDs in 11 WT (0.99 ± 0.4 SWDs/Hr), 6 Gabra1 KO (13 ± 3.4 SWDs/Hr) and 13 A322D KI (16.1 ± 4 SWDs/Hr) P30s mice and 9 WT (1.1 ± 0.5 SWDs/Hr), 12 Gabra1 KO (9.2 ± 3.3 SWDs/Hr) and 11 A322D KI (15.5 ± 3.2 SWDs/Hr) P120s mice is shown. Two-way ANOVA test was significant for genotype (*p value < 0.001*), but not for age (*p value < 0.727*). Post-hoc testing showed that there was no significant difference between Gabra1 KO and A322D KI mice (*p value = 0.090*)



Gabra1 KO and A322D KI mice only had rare spontaneous GTCS

The majority of JME patients have generalized tonic clonic seizures (GTCS)[12, 18]. Therefore, we determined if either the Gabra1 KO or A322D KI mutations conferred GTCS to P30s or P120s mice. We found that 1 Gabra1 KO and 1 Gabra1 A322D P120s mouse experienced a documented spontaneous GTCS during the 24 hour video/ EEG monitoring. No WT P120s mice or any of P30s mice experienced any documented GTCS during the 24hr hours video/EEG monitoring. These data suggest that if the Gabra1 KO and A322D KI mutations confer GTCSs, they are infrequent.

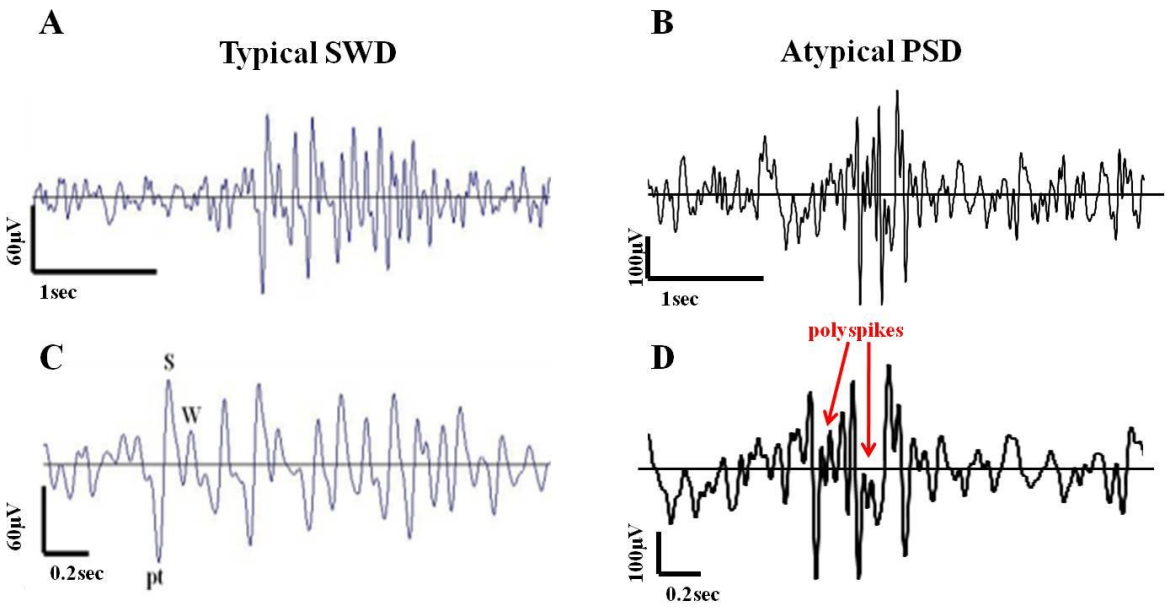
Gabra1 KO and A322D KI mice demonstrate atypical spontaneous polyspike discharges and myoclonic-like jerks later in development

It has been reported that 15% of CAE patients go on to develop JME later in life and 7% of these patients had a combination of myoclonic jerks and absence seizures[24]. Since patients with myoclonic jerks often have a 4-6 Hz irregular spike-wave and polyspike-wave pattern on EEG[12, 20, 21], we hypothesized that another epileptiform phenotype also existed in Gabra1 KO and A322D KI mice.

Examination of the synchronized video/EEG revealed rare but distinct brief poly-spike discharges (PSDs) in mutant mice, apart from SWDs. An example of these PSDs compared to a typical SWD is shown in figure 3-9. These events typically consist of poly-spike complexes that lasted less than half a second (average duration 0.35 ± 0.02 seconds) and have a high spike frequency (average spike frequency 18.5 ± 0.27 Hz). Due to the mouse movement and position of the recording camera, only a small number of these PSDs could be examined for any associated behavioral effect. Subtle, but visible, myoclonic jerks were observed in 15% and 10% of all the observed PSDs in Gabra1 KO and A322D KI mice, respectively. Sampling the video at times when PSDs were not present revealed that these behavioral jerks were not present in the absence of the electrographic PSDs. In order to further analyze the PSDs we decided on a

strict criteria of defining a PSD as a short event (<0.5 second) consisting of at least 2 positive and 2 negative spikes along with polyspikes.

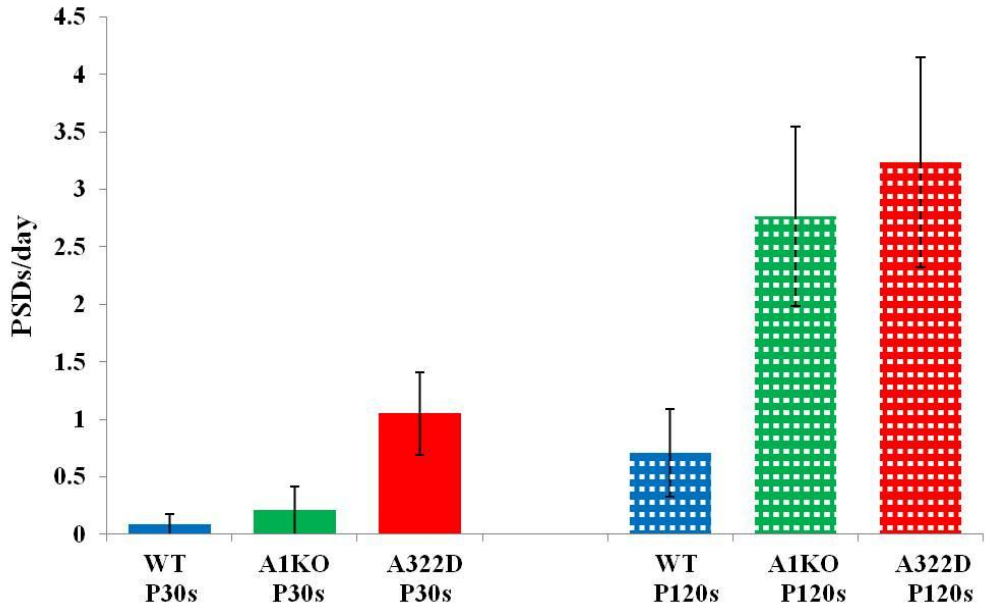
Figure 3-9: Examples of SWD and PSD. Example of a typical SWD discharge (A) compared to a PSD (B), seen in the same Gabra1 A322D KI mouse. Expanded time scale shows how the spike and wave pattern of a typical SWD (C) differs from the polyspike complex of a PSD (D).



The incidence of PSDs is significantly greater in Gabra1 KO and A322D KI mice, compared to WT, and more frequent at P120s than P30s

In order to determine the significance of age and genotype on the incidence of PSDs, we first performed a multi factor ANOVA test. Both genotype (*p value = 0.018*) and age (*p value < 0.001*) had a significant effect on the incidence of PSDs, but significant interaction of genotype with age was noted (*p value = 0.259*). Next, to compare the significance of each genotype on PSD incidence, we did a post-hoc pair wise comparison of means. Both Gabra1 KO and A322D KI mice differed significantly from WT mice (*p value <0.013*), but no significant difference was observed between Gabra1 KO and A322D KI mice (*p value = 0.860*).

Figure 3-10: The incidence of PSDs is significantly greater in mutant mice and more frequent at P120s than P30s. The incidence of PSDs/day in 13 WT (0.09 ± 0.09 PSDs/day), 5 Gabra1 KO (0.21 ± 0.21 PSDs/day) and 16 A322D KI (1.05 ± 0.36 PSDs/day) P30s mice compared to 9 WT (0.71 ± 0.38 PSDs/day), 13 Gabra1 KO (2.76 ± 0.78 PSDs/day) and 11 A322D KI (3.23 ± 0.91 PSDs/day) P120s mice is shown. Two-way ANOVA test was significant for both age (*p value < 0.001*) and genotype (*p value < 0.018*). Post-hoc test did not show a significant difference between Gabra1 KO and A322D KI mice at either age (*p value = 0.860*).

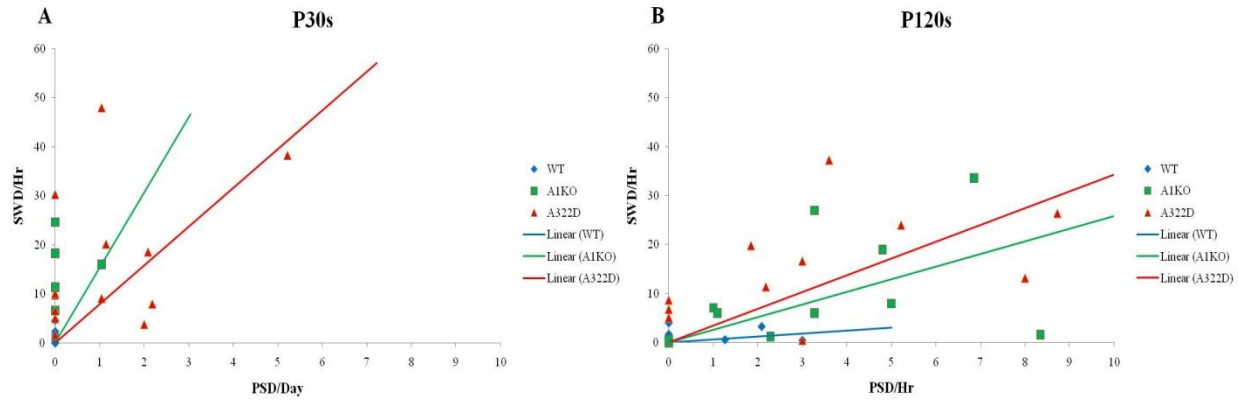


Incidence of SWDs does not correlate with PSDs

Previous studies have reported that 15% of CAE patients develop JME at a later age, while 7% of CAE patients go on to have a combination of myoclonic jerks and absence seizures[24]. Therefore we decided to investigate if the animals that were having more frequent SWDs also had more PSDs. We analyzed the EEGs recorded from both Gabra1 KO and A322D KI mice at P30s and P120s (the age at which PSDs

were significantly more common, along with SWDs). We saw no correlation between the incidence of SWDs and PSDs in either Gabra1 KO and A322D KI mutant or WT mice at P30s or P120s (figure 3-11)

Figure 3-11: The frequency of SWD does not correlate with the frequency of PSD at P30s or P120s. Scatter plot of PSD/ day vs SWD/ hr from (A) P30s (WT P30s $r^2 = 0.00$, Gabra1 KO P30s $r^2 = 0.00$ and Gabra1 A322D P30s $r^2 = 0.18$) and (B) P120s (Wt P120s $r^2 = 0.02$, Gabra1 KO P120s $r^2 = 0.24$ and A322D KI P120s $r^2 = 0.24$) is shown.



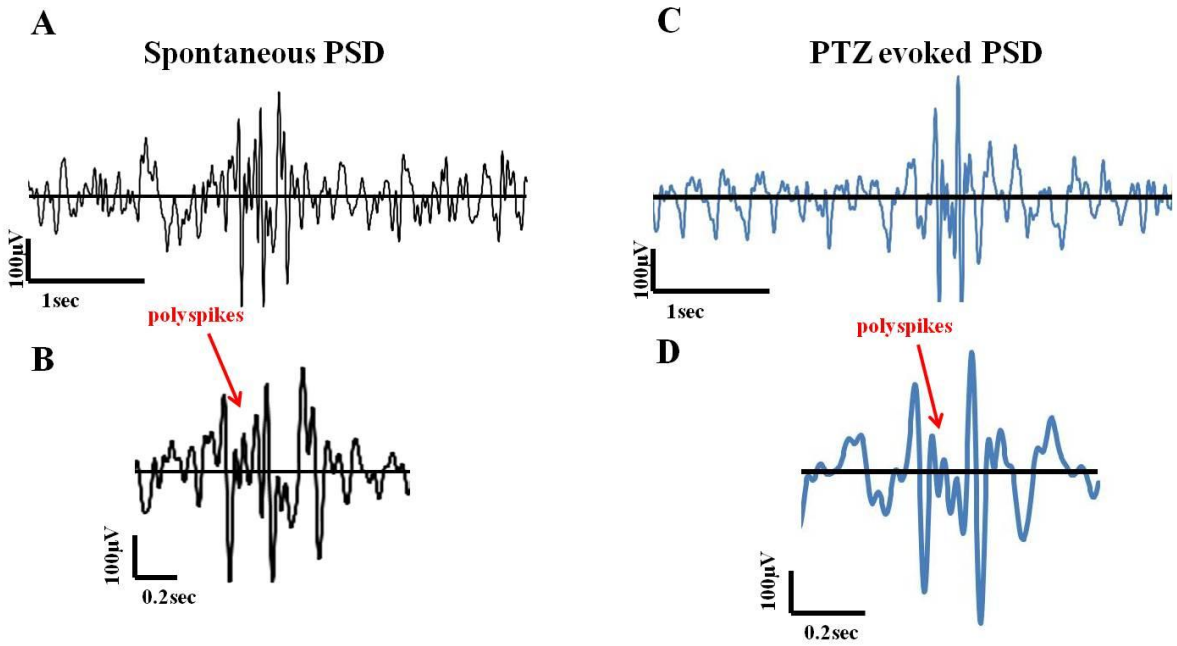
Gabra1 KO and A322D KI mice have a decreased latency of evoked PSDs and myoclonic seizures at P120s than WT mice

Our results showed that P120s Gabra1 KO and A322D KI mice had significantly greater frequency of spontaneous PSDs. Next, we set out to determine if genotype or age affects chemically evoked seizures. Pentylenetetrazole (PTZ), a drug that acts primarily by antagonizing GABAergic inhibition[43], has been shown to induce myoclonic jerks and tonic clonic seizures[44, 45]. PTZ is widely used experimentally to study seizure phenomena and to identify pharmaceuticals that may alter seizure susceptibility[46, 47]. Therefore we decided to continue our studies further using a previously developed low dose repeated administration of pentylenetetrazole (PTZ) protocol[31] to increase the incidence of PSDs.

Wong et.al. described a novel technique that produces myoclonic jerks along with GTCS, by administering repeated low doses of PTZ [31] (explained in the methods section). Following that protocol, we injected 25 mg/kg of PTZ intra-peritoneally (IP) and observed the mice for 45 minutes. Next we administered repeated doses of 10 mg/kg PTZ 10 minutes apart four times or until the mice developed GTCS. We determined the probability and latency to develop PSDs, myoclonic jerks and GTCS.

Upon administering PTZ, we observed PSDs that were similar to the spontaneous PSDs, observed previously, in duration and morphology (figure 3-12). Furthermore the subtle visible myoclonic jerks associated with the PTZ-induced PSDs were very similar to the myoclonic jerks seen with spontaneous PSDs. It was also noted that not all PTZ-induced PSDs were associated with a visible myoclonic jerk; another feature similar to the spontaneous PSDs, which were also not always associated with visible myoclonic jerks.

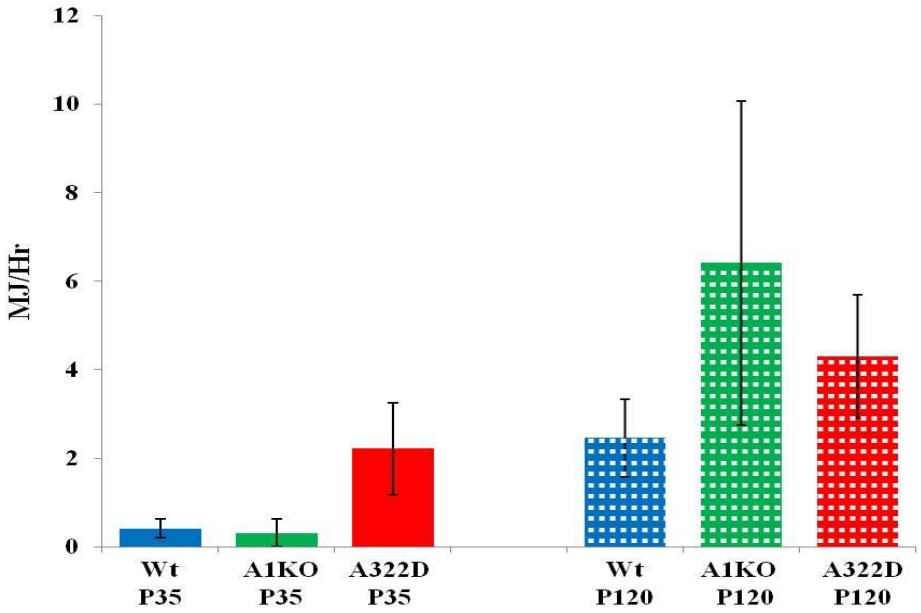
Figure 3-12: Spontaneous and PTZ-evoked PSDs have similar morphology. Example of (A) spontaneous PSD compared to (B) PTZ-evoked PSD, seen in Gabra1 A322D KI mice. Expanded time scale shows the similarity of the polyspike pattern of (C) spontaneous and (D) PTZ-evoked PSD.



The incidence of PTZ-evoked PSDs is greater at P120s than P30s for all genotypes

Next we determined the incidence of PSDs in WT, Gabra1 KO and A322D KI mice at P30s and P120s evoked with PTZ administration. Although there was no significant effect of genotype, there was an effect of age. At P30s the incidence of PSDs in WT, Gabra1 KO and A322D KI mice was low but at P120s the incidence of PSDs in WT, Gabra1 KO and A322D KI mice was higher, as shown by two-way ANOVA (*p value = 0.006*) (figure 3-13). Therefore, pharmacologically-evoked PSDs, like the spontaneous PSDs, had a substantially greater incidence in P120s than P30s mice, a result that suggests a developmentally-dependent change in neuronal circuitry.

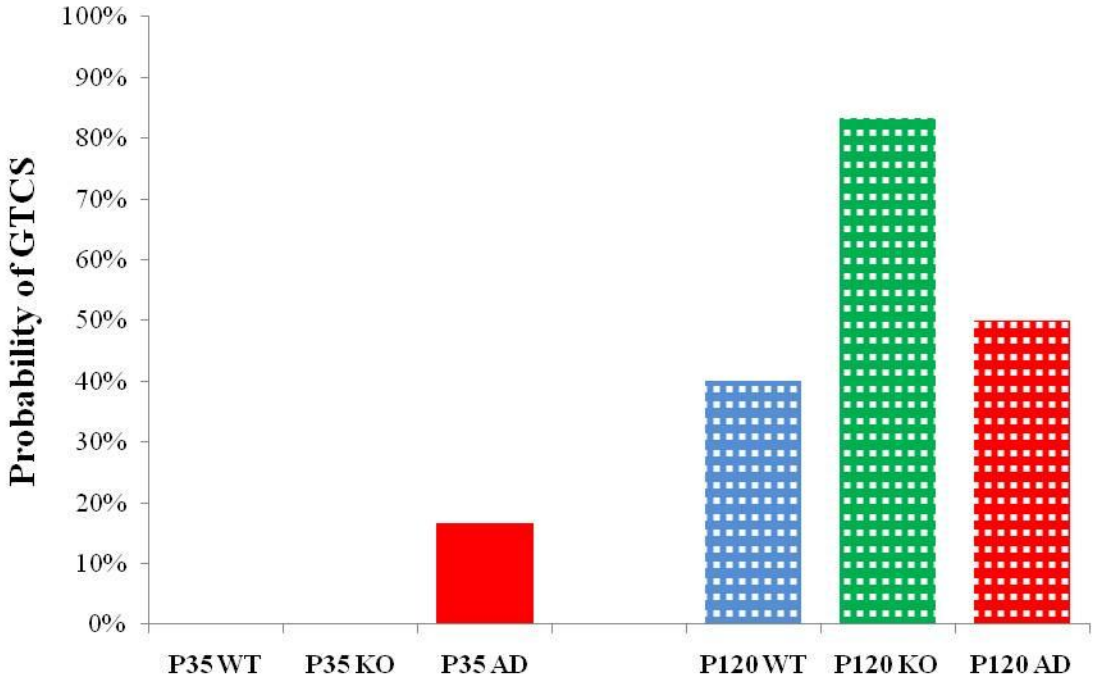
Figure 3-13: The incidence of PTZ-evoked PSDs is greater at P120s compared to P30s for all genotypes. The incidence of PTZ-evoked PSD/ hr for 4 WT(0.35 ± 0.2 PSDs/hr), 4 Gabra1 KO (0.18 ± 0.18 PSDs/hr) and 6 A322D KI (1.34 ± 0.56 PSDs/hr) mice at P30s and 5 WT (2.54 ± 0.9 PSDs/hr), 6 Gabra1 KO (10.4 ± 4.3 PSDs/hr) and 6 A322D KI (4.52 ± 1.4 PSDs/hr) mice at P120s is shown. The effect of age was significant on PTZ-evoked PSDs as shown by two-way ANOVA (*p value = 0.008*).



The probability of GTCSs evoked by PTZ is greater at P120s than P30s for all genotypes

We determined the probability of evoking GTCSs with PTZ administration in WT, Gabra1 KO and A322D KI mice at P30s and P120s. Using log-linear analysis for 3-way contingency table, we determined that the probability of evoking GTCSs with PTZ administration is significantly greater at P120s than P30s (*p value 0.021*) (figure 3-14). The effect of genotype interacting with age for this response to PTZ administration was not statistically significant.

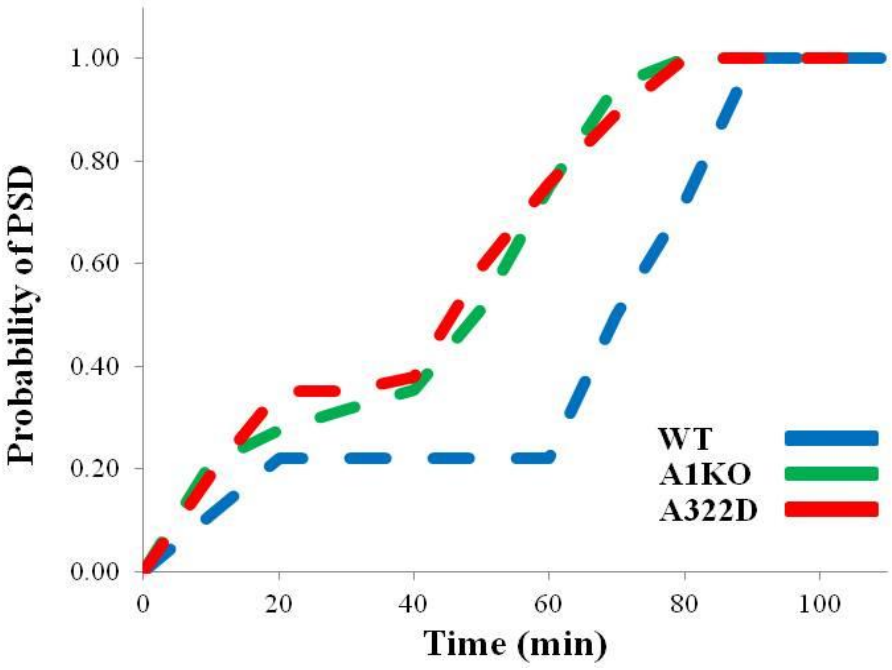
Figure 3-14: The probability of GTCSs evoked by PTZ is greater at P120s compared to P30s for all genotypes. The probability of PTZ-evoked GTCS for 4 WT(0%), 4 Gabra1 KO (0%) and 6 A322D KI (17%) mice at P30s and 5 WT (40%), 6 Gabra1 KO (83%) and 6 A322D KI (50%) mice at P120s is shown. The effect of age was significant on the probability of PTZ-evoked GTCS, as shown by modified chi-square test for age (*p value = 0.021*), but not significant for genotype interacting with age (*p value = 0.079*).



Latency of PTZ-evoked PSDs is shorter in Gabra1 KO and A322D KI mice compared to WT at P120s

We next determined whether the genotype affected the latency of PSDs (relative to the first PTZ injection). The cumulative probability of a PSD was plotted against 10 minute intervals from start of the experiment. Multiple K-S test with Bonferroni correction showed that Gabra1 KO and A322D KI mice had a significantly reduced latency for developing PSDs, compared to WT (figure 3-15). Therefore, while age, and not genotype, affected the number of PSDs during the entire experiment, genotype did affect the PSD latency. It should also be noted that neither age nor genotype was associated with a significant difference in the latency of GTCS.

Figure 3-15: The latency to develop PTZ induced PSDs is shorter in Gabra1 KO and A322D KI mice. The probability of developing PSDs is plotted against time from the start of the experiment. 5 WT, 6 Gabra1 KO and 6 A322D KI mice, aged P120s, were used for these experiments. Multiple K-S test with Bonferroni correction showed that Gabra1 KO and A322D KI mice had a significantly reduced latency for developing PSDs compared to WT mice (*p value < 0.001*).



In summary, the PTZ-evoked PSDs were similar in duration and morphology to spontaneous PSDs. In addition, the pharmacologically-evoked PSDs, like the spontaneous PSDs, demonstrated an age-dependent and genotype-dependent acquisition of the phenotype. In addition, post hoc analyses demonstrated that the two mutations (Gabra1 KO and A322D KI) did not confer different phenotypes from each other.

Discussion

Seizures remain uncontrolled or refractory in a significant proportion of patients suffering with epilepsy[48]. Recurring epileptic seizures have been associated with job loss, anxiety, driving restrictions, reduced self-esteem, depression, injury and even death[49-51]. Therefore understanding and studying epilepsy is of extreme importance to eventually counter the substantial economic and psychosocial burden on individuals and society.

Childhood absence epilepsy (CAE) is considered a relatively benign form of epilepsy with regards to seizure prognosis because 70-80% of these patients are well controlled with antiepileptic drugs[52], however, 40% of CAE patients also develop generalized tonic-clonic seizures[53]. Previous studies have also reported that 15-18% of CAE patients go on to have concomitant myoclonic jerks and absence seizures later in life[24, 54].

On the other hand juvenile myoclonic epilepsy (JME) patients present with myoclonic jerks, generalized tonic clonic seizures and absence seizures[55]. Due to the heterogeneity of presenting symptoms Martinez-Juarez et.al. suggested a subdivision of JME into four categories: 1) “Classic” JME which presents with adolescent onset of myoclonic jerks, tonic-clonic seizures and rare-to-infrequent absence seizures (seen in 72% of all reported JME cases), 2) CAE persists and evolves into JME and presents with absence seizure, myoclonic jerks and tonic clonic seizures (seen in 18% of all reported JME cases), 3) JME that presents with adolescent onset of frequent absence seizures along with myoclonic jerks and tonic clonic seizures (seen in 7% of all reported JME cases), and 4) JME presenting with myoclonic jerks, tonic clonic seizures

and astatic seizures (seen in 3% of all reported cases)[56]. The authors also reported that 91% of the category 2 patients (CAE persist and evolves into JME) did not achieve complete long term seizure remission[56]. In fact, a recent study also reported that JME patients who do not go into seizure remission with age have a significantly higher incidence of absence seizures[55].

Therefore, identification of an animal model that shows the characteristics of symptoms like absence seizures persisting and evolving into myoclonic jerks can be very useful in gaining a better understanding of this disease. The fact that the majority of patients who present with these symptoms never achieve seizure remission makes it even more important to study an animal model with these symptoms, to better understand JME and help develop better possible therapeutic interventions for it.

The A322D mutation in the $GABA_A$ receptor α1 subunit, discovered in a French-Canadian family suffering from an autosomal dominant form of JME[13], has been shown *in vitro* to cause a 88% reduction in the total and surface expression of the α1 subunit[33]. In chapter II we described how the Gabra1 KO mutation results in absence seizures in a mouse model[27]. We have now shown that like the Gabra1 KO mutation, the A322D KI mutation also causes absence seizure at P30s that persist into adulthood. The similarity of the typical SWD pattern associated with behavioral arrest, observed with synchronized video/EEG in Gabra1 A322D KI mice, indicates that these mutant mice have absence seizures similar to those seen in Gabra1 KO mice.

An important finding described in this chapter are the spontaneous polyspike discharges (PSDs) that are occasionally accompanied with subtle visible myoclonic jerks in the P120s Gabra1 KO and A322D KI mice. There are 4 major reasons to consider Gabra1 KO and A322D KI mice good models to study JME. 1) *Morphology of PSDs*: The atypical discharges seen in Gabra1 KO and A322D KI mice consists of short and fast polyspikes, similar to what has been reported in other models of myoclonic epilepsy[57]. Even in humans, the presence of 4-6Hz fast polyspike-wave complexes, lasting less than half a second, is a requirement for diagnosing JME[58, 59]. 2) *Concomitant behavior with PSDs*: The rapid brief contractions of

body muscles observed with some PSDs was very similar to what have been reported in humans[59] and rodent models[57]. 3) *Age of onset*: The peak age of onset of symptoms in CAE patients is 6-7 years and a subset of these patients develop concomitant myoclonic jerks 1 to 6 years after the onset of absence seizures[24]. The fact that we observed PSDs and myoclonic jerks later in development (P120s), while SWDs and absence seizures were seen much early in development (P30s), further shows the similarity between human patients and Gabra1 KO and A322D KI mice.

However one limitation of this model is that spontaneous GTCS are observed only rarely in these mutant mice, however, this is not a very critical factor because even human JME patients have been reported to just have myoclonic jerks for years before the onset of GTCS (the most common reason for presentation)[59]. Also monitoring for only 24 hours would not be expected to capture GTCS in human JME patients too. Future experiments with long term monitoring of mutant mice (perhaps with noninvasive video analysis) may capture more GTCS.

Our data also demonstrated that even though the Gabra1 A322D KI mutation exhibited a small but dominant negative effect on wild type $GABA_A$ receptor expression *in vitro*[28], Gabra1 KO and A322D KI mutations resulted in similar incidence of spontaneous and evoked SWDs and PSDs in mice. This could possibly happen because (1) *in vitro*, under endogenous conditions, there is a greater reduction in the expression of mutant Gabra1 (A322D) subunit than in the *in vivo* model or (2) the dominant negative effect, seen *in vitro*, is not significant *in vivo*. Nevertheless, these data demonstrate that heterozygous loss of $GABA_A$ receptor α1 subunit is sufficient to cause the myoclonic-like phenotype in the absence of a dominant negative effect.

We have discovered a good animal model for studying the CAE and JME. Typical rodent models used to study absence epilepsy, for example WAG/Rij strain of rats[60], and myoclonic epilepsy, for example *Efhc1*-deficient mice[26], present with only one symptom. The animal model we described here shows an age-dependent evolution of behavioral phenotype that first presents with absence seizures but then goes

on to have myoclonic jerks and absence seizures as the animal ages, a feature seen in some human patients[56]. This model can help us reveal how the epilepsy-network in brain evolves throughout development. A complete characterization of the structure and function of human brain networks promises important insights for understanding normal and pathological brain activity[61, 62]. Changes in functional connectivity have been reported in epileptic patients[63, 64], but no study has described how the functional connectivity evolves, as the different types of seizures evolve in epileptic patients or animal models. Further studies on Gabra1 KO and A322D KI mice, as a model of human JME subclass (CAE persisting and evolving into JME), can be extremely useful for developing a better understanding of this disease. Understanding the evolution of the underlying brain circuitry, using this model, can help us develop possibilities for better therapeutic interventions for this treatment resistant disease.

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Chapter IV

$GABA_A$ receptor subunit expression in the cortex is modulated with age in Gabra1 KO and A322D KI mutant mice

Introduction

Seizures frequently result from an imbalance of excitation and inhibition due to a failure of inhibitory neurotransmission, which is mainly mediated by the neurotransmitter GABA[1]. In fact $GABA_A$ receptors are the target of many anti-seizure medications like benzodiazepines, tiagabine and vigabatrin[1, 2]. Furthermore, blockade of the excitatory function of GABA, seen in early development[3], has been reported to reduce the epileptiform activity *in vitro* and *in vivo* in neonatal rats[4]. Therefore, alteration in the $GABA_A$ receptor physiology can have a significant effect on the development of an epileptiform phenotype.

The subunit composition of $GABA_A$ receptors changes with age[5]. During early postnatal development a gradual parallel decrease in α2 and α3 subunits and an increase in α1 subunit has been reported in rodent brains[6-8] (figure 1-9 chapter 1). The subunit composition of $GABA_A$ receptor governs the intrinsic properties of the channel such as the affinity for GABA, receptor kinetics, conductance and allosteric modulation[9]. $GABA_A$ receptors in very young animals, containing α2 and α3 subunits mostly, mediate relatively long lasting inhibitory post-synaptic currents (IPSCs)[10-12], while $GABA_A$ receptors in adult animals, containing mainly α1 subunits, mediate relatively short IPSCs[10, 13, 14].

To date, four mutations in the $GABA_A$ receptor α1 (Gabra1) subunit, A322D, S326fs328X, D219N, and K353delins18X, have been associated with genetic generalized epilepsy[15-17]. All 4 of these mutations have been associated with reduced Gabra1 subunit expression in vitro[16, 18, 19]; but in particular the S326fs328X mutation resulted in complete elimination of the α1 subunit[19]. Consistent with this, we have reported that α1 subunit expression is reduced in the cortex of P30s heterozygous $GABA_A$ receptor α1 subunit knockout (KO) mice[20], that also have absence seizures[21]. We also reported an abnormally high

expression of α3 subunits in Gabra1 KO mice[20]. A preservation of the long lasting $GABA_A$ receptor-mediated IPSCs has also been reported in the mice lacking α1 subunits[10], indicating a possible persistent high expression of α2 and α3 subunits. These findings stress the fact that abnormal expression of $GABA_A$ receptor subunits can have significant effects at both cellular and behavioral levels.

Changes in $GABA_A$ receptor composition and function have been associated with acute and chronic seizures[1]. In Chapter 3, we characterized the developmental evolution of the seizure phenotype in Gabra1 KO and A322D knock-in (KI) mice, from absence seizures only in P30s mice, to persistent absence seizures along with myoclonic jerks in P120s mice. Here we hypothesized that the modulation of the $GABA_A$ receptor subunits may be a mechanism for the evolution of the seizure phenotype with age. Therefore, we characterized the $GABA_A$ receptor subunits expression at the cellular and cortical level in P30s and P120s Gabra1 KO and A322D KI mice, as a possible mechanism for the evolution of seizure phenotype with age.

Methods and Materials

Generation and maintenance of Gabra1 KO and A322D KI mice in congenic strains

We obtained the Gabra1 KO mutant mice from Jackson Laboratories (B6.129(FVB)Gabra1tm1Geh/J; stock 004318) (for details refer to chapter2) and had the Gabra1 A322D KI mouse line made using the commercially available service of the University of Connecticut Health Center (for details refer to chapter 3). In order to compare the effects of Gabra1 KO and A322D KI mutations in the same litter, heterozygous (het) Gabra1 KO mice were mated with het Gabra1 A322D KI mice to produce wild type (WT), het Gabra1 KO, het A322D KI and Gabra1 KO/A322D KI double mutant mice in equal Mendelian proportions (figure 3-3 chapter 3). Only female WT, Gabra1 KO and A322D KI mice aged P33-37 (referred to as P30s from here on) and P120-130 (referred to as P120s from here on) were used for further experiments.

All procedures were performed in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Mice were housed in a temperature and humidity controlled environment, with a 12 hour light/dark schedule. Water and food was provided ad libitum.

Determining total expression of $GABA_A$ receptor subunits with western blots

We conducted western blot experiments using published protocols[20]. Briefly, mice were anesthetized with isoflurane and decapitated with sharp scissors. Brain was removed from the skull and coronal sections were obtained using a vibratome. The cutting solution contained 210 mm sucrose, 20 mm NaCl, 2.5 mm KCl, 1.2 mm NaH_2PO_4, 1 mm $MgCl_2$, and 10 mm d-glucose maintained at pH 7.4. The slices were then transferred to artificial cerebrospinal fluid (aCSF), containing 126 mm NaCl, 2.5 mm KCl, 1.25 mm NaH_2PO_4, 2 mm $CaCl_2$, 1 mm $MgCl_2$, and 10 mm d-glucose maintained at pH 7.4. Cortices from these slices were dissected and sonicated in radioimmunoprecipitation assay (RIPA) solution (20 mm Tris, pH 7.4, 1% Triton X-100, 250 mm NaCl) that also contained protease inhibitor mixture (1:100; Sigma-Aldrich), 0.5% deoxycholate, and 0.1% SDS. Protein concentrations were determined using a bicinchoninic acid-based assay (Thermo Scientific).

Proteins were fractionated on 10% SDS-polyacrylamide gels and then electrotransferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween, pH 7.4. We incubated the blots with primary antibody at 4 °C overnight and then with secondary antibody at room temperature for 1 h. The blots were imaged on an infrared fluorescent imaging system (LI-COR Biosciences).

Antibodies used for western blots

We obtained the antibodies from the following sources and listed the clone or catalogue number and the concentrations used for the Western blots in parentheses. The purified mouse monoclonal anti-$GABA_AR$ α1 subunit antibody (catalogue number N95/35; concentration 1:250) was obtained from the University of California Davis/National Institutes of Health NeuroMab Facility. The anti-α3 subunit antibody was from Alomone (catalogue number AGA-003; concentration 1:500). The anti-α3 subunit antibody was from Alomone (catalogue number AGA-003; concentration 1:500). The anti-γ2 subunit antibody was from Millipore (catalogue number AB5559; concentration 1:1000). The anti-β2/3 subunit antibody was from Millipore (catalogue number 05-474; concentration 1:300). The anti-actin antibody was from Millipore (catalogue number JLA20; concentration 1:1000). The fluorescently conjugated goat anti rabbit-680 (catalogue number 926-32221) and goat anti mouse-800 (catalogue number 926-32210) secondary antibodies were from LI-COR (concentration 1:10,000).

Measuring regional expression with immunofluorescence and confocal microscopy

Immunofluorescence experiments were performed using the protocol described by Gasser et.al. previously[22]. Briefly, mice were anesthetized with isoflurane and decapitated with sharp scissors. Brains were removed from the skull and 2mm thick coronal slices were obtained using mouse brain slicer matrix. These thick coronal slices were washed briefly with ice-cold phosphate buffered saline (PBS) before fixation in ice-cold 4% paraformaldehyde, for 30 minutes. After this the thick coronal slices were washed three times with ice-cold phosphate buffered saline (PBS), before being transferred to cryoprotectant (30% sucrose) and stored overnight at 4°C. Next day, 15μm thin coronal sections were cut from these weakly perfused slices using a cryostat (Leica) and transferred onto Shandon Colorfrost Plus glass slides (Thermo Scientific). The slides were air dried for a minimum of 30 seconds and stored at -20°C over night before using them for immunohistochemistry (IHC).

Cryostat slices were blocked with blocking buffer (10% donkey serum, 2% Triton X-100 in PBS) for 1 hour at room temperature. They were then incubated overnight at 4°C with rabbit anti-$GABA_A$ receptor α1 subunit (Millipore, 06868, 1:250), guinea pig anti-$GABA_A$ receptor α3 subunit (Synaptic Systems, 224304, 1:500) and mouse anti-gephrin antibodies (Synaptic Systems, 147021, 1:100). The following day, the slides were washed with PBS and incubated for 1 hour at room temperature with Cy3-conjugated donkey anti-rabbit (Jackson Immuno Research Laboratories, 711-165-152, 1:500), Alexa 488 conjugated donkey anti-guinea pig (Jackson Immuno Research Laboratories, 706-545-148, 1:500) and Alexa 647 conjugated donkey anti-mouse (Jackson Immuno Research Laboratories, 715-605-150, 1:500) antibodies. Slides were washed again with PBS and a coverslip was applied using Vectasheid mounting medium (Vector Laboratories), that also contained 4′,6-diamidino-2-phenylindole (DAPI) to label cellular nuclei. Finally the coverslip was sealed by applying quick dry clear nail polish (Sally Hensen).

The slides were first imaged on a Zeiss Axio Observer.Z1 epi-fluorescence microscope using Ph1 Plan-NeoFluar 5x/0.15 and LD Plan-Neofluar 20x/0.4 Corr Ph2 M27 objectives. Scan settings were adjusted to utilize the full dynamic range of the photomultipliers. The same scan settings were used for all of the images acquired within an experiment. We obtained images from the motor cortex, 1 μm below the surface of the tissue in cortical layers II/III and 1 μm above the subcortical white matter in cortical layer VI.

The slides were also imaged on Olympus FV-1000 confocal microscope using a 100x / 1.40 SPlan-UApo objective. Scan settings were adjusted to utilize the full dynamic range of the photomultipliers and to provide a scan resolution of 97 nm/pixel and a slice thickness of 1 μm. The same scan settings were used for all of the images acquired within an experiment. We obtained images in the somatosensory cortex and motor cortex 1 μm below the surface of the tissue in cortical layers II/III and VI as well as in the subcortical white matter just below the edge of layer VI.

Analysis of images from IHC

Images were analyzed using Image J and Olympus FV1200/FV1000 Viewer software. The background was defined as the average (among all of the slices imaged in a single experiment) of the mean pixel intensity of the white matter just below the somatosensory cortex. The same background value was used for all of the images in the experiment. We then calculated the mean background-subtracted intensity of $GABA_A$ receptor α1 and α3 subunit and gephyrin staining.

Results

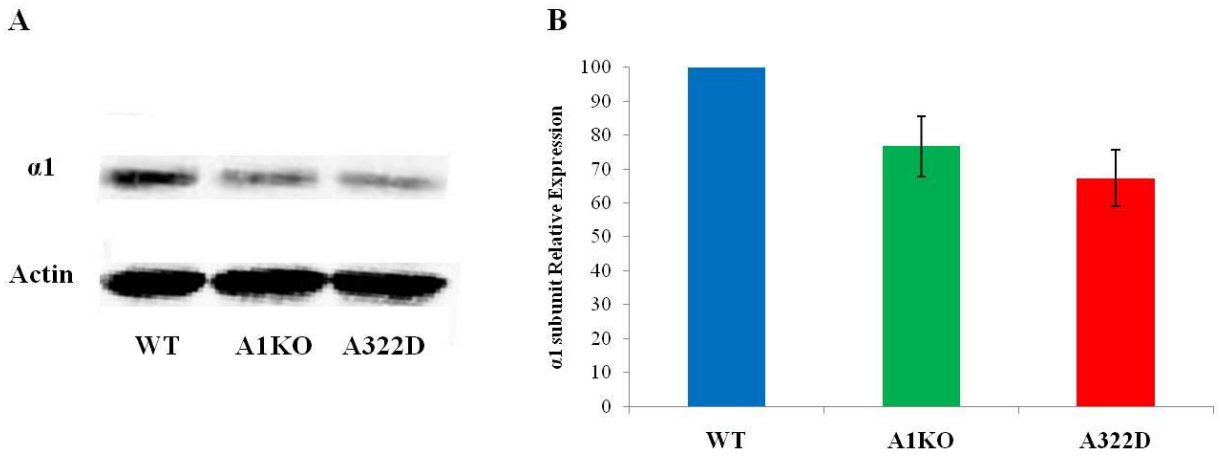
The Gabra1 KO and A322D KI mutations cause modulation of $GABA_A$ receptor subunit expression

The subunit composition of $GABA_A$ receptors confers a unique pharmacology that dictates the binding characteristics, functional capacity and the role of the receptor in maintaining the inhibitory tone of the CNS[23]. We have shown previously that in the P30s Gabra1 KO mouse cortex, expression of the α1 subunit is reduced (as expected), while α3 subunit expression is increased[20]. We have also shown *in vitro* that the Gabra1 A322D mutation results in decreased expression of α1 subunits[18], but no report of the effect of the Gabra1 A322D mutation on $GABA_A$ receptor expression *in vivo* is available. Therefore, we decided to compare the effects of Gabra1 A322D and Gabra1 KO mutations on $GABA_A$ receptor subunits expression *in vivo*.

Changes in the expression of α1 and α3 subunits are similar in Gabra1 KO and A322D KI mice

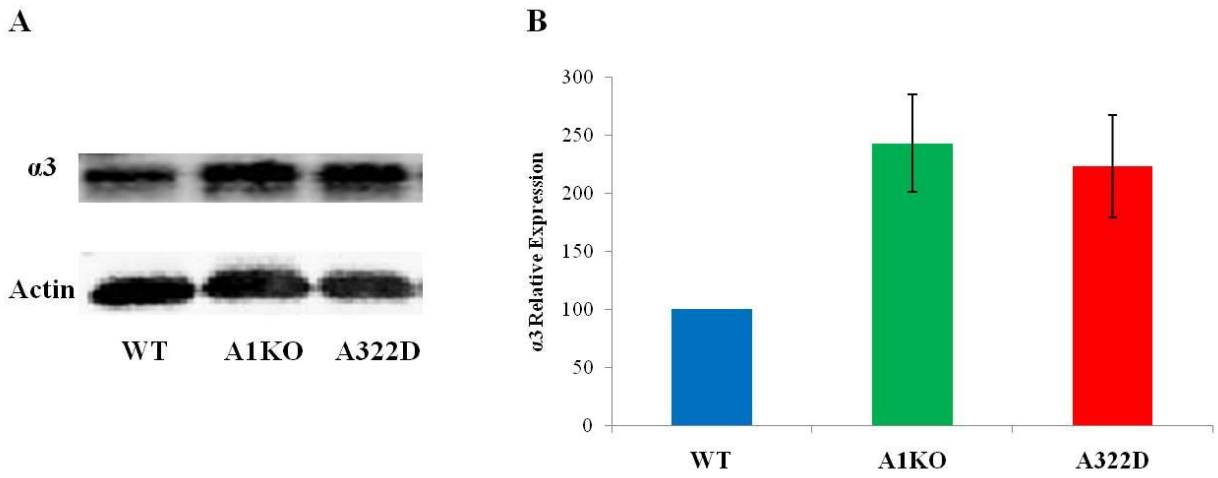
The total expression of α1 subunit in the cortex was reduced in P30s Gabra1 KO and A322D KI mutant mice as compared to WT (figure 4-1). In our previous study we showed that the A322D KI mutation caused a small significant reduction in the expression of WT Gabra1 subunit *in vitro*[18]. Although the expression of α1 subunit in Gabra1 KO and A322D KI mice was significantly different compared to WT, no dominant negative effect was seen.

Figure 4-1: Relative expression of α1 subunit in reduced in Gabra1 KO and A322D mice. (A) Sample of western blot stained for α1 subunit and actin (loading control) is shown. (B) Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI mice is shown. ANOVA testing shows a significant genotype-dependent difference in α1 expression (*p value = 0.01*).



In our previous study we have shown that the α3 subunit expression is increased in the cortex of Gabra1 KO mice[20]. Similar to our previous finding we saw that the expression of α3 subunit was increased in both Gabra1 KO and A322D KI mice compared to WT (figure 4-2). No difference in the expression of α3 subunit was observed between Gabra1 KO and A322D KI mice.

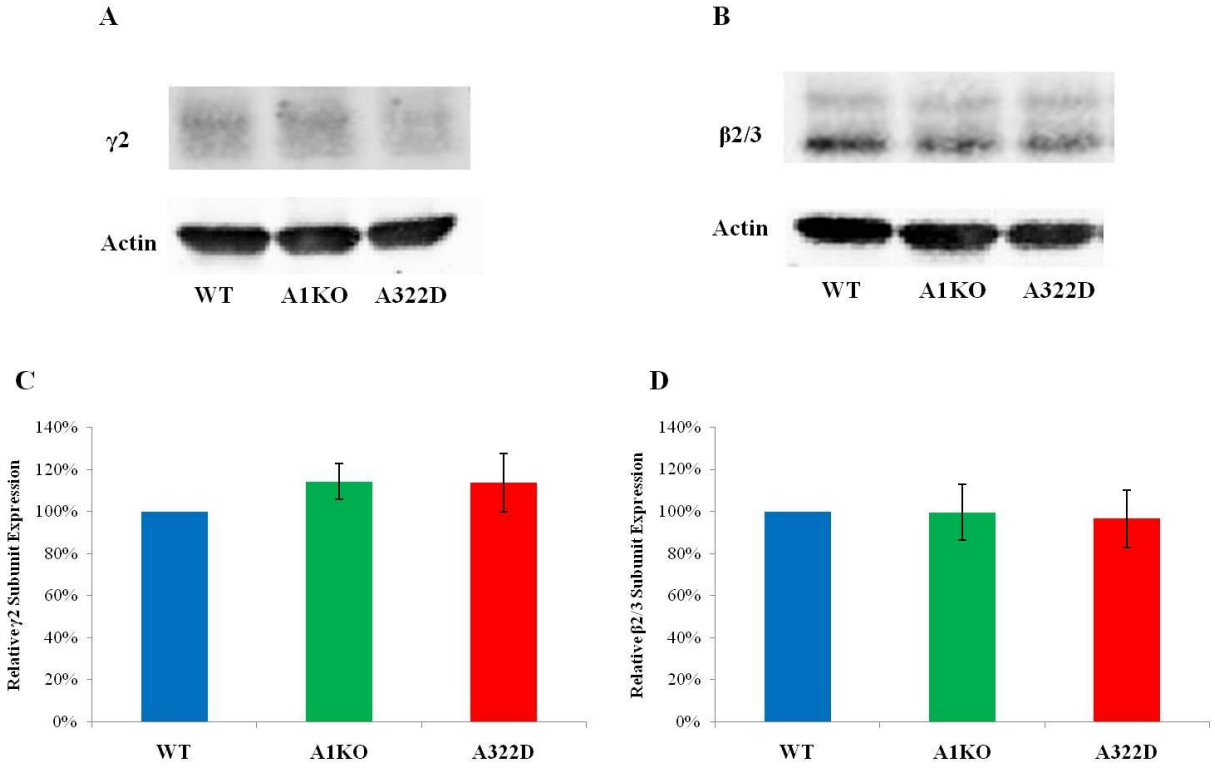
Figure 4-2: Relative expression of α3 subunit is increased in Gabra1 KO and A322D mice. (A) Sample of western blot stained for α3 subunit and actin (loading control) is shown. (B) Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI mice is shown. ANOVA testing shows a significant genotype dependent difference in α1 expression (*p value = 0.02*).



Total $GABA_A$ receptor expression is not different between WT, Gabra1 KO and A322D KI mice

Next we determined if total expression of $GABA_A$ receptor was also reduced in the Gabra1 KO and A322D KI mutant mice compared to WT. The relative expression of β2/3 and γ2 subunits was not statistically different in Gabra1 KO and A322D KI mice compared to WT (figure 4-3). These data indicate the decrease in α1 subunit and increase in α3 subunit, seen in the Gabra1 KO and A322D KI mice does not affect the total expression $GABA_A$ receptors.

Figure 4-3: Relative expression of β2/3 and γ2 subunits subunit is not different in Gabra1 KO and A322D mice compared to WT. Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI mice was analyzed. Examples of (A) γ2 and (B) β2/3 subunit expression from WT, Gabra1 KO and A322D KI mice. Relative expression of (C) γ2 and (D) β2/3 subunits in Gabra1 KO and A322D KI mice compared to WT is shown. ANOVA testing shows no significant genotype dependent difference in β2/3 and γ2 subunits expression (γ2 subunits *p value = 0.484*; β2/3 *p value = 0.970*).



Relative expression of α1 subunit in Gabra1 KO and A322D KI mice is not different from WT mice at P120s

As explained in chapter 1, in rodents the expression of α1 subunit is low early in development but it increases dramatically in the first postnatal week[6]. On the other hand, the expression of α3 subunit is high early in development but declines with age[24]. We have shown, in our findings discussed in chapter 3, that the epilepsy phenotype evolves in the Gabra1 KO and A322D KI mice from absence seizures only at P30s to absence seizures along with myoclonic jerks at P120s. Therefore, we decided to investigate if the change in epilepsy phenotype is accompanied by an alteration of $GABA_A$ receptor subunit expression.

We compared the total expression of α1 subunit in WT, Gabra1 KO and A322D KI mice at P30s to P120s. In contrast to the relatively reduced expression of α1 subunit in Gabra1 KO and A322D KI mice compared to WT mice at P30s, there was no statistical difference in the expression of α1 subunit at P120s (figure 4-4). Interestingly, a significant reduction of relative expression of α1 subunit in P120s WT, Gabra1 KO and A322D KI is seen when compared to P30s WT (figure 4-5). 2 factor ANOVA showed a significant effect of age and interaction of age and genotype on α1 subunit expression.

Figure 4-4: Relative expression of α1 subunit is not different in Gabra1 KO and A322D mice compared to WT at P120s. (A) Sample of western blot stained for α1 subunit and actin (loading control) is shown. (B) Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI mice is shown. ANOVA testing shows no significant effect of genotype on α1 expression (*p value = 0.285*).

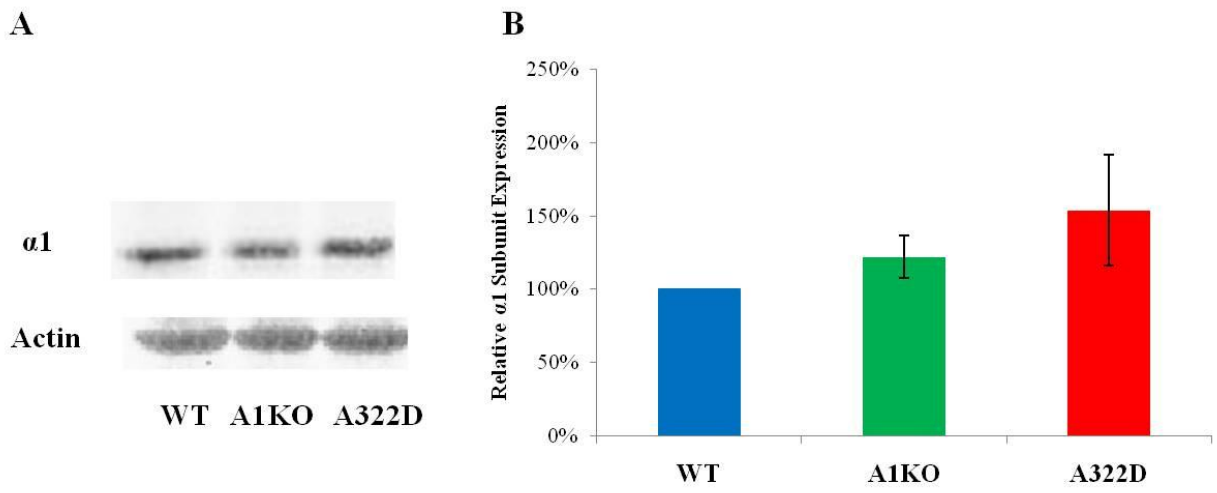
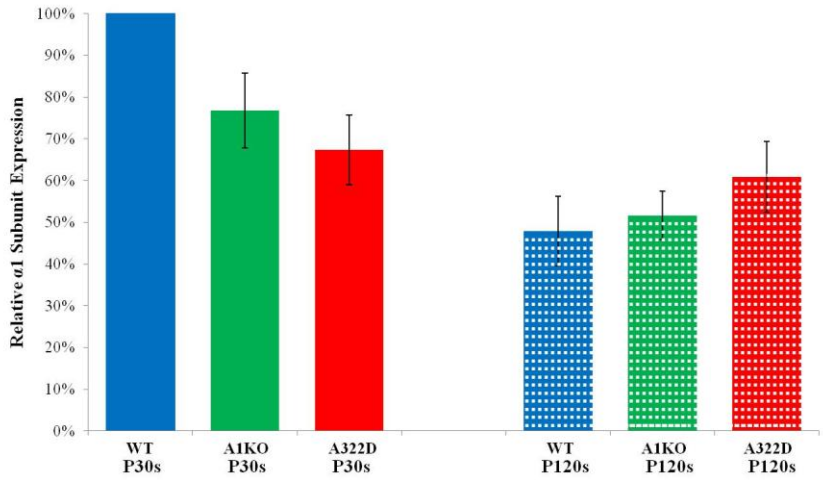


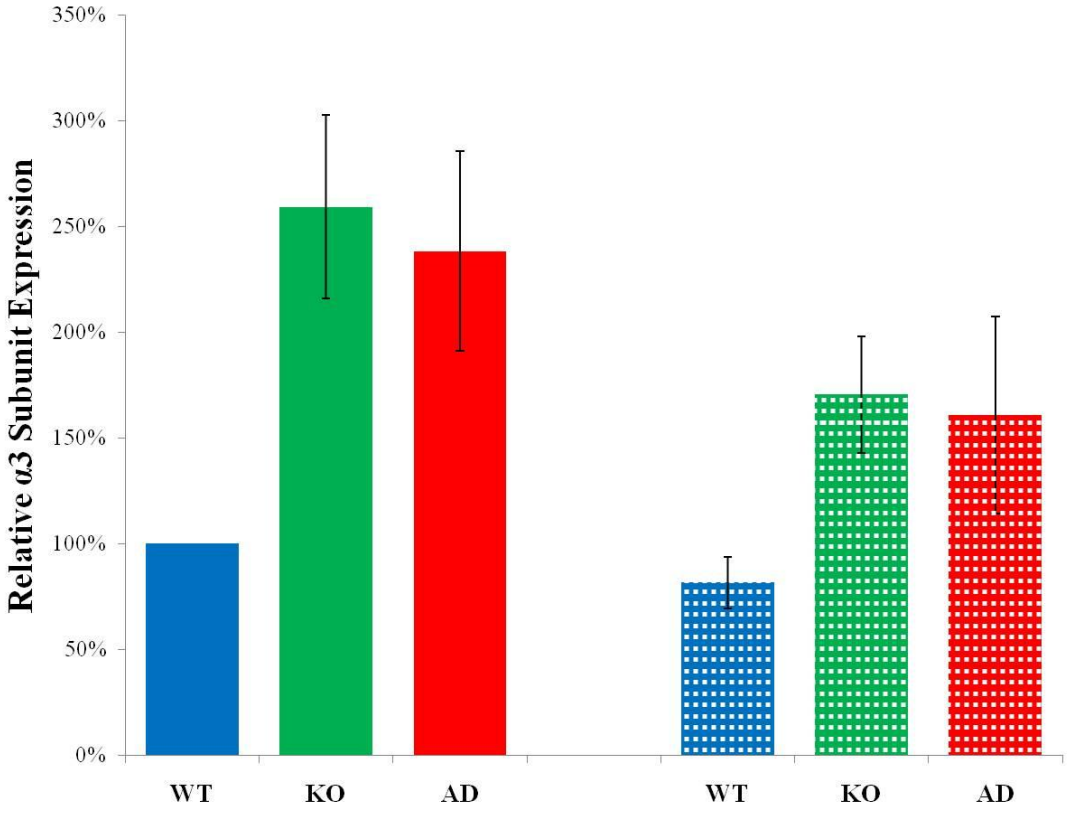
Figure 4-5: Quantification of relative expression of α1 subunit is reduced in P120s WT, Gabra1 KO and A322D mice compared to P30s WT. Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI P120s mice and 3 WT, 3 Gabra1 KO and 3 A322D KI P30s mice are shown. 2 pair ANOVA testing shows a significant effect of age (*p value < 0.001*) and interaction of age and genotype (*p value < 0.001*).



Relative expression of α3 subunit in Gabra1 KO and A322D KI mice continues to be greater than WT at P120s

We compared the total expression of α3 subunit in WT, Gabra1 KO and A322D KI mice at P30s to P120s. Although the total expression of α3 subunit in P120s WT, Gabra1 KO and A322D KI mice is reduced compared to P30s, the relative expression of α3 subunit in Gabra1 KO and A322D KI mice, compared to WT, was greater (figure 4-6). 2 factor ANOVA showed a significant effect of age and genotype on α3 subunit expression.

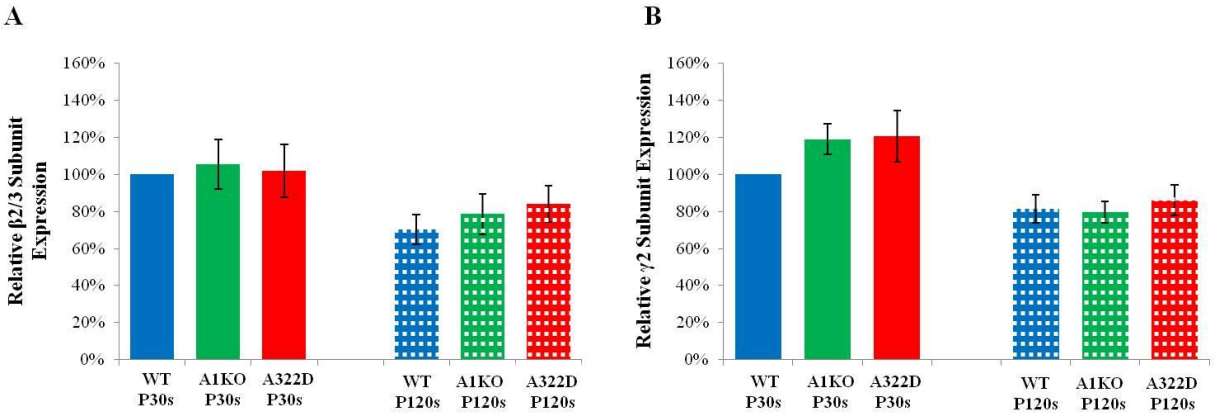
Figure 4-6: Relative expression of α3 subunit is reduced at P120s compared to P30s but continued to high in Gabra1 KO and A322D KI mice compared to WT. Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI P120s mice and 3 WT, 3 Gabra1 KO and 3 A322D KI P30s mice are shown. 2 pair ANOVA testing shows a significant effect of age (*p value = 0.003*) and genotype (*p value = 0.002*).



Total $GABA_A$ receptor expression is reduced at P120s compared to P30s

Finally we determined if the total expression of $GABA_A$ receptor was also affected in the Gabra1 KO and A322D KI mutant mice, compared to WT mice, at P120s. The relative expression of β2/3 and γ2 subunits was significantly reduced at P120s in WT, Gabra1 KO and A322D KI mice compared to P30s WT (figure 4-7). These data indicate that the total $GABA_A$ receptor expression is reduced in P120s mice regardless of their genotype.

Figure 4-7: Relative expression of β2/3 and γ2 subunits subunit is significantly reduced in P120s WT, Gabra1 KO and A322D mice compared to P30s WT. Results from repeated quantification of 3 WT, 3 Gabra1 KO and 3 A322D KI P120s mice and 3 WT, 3 Gabra1 KO and 3 A322D KI P30s mice are shown. 2 pair ANOVA testing shows a significant effect of age for both (A) β2/3 (*p value < 0.001*) and (B) γ2 subunits expression (*p value < 0.005*).



Regional expression

The regional and temporal expression of different $GABA_A$ receptor subunits has been discussed in detail in chapter I. However it must be remembered that the $GABA_A$ receptor composition is distinct in different regions of the brain and changes with age[25]. For example the expression of α1 subunit in major brain areas, like neocortex, thalamus and hippocampus, is low early in development[25], but its expression increases sharply as the animal develops and its absence in certain regions, for example the reticular nucleus of the thalamus, becomes sharply distinct, compared to the surrounding[26].

Apart from the regional and developmental variability of $GABA_A$ receptor subunits, the receptor composition may vary in certain disease states[27]. For example, the α1 subunit is significantly reduced in the dentate gyrus of the hippocampus of human temporal lobe epilepsy (TLE)[28] and a rodent model of TLE[29]. Whereas in the same brain region of stargazer mutant mice (a model of absence epilepsy[30]), the α1 subunit is unaffected[31].

We showed that the expression of α3 subunit is increased in layer II/III and VI of the somatosensory cortex of P30s Gabra1 KO mice (that have absence seizures[21]), compared to WT mice[20]. Therefore we hypothesized that α subunit expression and distribution may be similar in Gabra1 A322D KI mice at P30s and evolved as the mice aged.

Expression of α3 subunit is increased in layer II/III and VI of cortex in Gabra1 KO and A322D mice

Consistent with our results from western blot experiments, we saw a significant increase in the expression of α3 subunit in layer II/III of the motor cortex in Gabra1 KO and A322D KI mice, compared to WT, at both P30s and P120s (figure 4-8) (*p value < 0.003*). Similar to western blot experiment results, no effect of age was noted (p value = 0.829). In layer VI, the expression of α3 subunit appeared to be greater in Gabra1 KO and A322D KI mice, but it was not statistically significant (*p value = 0.095*) (figure 4-9).

Figure 4-8: Expression of α3 subunit in layer II/III is greater in Gabra1 KO and A322D KI mice. Examples of IHC results from layer II/III of (A) P30s and (B) P120s of WT, Gabra1 KO and A322D KI mice are shown. Analysis using 2 pair ANOVA shows a significant difference in the (C) quantified expression of α3 subunit expression (*p value < 0.003*).

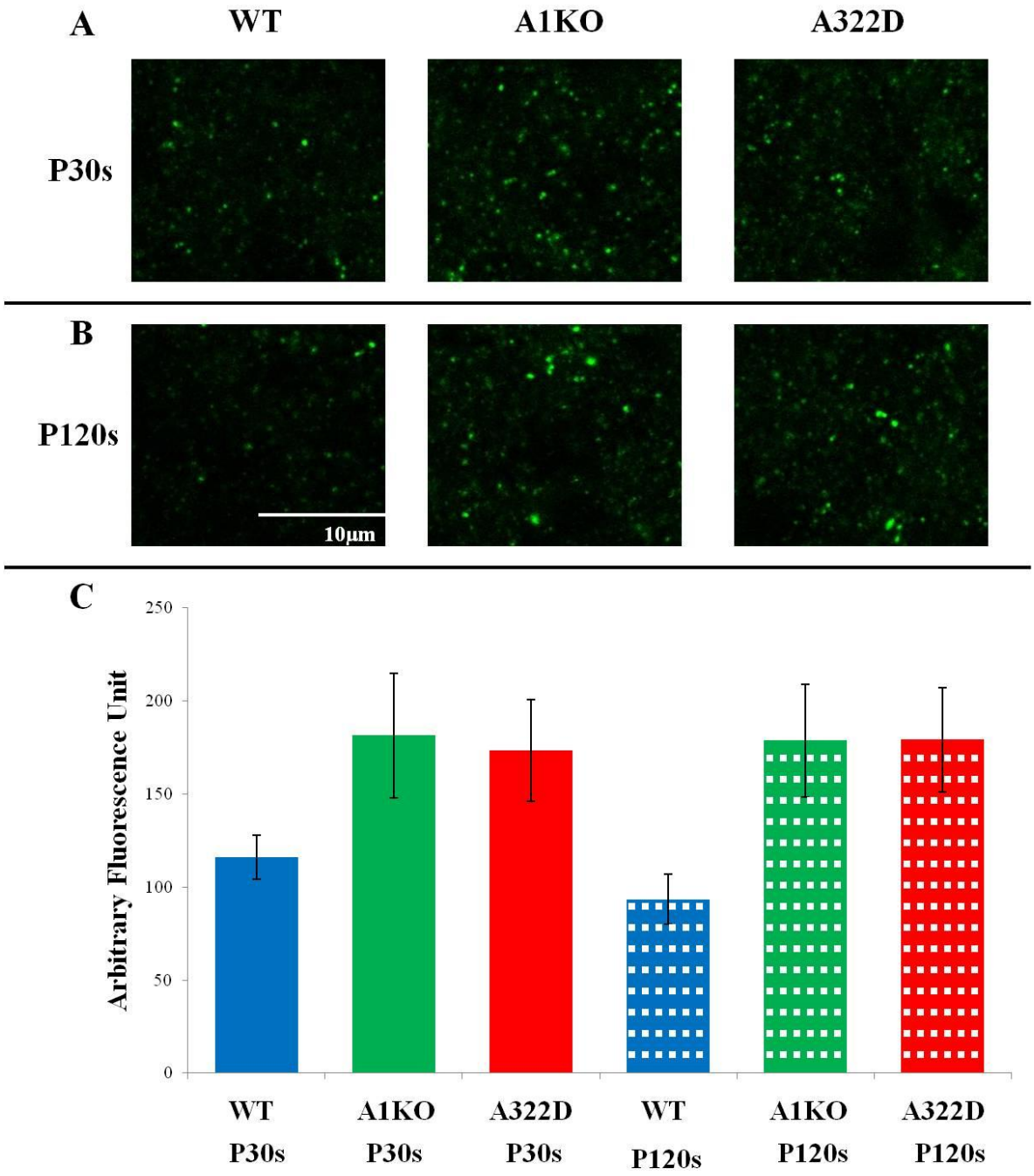
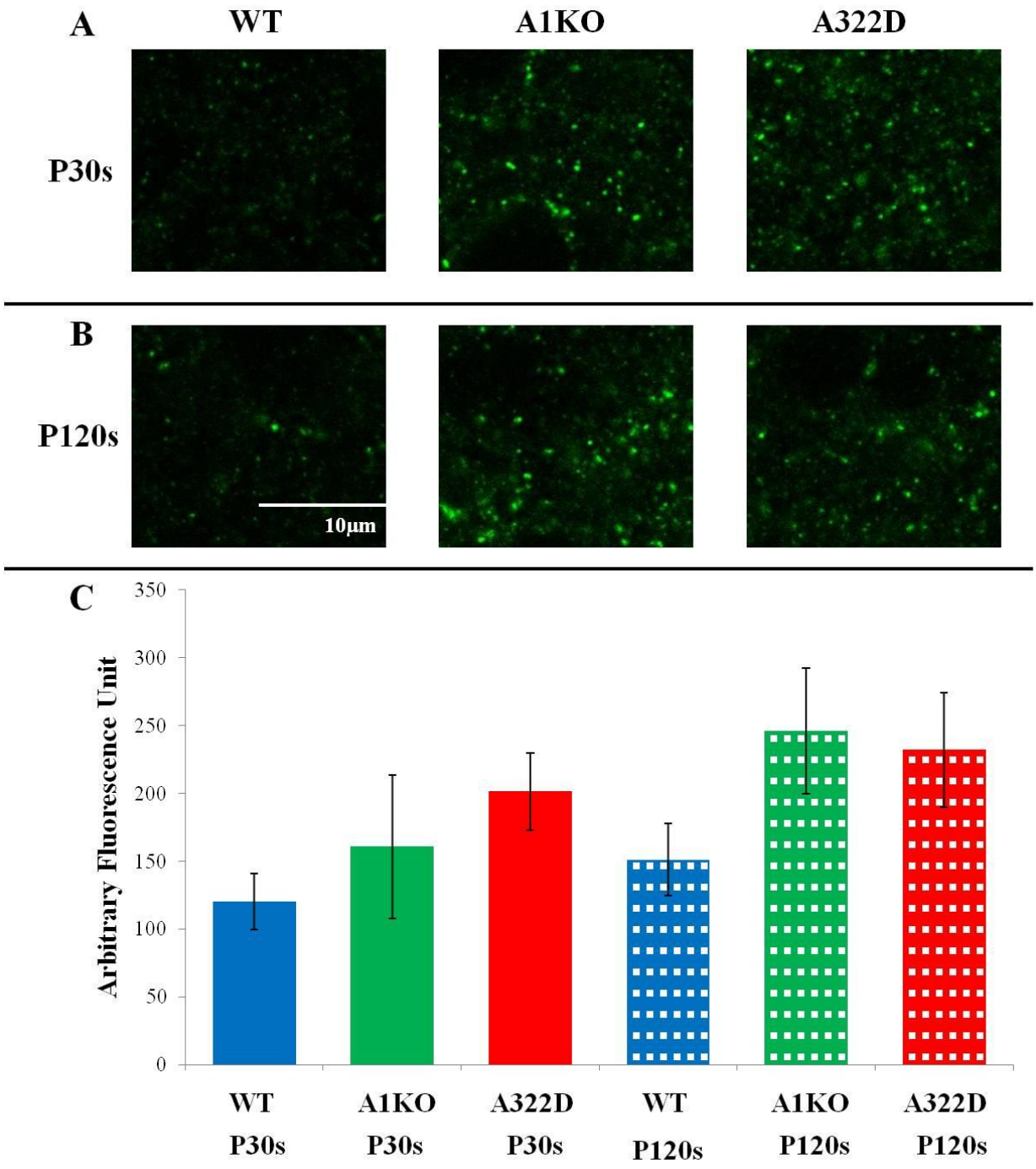


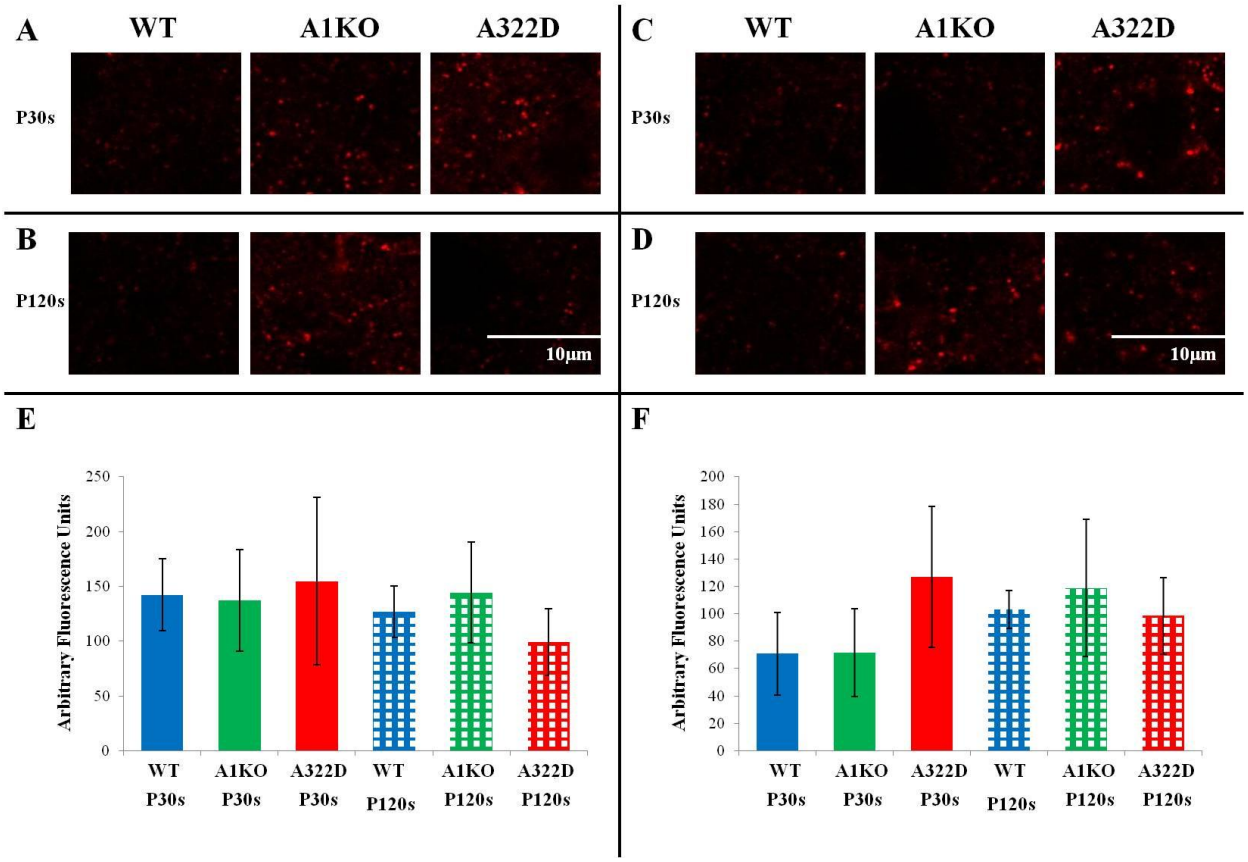
Figure 4-9: Expression of α3 subunit in layer VI appeared to be greater in Gabra1 KO and A322D KI mice. Examples of IHC results from layer VI of (A) P30s and (B) P120s of WT, Gabra1 KO and A322D KI mice are shown. Analysis using 2 pair ANOVA does not show a significant difference in the (C) quantified expression of α3 subunit expression (*p value = 0.095*).



Expression of α1 subunit is not significantly different in layer II/III and VI of cortex in Gabra1 KO and A322D mice

There was significant difference in the expression of α1 subunit in layer II/III or VI of the motor cortex in Gabra1 KO and A322D KI mice, compared to WT, at both P30s and P120s (figure 4-10) (layer II/III *p value = 0.908*; layer VI *p value 0.837*). The results of P120s Gabra1 KO, A322D KI and WT were consistent with the western blot experiment results; however no significant difference between these animals at P30s was observed. This difference in the findings from IHC and western blot experiments could be because the changes in α1 subunit expression occurs in cortical layers other than layer II/III and VI or in a region other than the motor cortex, for example somatosensory cortex.

Figure 4-10: Expression of α1 subunit is not different at P30s and P120s between Gabra1 KO and A322D KI mice and WT mice. Examples of IHC results from layer II/III of (A) P30s and (B) P120s and layer VI of (C) P30s and (D) P120s WT, Gabra1 KO and A322D KI mice are shown. Analysis using 2 pair ANOVA does not show a significant difference in the quantified expression of α1 subunit expression in (E) layer II/III and (F) layer VI (layer II/III *p value = 0.908*; layer VI *p value 0.837*).



Gabra1 KO and A322D mutant mice show a persistent co-localization of α3 subunit and gephyrin during development

Gephyrin binds to the intracellular loop between the third and fourth transmembrane domain of $GABA_A$ receptor α subunits to form a stable ternary structure[32]. *In vitro* studies have also demonstrated a direct interaction between gephyrin and α1[33] and α3 subunits[34]. We did IHC experiments to determine the

number of co-localized gephyrin and α1 or gephyrin and α3 particles in the Gabra1 KO, A322D KI and WT mice. The number of co-localized gephyrin and α1 particles was not significantly different in Gabra1 KO, A322D KI and WT mice, at either P30s or P120s (figure 4-11). However the number of co-localized gephyrin and α3 particles was reduced significantly in P120s WT mice. No such decrease was observed in Gabra1 KO and A322D KI mice, indicating a persistence of co-localized gephyrin and α3 particles during development (figure 4-12).

Figure 4-11: Number of co-localized gephyrin and α1 particles is not significantly different between Gabra1 KO, A322D KI and WT mice at P30s and P120s. Examples of co-localized gephyrin and α1 particles are shown for (A) P30s and (B) P120s Gabra1 KO, A322D KI and WT mice. (C) Quantification of the co-localized gephyrin and α1 particles is shown. *p value* = 0.995

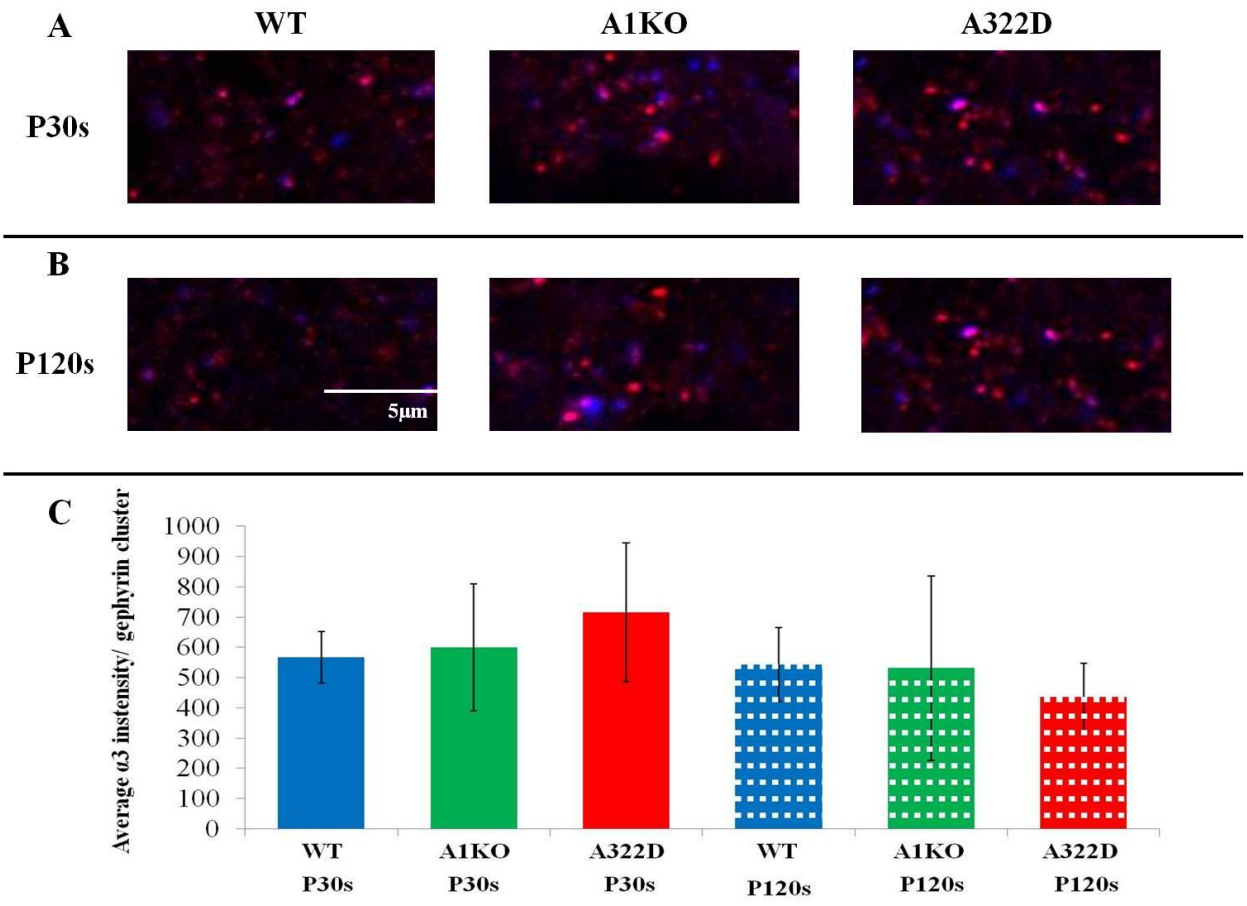
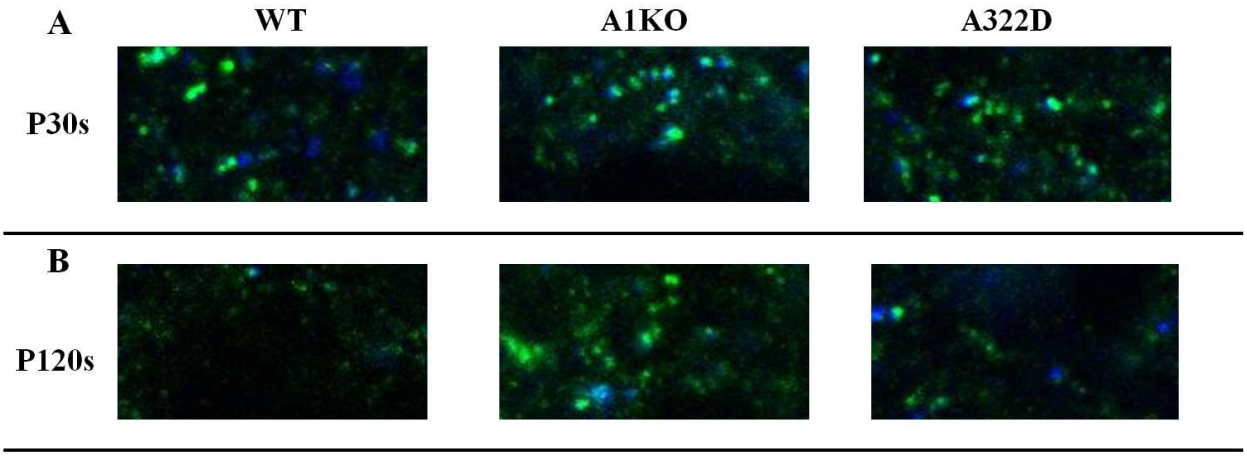
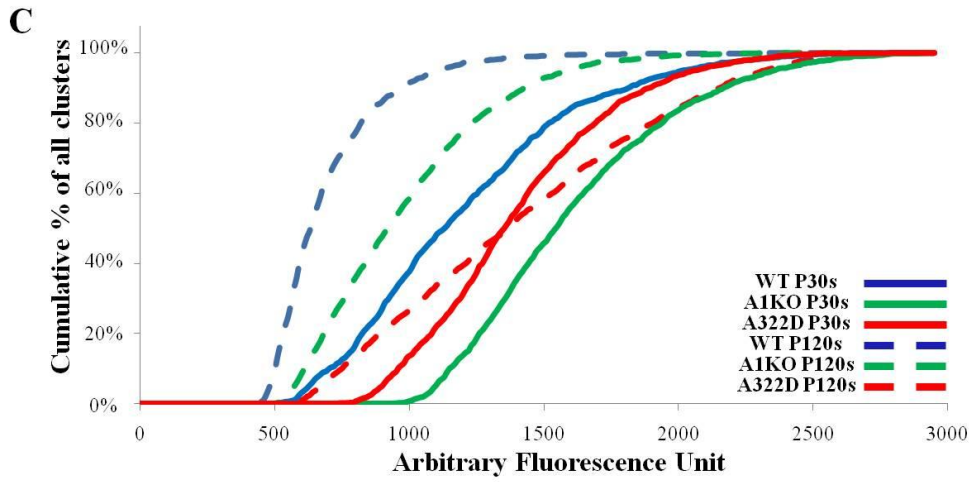


Figure 4-12: Number of co-localized gephyrin and α3 particles is significantly reduced in WT P120s mice but not in Gabra1 KO and A322D KI mice. Examples of co-localized gephyrin and α3 particles are shown for (A) P30s and (B) P120s Gabra1 KO, A322D KI and WT mice. (C) Quantification of the co-localized gephyrin and α3 particles is shown. *p value* < 0.001





Discussion

Epilepsy is fundamentally a circuit phenomenon[35]. It is increasingly recognized that seizures may leave their imprint on the developing brain by altering the way that neurons differentiate, connect and communicate with each other, even if, in many cases, such changes may be ultimately compensated for[36]. Therefore, understanding the biochemical mechanisms underlying seizures and epilepsy is crucial for gaining a better understanding of this disease and developing better treatment strategies for it.

GABA is the major inhibitory neurotransmitter in mammalian central nervous system[37] and mutations in its receptors have been associated with epilepsy[38]. The composition of the pentameric $GABA_A$ receptor, consisting of α1–6, β1–3, γ1–3, δ, ϵ, θ, π, and ρ1–3 subunit, governs the intrinsic properties of the channel, such as the affinity for GABA, receptor kinetics, conductance and allosteric modulation[9, 39]. The regional and total expression of $GABA_A$ receptor α subunits changes significantly during development[25]. Since the identity of the α subunit incorporated into $GABA_A$ receptors plays a significant role in its physiological properties, any alteration in the subtype of α subunit can significantly alter the physiologic circuitry in the brain and potentially lead to abnormal activity, including seizures. In fact, alterations in the expression and function of $GABA_A$ receptor subunits have been documented in animal models of human cases of temporal lobe epilepsy[39].

Under physiologic conditions, the expression of α1 subunit is low early in development and restricted to a few areas, but it increases dramatically with age[25, 40]. On the other hand, α3 subunit expression is high early in development but declines with age and gets restricted to a few areas; for example reticular nucleus of the thalamus[24, 41]. In chapter II and our previous study[21], we have shown that P30s het Gabra1 KO mice have absence seizures. We have also shown that Gabra1 KO mice have an expected decrease in the expression of α1 subunit along with a robust increase in α3 subunit[20]. In chapter III, we showed that the Gabra1 A322D KI mice also have a similar incidence of absence seizure at P30s. We have now shown that the decrease in α1 and increase in α3 subunit expression observed in Gabra1 KO mice also occurs in

Gabra1 A322D KI mice. Therefore no difference in the cellular or behavioral level was seen between Gabra1 KO and A322D KI mice at P30s.

In chapter III, we reported a significant finding that at P120s the Gabra1 KO and A322D KI mice not only continue to have absence seizure but also start to have polyspike discharges, which were occasionally associated with subtle, but visible, myoclonic jerks. Interestingly, the decrease in the expression of α1 subunit in Gabra1 KO and A322D KI, compared to WT mice, at P30s was not seen in P120s. Although no significant difference was observed in the expression of α1 subunits, the expression of α3 subunits continued to be significantly higher in Gabra1 KO and A322D KI mice compared to WT mice. How does this compensation for the loss of α1 subunit, along with the continued over expression of α3 subunit, affect the functioning of the brain?

The pharmacological and physiological properties of $GABA_A$ receptors, like affinity for GABA, receptor kinetics, conductance and allosteric modulation is determined by their subunit composition[9]. For example, $GABA_A$ receptors that contain α1 subunits mediate relatively short inhibitory post-synaptic currents (IPSCs), the $GABA_A$ receptors that contain α2 or α3 mediate relatively long lasting IPSCs[10, 13, 14]. The proportion of $GABA_A$ receptors containing any particular α subunit can have a significant effect on the overall functioning of the $GABA_A$ receptor expressing neurons. Perturbation of $GABA_A$ receptor signaling may facilitate seizures[36].

We have recently shown that the α3 subunit was up-regulated in the P30s Gabra1 KO mice, compared to WT mice[20]. This change was accompanied with reduced peak amplitude and increased rise and decay time of mIPSCs, recorded from somatosensory cortex layer VI pyramidal neurons P30s Gabra1 KO mice[20]. Based on the comparison of our findings for behavior and protein expression of Gabra1 KO and A322D KI mice, it is not unreasonable to expect that electrophysiological findings would be similar in both Gabra1 KO and A322D KI mice.

We have reported changes in the seizure phenotype (polyspike discharges evolve along with persistent absence seizures) and the protein expression (expression of α1 subunit is no longer decreased compared to WT) at P120s in Gabra1 KO and A322D KI mice, compared to P30s mice, in this thesis. Two key questions come up in regards to these finding: (1) are the electrophysiological properties of layer VI pyramidal neurons different at P120s in Gabra1 KO and A322D KI mice, compared to P30s and (2) do a unique class of $GABA_A$ receptors exists at P120s in Gabra1 KO and A322D KI mice, that is involved in these behavioral changes.

It has been reported that 18% of juvenile myoclonic epilepsy patients have a history of persistent absence seizures, and this group has been called "childhood absence epilepsy evolving into juvenile myoclonic epilepsy"[42]. In this group of patients, absence seizures alone or in combination with myoclonic jerks persisted in 63% of patients for as long as 11-50 years, in spite of antiepileptic drug treatment[42]. Behavioral finding in Gabra1 KO and A322D KI mice, of absence seizures persisting and evolving into polyspike discharges, makes them a good model for studying this treatment resistant class of JME. Based on our results from western blot and immunohistochemistry studies, we hypothesize that a different class of $GABA_A$ receptor exists later in development of Gabra1 KO and A322D KI mice. Identification of this class of $GABA_A$ receptors can lead to valuable insights into molecular basis of this treatment resistant class of JME and possibly lead to the development of better therapeutic interventions.

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Chapter V

Discussion and future Directions

The novel findings of these studies include: (1) Loss of $GABA_A$ receptor α1 subunit via $GABA_A$ receptor α1 subunit (Gabra1) resulting from knock-out (KO) and A322D knock-in (KI) mutations, is sufficient to cause an absence epilepsy phenotype in genetically modified mice. (2) Absence seizures persist and evolve into myoclonic-like epilepsy in Gabra1 subunit KO and A322D KI mice at postnatal day (P) 120. (3) Expression levels of $GABA_A$ receptor subunits change dynamically with age in Gabra1 KO and A322D KI mice.

Gabra1 KO and A322D KI mutations cause an absence epilepsy phenotype in genetically modified mice

Two mutations in the α1 subunit of $GABA_A$ receptor, S326fs328X and A322D, are associated with childhood absence epilepsy and juvenile myoclonic epilepsy, respectively[1, 2]. *In vitro* studies conducted on S326fs328X mutation showed a complete reduction in the expression of $GABA_A$ receptor α1 subunit[3], while A322D mutation reduced the $GABA_A$ receptor α1 subunit expression by 88%[4]. These findings make the $GABA_A$ receptor α1 subunit (Gabra1) KO mouse a relevant model to study epilepsy. Interestingly, previous studies conducted on Gabra1 KO mice reported the lack of visually apparent seizures[5, 6]. But one must keep certain factors under consideration when studying any animal model for epilepsy.

Firstly, epilepsy is a complex disease that has both genetic and environmental causes. Although the effects of environmental causes can be controlled by strict experimental protocols or overcome by increasing sample size, it is the genetic complexity that presents the greatest obstacle[7]. An important strategy used to control the genetic complexity, in mouse based studies, is the use of congenic strains[8]. Ever since the development of the congenic strains, by the Nobel Laureate George Snell[9], their use has been emphasized to get reproducible and consistent results. We used congenic strains of mice for all of

our experiments, but previous studies that reported a lack of visually apparent seizures did not use congenic strains[5, 6].

Secondly, the definition of seizure in animal models is challenging. It has been suggested that investigators should define objectively and quantitatively, what a seizure is and also show sufficient number of examples of the seizures[10]. Most definitions of seizures in animals require that they are detected by electroencephalogram (EEG) recording[11]. In particular, the use of EEG is essential for distinguishing nonconvulsive seizures from normal behavior of animals[11].

Therefore, we conducted synchronized video/EEG on Gabra1 KO and A322D KI mice maintained in a congenic background and discovered spike wave discharges (SWDs) that were associated with behavioral arrest. Furthermore, these SWDs had bihemispheric origin. Finally, treatment with ethosuximide (a drug used to treat absence epilepsy) resulted in reducing the incidence of SWDs in Gabra1 KO mice. With all of these findings, we have provided objective evidence that both Gabra1 KO and A322D KI mice are good models of absence epilepsy. Furthermore, we showed that the incidence of SWDs in female C57BL/6 het Gabra1 KO mice was significantly greater than males. A similar sex dependent discrepancy has also been reported in human female epilepsy patients, termed catamenial epilepsy[12]. In particular, the incidence of childhood absence epilepsy is more common in females[13-15]. Therefore, C57BL/6 Gabra1 KO female mutant mice can be used to study catamenial epilepsy.

The concept of childhood absence epilepsy (CAE) as a benign epilepsy syndrome with high remission rates and few long-term neuropsychiatric consequences, is changing[16]. Previous studies give a 65-80% remission rate for childhood absence epilepsy[17-19]. But persisting and evolving seizure phenotype with associated subtle cognitive deficits, linguistic difficulties and psychiatric diseases, have also been reported[17-19]. Therefore, the persistence of absence seizures and evolution into myoclonic-like jerks in Gabra1 KO and A322D KI mice was an essential component of our investigation.

Absence seizures persist and evolve into myoclonic-like epilepsy in Gabra1 subunit KO and A322D KI mice at P 120

CAE is considered a relatively benign disease due to its high remission rates[20]. But a significant proportion of CAE patients have been reported to have absence seizures that persist and evolve into juvenile myoclonic epilepsy (JME) and generalized tonic-clonic seizures (GTCS)[17, 21]. The peak age of onset of concomitant myoclonic jerks, in these CAE patients, is 1 to 6 years after the onset of absence seizures[22]. 91% of such patients do not achieve complete long term seizure remission[23]. In another study, all of the treatment-resistant JME patients were reported to have other forms of seizures, along with myoclonic jerks[24].

We discovered that Gabra1 KO and A322D KI mice continue to have SWDs and absence seizures at P120s. We also observed that the Gabra1 KO and A322D KI mice had spontaneous polyspike discharges (PSDs), that were occasionally accompanied by subtle but visible myoclonic-like jerks. Rare GTCS were also noticed in mutant mice at P120s. Such an age dependent evolution of behavioral phenotype has not been reported in other rodent models of absence epilepsy. The characteristics of Gabra1 KO and A322D KI mice we report here, that include absence seizures persisting and evolving into myoclonic-like jerks, make them a very useful model to study this treatment resistant form of epilepsy.

Since the incidence of PSDs was significant, but low, we decided to employ a previously described protocol of administering repeated low doses of pentylenetetrazole (PTZ) (a $GABA_A$ receptor antagonist[25]) to induce more frequent myoclonic jerks[26]. We noticed a reduced latency to develop PSDs in Gabra1 KO and A322D KI mice compared to WT mice at P120s that were associated with visible myoclonic-like jerks. The Gabra1 KO and A322D KI mice also showed a trend towards increased probability of developing generalized tonic-clonic seizures (GTCS) with PTZ administration. The spontaneous and PTZ-induced myoclonic-like jerks observed in these P120s Gabra1 KO and A322D KI mice were similar in characteristics with other known models of myoclonic epilepsy. For example, in

Efhc1 mutant mice (a known model of myoclonic epilepsy), episodes of myoclonic jerks have been reported at 7-8 months of age[27]. Furthermore, the spontaneous, quick (<200ms), high amplitude multi-spikes observed on the EEG recording of Efhc1 mutant mice[27] were similar to the spontaneous PSDs we observed in P120 Gabra1 KO and A322D KI mice.

Expression levels of $GABA_A$ receptor subunits change dynamically with age in Gabra1 KO and A322D KI mice

The kinetics, conductance and allosteric modulation of $GABA_A$ receptor is significantly affected by the type of α subunit it contains[28]. The expression of α3 subunit declines, while α1 subunit increases with age in rodent brains[29]. Abnormal temporal or spatial expression of $GABA_A$ receptor α subunits can have a significant effect on the physiology of $GABA_A$ receptor expressing neurons. We have recently shown that the expression of α3 subunit is abnormally high in P30s Gabra1 KO mice, while the expression of α1 subunit was reduced as expected[30]. The electrophysiological finding of layer VI cortical neurons of Gabra1 KO mice, including peak amplitude and decay rate, was also significantly different compared to WT mice[30]. These Gabra1 KO mice also had absence seizures[31].

When we determined the expression of α1 and α3 subunits in Gabra1 A322D KI mice at P30s using western blot analysis, we discovered a similar decrease in α1 subunits and increase in α3 subunits, while no significant difference was observed between Gabra1 KO and A322D KI mice. Interestingly, at P120s, although the expression of α3 subunit remained high in Gabra1 KO and A322D KI mice, the difference in the expression of α1 subunit no longer existed between mutant and WT mice.

We also quantified the expression of α1 and α3 subunits in layer II/III and VI of motor cortex of WT, Gabra1 KO and A322D KI mice, using immunohistochemistry (IHC). Consistent with our results from western blot experiments, we report an increase in α3 subunit expression in layers II/III and VI, at both P30s and P120s. Also consistent with our results from western blot experiments, we didn't see a significant difference in the expression of α1 subunit in Gabra1 KO and A322D KI mice compared to

WT, at P120s. However at P30s we didn't see the decrease in the expression of α1 subunit in Gabra1 KO and A322D KI mice that we saw in our western blot results. But this finding from IHC experiments could possibly happen if the changes in α1 subunit expression occurs in cortical layers other than layer II/III and VI or in a region other than the motor cortex, for example somatosensory cortex.

In vitro studies have demonstrated a direct interaction between gephyrin and α1[32] and α3[33] subunits that helps in the formation of a stable ternary structure. In our IHC experiments to stain for gephyrin and α1 and α3 subunits, we did not see any significant change in the number of gephyrin and α1 co-localized particles between Gabra1 KO, A322D KI and WT mice at either P30s or P120s, but interestingly, the number of α3 and gephyrin co-localized particles at P120s was significantly reduced compared to P30s in the WT mice. But no significant difference was observed in the number of α3 and gephyrin co-localized particles at either P120s or P35s in Gabra1 KO and A322D KI mice, indicating a persistent expression during development.

These findings suggest a potential role of Gabra1 KO and A322D KI mutations on the function of the $GABA_A$ receptor. Since the electrophysiological properties of $GABA_A$ receptors containing α1 subunits is significantly different than those that contain α3 subunit, these changes in expression pattern of α subunits, seen at P120s, can have a significant impact on the electrophysiological properties of cortical neurons, for example peak amplitude and decay rate. These changes in expression pattern, and possibly electrophysiology, can have a significant impact on the brain circuitry that involves $GABA_A$ receptors. Such a modulation of circuitry can be part of the etiology of the evolving seizure phenotype that we have reported in this thesis.

Future directions

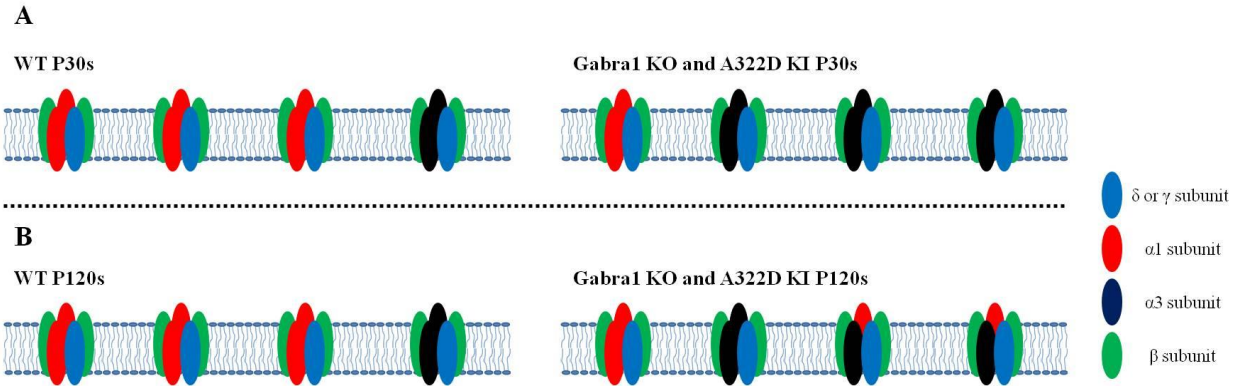
To better understand the role of the Gabra1 KO and A322D KI mutation in the etiology of childhood absence epilepsy (CAE) and juvenile myoclonic epilepsy (JME), further experiments need to be conducted. Our results have shown that Gabra1 KO and A322D KI mutant mice can be an extremely useful model to study this complicated and treatment resistant form of CAE that persists and evolves into JME.

Hypothesis and strategies for the future

Future hypothesis 1: Unique $GABA_A$ receptors exist at P120s in mutant mice

We hypothesize that at P30s, the absence seizures occur due to the different electro-physiological properties of the over expression of $GABA_A$ receptors containing α3. But the increase in α1 subunit seen in older (P120) mutant mice, could possibly result in the formation of unique $GABA_A$ receptors (containing both α1 and α3 subunits) that have unique electro-physiological properties compared to $GABA_A$ receptors containing either α1 or α3 subunits. The expression of this novel $GABA_A$ receptor may result in altered circuitry of the brain and consequently lead to an abnormal, epileptic phenotype. This hypothesis is summarized in figure 5-2.

Figure 5-2: Construct of $GABA_A$ receptors containing different α subunits in mutant mice at P30s and P120s. (A) Based on previous reports and our results, the expression of α1 subunit is greater in WT mice, while the expression of α3 subunit is higher in the Gabra1 KO and A322D KI mice at P30s. (B) Based on our hypothesize, unique $GABA_A$ receptors (that contain both α1 and α3 subunits) exist in Gabra1 KO and A322D KI mice at P120s.



Strategies to test future hypothesis 1:

A) The increased expression of $GABA_A$ receptors that contain both α1 and α3 subunits at P120s result in unique electrophysiological properties $GABA_A$ receptors in neurons of Gabra1 KO and A322D KI mice

The kinetics and pharmacology of $GABA_A$ receptors depends on their α subunit composition[35, 36]. *In vitro* studies conducted in HEK293 cells show that, compared to α1 subunit-containing $GABA_A$ receptors, α3 subunit-containing $GABA_A$ receptors have slower activation and deactivation kinetics, along with decreased sensitivity to GABA[37]. We recently showed, by recording from brain slices of Gabra1 KO mice (that express more α3 subunit compared to WT), that the peak current amplitude is reduced and the rise and decay time of mIPSCs is increased in layer VI cortical neurons of Gabra1 KO (that express more α3 subunit) than WT[30].

We have reported that the seizure phenotype evolves in the Gabra1 KO and A322D KI mice from only absence seizures at P30s to absence seizure and myoclonic-like jerks at P120s. This change in seizure phenotype is accompanied by an increase in α1 subunit expression in Gabra1 KO and A322D KI mice at P120s compared to P30s. As explained in my hypothesis above, it is possible that at P120s $GABA_A$ receptors are present in Gabra1 KO and A322D KI mice that express both α1 and α3 subunit and have completely different kinetics and pharmacology than we see due to the activity of $GABA_A$ receptors that express either α1 or α3 subunits alone. In order, to test this hypothesis we need to conduct whole-cell patch clamp recording on pyramidal neurons of both layer I/II and layer VI of somatosensory and motor cortex of WT, Gabra1 KO and A322D KI mice and see if the characteristics of mIPSCs are different between the two age groups.

B) Using co-immunoprecipitation, we can test if α3 and α1 subunit are part of the same protein complex

Co-immunoprecipitation is a popular technique used to identify protein complexes. Many studies have used this technique to detect the association of surface expressed $GABA_A$ receptors with other proteins, for example gephyrin[38] and potassium and chloride channel[39]. To determine the presence of a unique population of $GABA_A$ receptors that contains of both α1 and α3 subunits, we would need to isolate surface protein from biotinylated brain slices of WT, Gabra1 KO and A322D KI mice at P30s and P120s. These biotinylated proteins would be immunoprecipitated using antibody against α1 subunit and then stained for α3 subunit. Separately, the biotinylated proteins will be immunoprecipitated using antibody against α3 subunit and then stained for α1 subunit. If a population of $GABA_A$ receptors that contain both α1 and α3 subunits exist (figure 5-2 B), immunoprecipitation using either α1 or α3 subunits will be able to give α3 and α1 subunits, respectively.

Future hypothesis 2: The change in $GABA_A$ receptor subunit expression is not due to seizures, but only the result of Gabra1 KO and A322D KI mutations

Previous studies have shown that chemically-induced seizures cause a reduction in the expression of α1, α4, β2/3 and γ2 subunits in the CA1 region of hippocampus[40, 41]. However, following γ-hydroxybutyric acid (GHB) induced absence seizures in rats, a significant increase in α1 subunit mRNA and decrease in α4 subunit mRNA was observed in the thalamic relay neurons[42]. In another study, hyperthermia-induced seizures resulted in decreased expression of α3 and α2 subunit mRNA in the dentate gyrus of neonatal rats[43]. Therefore, artificially-evoked seizures can alter the expression of $GABA_A$ receptor subunit expression. But do absence and myoclonic-like seizures occurring in Gabra1 KO and A322D KI mice also play a role in alteration of $GABA_A$ receptor subunit expression? We hypothesize that the changes in $GABA_A$ receptor subunit expression are not due to seizures, but only the result of Gabra1 KO and A322D KI mutations.

Strategy to test future hypothesis 2:

In order to test this hypothesis, we can chemically or electrically induce seizures in WT, Gabra1 KO and A322D KI mice, at P30s (age at which only absence seizures occur) and P120s (the age at which persistent absence seizures and myoclonic-like jerks occur). If our hypothesis is correct, Gabra1 KO and A322D KI mice will have similar levels of $GABA_A$ receptor subunit expression after chemically- or electrically-induced seizures, as those seen in unprovoked mutant mice (results of unprovoked mutant mice are shown in chapter 4). But the $GABA_A$ receptor subunit expression will be drastically different in WT mice after chemically- or electrically-induced seizures, as expected based on previous reports.

Future hypothesis 3: Gabra1 KO and A322D KI mice are models of attention deficit hyperactivity disorder.

Psychiatric disorders are very common in CAE and JME patients. Children affected by CAE are known to have difficulty in visual sustained attention, verbal and nonverbal attention, and memory, despite a good response to antiepileptic medications and normal intelligence[44]. 61% of CAE patients have also been diagnosed with a psychiatric disorder, in particular attention deficit hyperactivity disorder (ADHD)[19]. Attention problems in CAE patients can also interfere with children's academic performances[45]. Similarly, 49% of JME patients also suffer from psychiatric disorders, which include ADHD[46]. JME patients also have problems with verbal and visual memory[47] and social adjustment[48]. Based on our findings that Gabra1 KO and A322D KI mice are a good model for absence epilepsy and myoclonic-like jerks, we hypothesize that Gabra1 KO and A322D KI mice are also a model for ADHD.

Strategies to test future hypothesis 3:

To test for ADHD phenotype in mutant mice, we can use the commonly employed strategies that include, water maze test[49], aggressive behavior[50], Y-maze test[51], novel object recognition test[50], elevated zero maze test[52] and light/dark test[53]. Findings from these studies can help us better understand the severity of psychiatric comorbidies in patients suffering with this complicated version of CAE persisting and evolving into JME.

Future hypothesis 4: Gabra1 KO and A322D KI mice have altered circadian rhythm

A characteristic sleep/wake cycle has been reported in JME patients: they fall asleep late and get up late in the morning, with prolonged drowsiness in the morning[54]. It has been reported that JME patients have more frequent myoclonic jerks on awakening, which occasionally terminate in a convulsive seizure[55]. But this relationship between sleep-wake cycle and JME has been the subject of controversy. There are two

hypotheses for this circadian dysrhythmia and seizures: (A) an epileptic patient with self inflicted distorted lifestyle is more likely to become symptomatic with altered circadian rhythm or (B) the circadian dysrhythmia and seizures are a symptom of JME and a symptom of underlying subcortical/cortical disregulation[54]. Based these reports, we hypothesize that the Gabra1 KO and A322D KI mice have different circadian rhythm compared to WT.

Strategy to test future hypothesis 4:

To test for altered circadian rhythm, mice should be placed in special cages, kept in light tight ventilated cabinets (commonly used for such circadian rhythm studies), where the general activity of mice will be recorded individually and continuously with passive infrared movement detectors and wheel running activity[56]. Mice should be observed for 21 days in 12hour light/dark cycle, followed by 2weeks of total darkness cycle and finally 2 weeks of continuous light, as is routinely done for similar experiments. If our hypothesis is correct then the mutant mice will register movements significantly late in night and stop significantly late in morning, compared to WT mice.

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