MECHANISMS OF PERSISTENTLY DECREASED INHIBITION OF AREA CA1 OF THE HIPPOCAMPUS FOLLOWING PERINATAL HYPOXIA-INDUCED SEIZURES

A Dissertation

By

JASON ARNOLD JUSTICE

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Chair of committee,	Russell Sanchez
Committee members,	Sharon Demorrow
	Dave Dostal
	Shenyuan Zhang
Head of Department,	Harry Papaconstantinou

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ABSTRACT

Hypoxia in the neonate is one of the leading causes of encephalopathy and seizures. Sadly, a significant subset of these children will acquire epilepsy, as their risk increases several-fold even with recovery from the initial encephalopathy. Epilepsy can be debilitating and potentially fatal, and can be considered as a condition of hyper-excitable and hyper-synchronistic neural circuitry. Cellular and molecular changes consequent to neonatal seizure-inducing hypoxia are not fully understood, but multiple pro-epileptogenic candidate mechanisms exist. For example, acutely decreased inhibition has been shown at 1 hour post-hypoxia induced seizures, but it is unknown whether this effect is persistent. Such persistent effects on inhibition could promote the development of epilepsy. We designed three specific aims to determine mechanisms of persistently decreased inhibition of area CA1 of the hippocampus following perinatal hypoxia-induced seizures.

Specific Aim One was to determine whether the acute (P10) decrease in GABA mediated inhibitory currents, IPSCs, persists beyond the initial 24 hours reported previously. We hypothesized that there would be a persistent increase in inhibition at least one week post hypoxia. Whole-cell voltage-clamp data indicates persistently decreased action potential-dependent GABA-mediated inhibitory post synaptic currents that may possibly be due to decreased interneuron firing.

Specific Aim Two was to determine whether there are persistent changes to the composition/expression of the $GABA_A$ receptor in hippocampus. We hypothesized that there would be persistent decreases in the overall expression and or composition of the

$GABA_A$ receptor. Western blot analysis suggests that no change in the expression of α1, α2, β2/3 or γ2 $GABA_A$ subunits occurs, at any time point studied, following perinatal hypoxia-induced seizures.

Specific Aim Three was to determine whether there are persistent changes in calcineurin expression/activity. We hypothesized that there would be an increase in hippocampal calcineurin expression and/or activity persistently following hypoxia-induced seizures. Western blot data suggests that no change in the expression of calcineurin occurs beyond the initial 24 hours post hypoxia-induced seizures previously reported. Furthermore, calcineurin activity did not increase at 24 hours and 48 hours post hypoxia-induced seizures. Preliminary data suggests activation of an alternative enzymatic pathway, possibly Interleukin-1 receptor dependent activation of casein kinase II, possibly contributing to altered GABAergic inhibition of area CA1 of the hippocampus.

This work is dedicated to my loving wife, Laura.

Without your love and support this would not have been possible.

I love you!

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CHAPTER I

INTRODUCTION

Significance

The incidence of seizures is highest in the neonatal period and most commonly caused by hypoxic encephalopathy (Volpe 2001). Clinically, infants are exposed to hypoxia during a number of potential conditions, including perinatal asphyxia, respiratory arrest, near-miss sudden infant death syndrome, and during corrective heart surgery. Perinatal cerebral hypoxia greatly increases the risk of developing cerebral palsy, developmental delay and seizure disorders (Volpe 2001, Bernes and Kaplan 1994, Bergamasco et al 1984). Unfortunately, neonatal hypoxia induced seizures can be refractory to conventional anti-epileptic drug treatment. Understanding how perinatal cerebral hypoxia occurs and increases the susceptibility to develop epilepsy is critical if we hope to successfully treat seizures in this setting and prevent the sequence of events that lead to increased seizure susceptibility later in life.

In the early 1990s, researchers developed a neonatal animal model of global central nervous system hypoxia (Jensen et al 1991). This animal model replicates the human condition in key elements including; (I) age dependent susceptibility to hypoxia induced seizures, (II) refractoriness to anti-epileptic drug treatment, and (III) long-term increases in seizures susceptibility (Rakhade et al 2011, Jensen et al 1991). In 2005, Sanchez et al, demonstrated that following hypoxia-induced seizures at P10, rats exhibited decreased frequency and amplitudes of GABA-mediated inhibitory post-synaptic potentials (IPSCS) in CA1 hippocampal pyramidal neurons. Furthermore,

antagonism of calcineurin and or blockade of AMPA receptors (a type of glutamate receptor) caused an increase in the frequency and amplitudes of GABA-mediated inhibitory post synaptic currents in area CA1 of the hippocampus, apparently opposing the effects of hypoxia. During this same time period the phosphorylation of the β2/3 subunit of the GABA receptor was decreased suggesting that calcineurin may act on GABA mediated IPSCs by dephosphorylation of the $GABA_A$ receptors, causing increased internalization, and hence decreased amplitudes and frequency of IPSCs. Probably the most interesting aspect of this paper was that pre administration of FK-506, an antagonist of calcineurin, significantly inhibited hypoxia-induced perinatal seizures, emphasizing the importance of this pathway in this model.

Many unknowns remain to be explored in this animal model. For example, whether or not GABA mediated inhibition remains persistently decreased beyond the first day following hypoxia-induced seizures is unknown. Furthermore, since acute changes occur in both amplitude and frequency of IPSCs, the potential for pre- and post-synaptic mechanisms is probable. Finally, it remains to be determined whether calcineurin activity/expression remains increased. We have proposed 3 specific Aims to address these questions.

Specific Aim One will determine whether the acute (P10) decrease in GABA mediated inhibitory currents, IPSCs, persists beyond the initial 24 hours reported previously (Sanchez 2005). We hypothesized that a persistent increase in inhibition would occur at least one week post-hypoxia. Specific Aim Two will determine whether there are persistent changes to the composition/expression of the $GABA_A$ receptor in

hippocampus. We hypothesized that a persistent decrease in the overall expression and or composition of the $GABA_A$ receptor would occur. Specific Aim Three will determine whether persistent changes in calcineurin expression/activity occur. We hypothesized that an increase in hippocampal calcineurin expression and/or activity would occur following hypoxia-induced seizures.

The following chapters will contain a discussion of the results and the implications of those results in the context of increased seizure susceptibly in the rat model of hypoxia-induced seizures. The introductory chapter will explain, in more depth, the importance of each component of which this dissertation is focused, in order to better understand the unique nature of neonatal hypoxia-associated epilepsy.

Background

Epilepsy

Epilepsy is a common neurological disorder, affecting approximately 1% of the world's population (Theodore et al 2006). Epilepsy is a pathological condition of hyperexcitable and hypersynchronous neural circuitry that manifests itself clinically as seizures. Seizures can cause neuronal cell loss, rewiring of neural circuitry, and redistribution of ion channels when excessive electrical activity occurs for long periods. This has been shown in numerous animal models and among others, altered $GABA_A$ and hyperpolarization activated cyclic nucleotide gated ion channels (HCN channels) may be involved in promoting epilepsy. For example, the expression of HCN channel has been shown to be modified in many animal models of epilepsy (Chen 2001, Budde 2005, and Shah et al 2004). HCN2 knockout mice display an altered I_H and an absence-like seizure phenotype (Ludwig et al 2003). Also, decreased neocortical HCN1 expression has been found to be associated with a rat genetic absence epilepsy model (Strauss et al 2004). Our laboratory recently showed that I_H is attenuated in pyramidal neurons in a neonatal hypoxia model of epilepsy (Zhang et al 2006). Although several studies have indicated an indirect role of HCN channels in regulating inhibition (Macaferri and McBain 1996, Lupica et al 2001), this is beyond the scope of this dissertation. This dissertation will focus on direct regulatory GABAergic mechanisms.

Alterations in GABAergic inhibition have been implicated in both human epilepsies and rat models of epilepsy. The expression and localization of several $GABA_A$ receptor subunits have been reported to be altered by seizures (. The functional

consequences of these alterations include changes in phasic and tonic GABA currents, as well as, alterations in pharmacological sensitivity to benzodiazepines (Mtchedlishviliet al 2001, Kapur and Macdonald 1997). Despite GABA's well-established role in epilepsy, the regulatory mechanisms governing pathological GABAergic inhibition are still being elucidated.

Neonatal Hypoxia

As mentioned above, hypoxia in the neonate is one of the leading causes of encephalopathy and seizures (Volpe et al 1989) and a significant subset of these children will acquire epilepsy, as their risk increases five-fold even with recovery from the initial encephalopathy (Bergamasco et al 1984, Connell et al 1989, Volpe et al 1989). Cellular and molecular neural changes consequent to neonatal seizure-inducing hypoxia are not fully understood, but multiple pro-epileptogenic candidate mechanisms exist. In the early 1990s Jensen et al began characterizing an animal model of neonatal hypoxia-induced seizures that mimics the acute and long-term epileptogenic effects of neonatal hypoxia observed clinically (Jensen et al 1991).

Rat pups exposed to global hypoxia (5-7% O_2 for 15 minutes) on postnatal day 10 exhibit spontaneous seizures and later exhibit chronically increased seizure susceptibility. Hippocampal slices obtained from these animals several days after the hypoxia-induced seizures exhibit an increased propensity for electrographic seizure-like activity, thus providing a convenient in vitro preparation to identify cellular mechanisms that underlie this hippocampal hyperexcitability. For example, acutely decreased inhibition has been shown in slices 1 hour post-hypoxia induced seizures, but it is

unknown whether this effect is persistent (Sanchez et al 2005). Such persistent effects on inhibition could promote the development of epilepsy.

Decreases of inhibitory neurotransmission could be due to several mechanisms including changes in the release of GABA from presynaptic stores, the composition/expression of the $GABA_A$ receptor, and the state of $GABA_A$ receptor modulation through various post-translational processes, especially calcineurin, a highly conserved Ca^{2+} and calmodulin-dependent serine/threonine phosphatase, which has been shown to be acutely up-regulated after hypoxia-induced seizures (Sanchez et al 2005). Altered $GABA_A$ receptor subunit expression and pharmacological sensitivity have been reported in neonatal, as well as, adult rodent models of seizures and human epileptic hippocampi (Zhang et al 2004, Schwarzer et al 1997, Bouilleret et al 2000, Raol et al 2006, Loup et al 2000). However, it is not known whether changes in expression of GABA receptor subunits occur following neonatal hypoxia-induced seizures.

Calcineurin is a serine threonine specific phosphatase involved in many essential cellular processes. Calcineurin is a key phosphatase involved in neonatal hypoxia-induced seizures, as demonstrated by an acute increase in calcineurin expression/activity following neonatal hypoxia-induced seizures and most importantly, blockade prevented hypoxia-induced neonatal seizures (Sanchez et al 2005). This increased calcineurin activity was associated with decreased phosphorylation of the β2/3 subunit of the $GABA_A$ (Sanchez et al 2005). Still, whether calcineurin expression/activity persists and whether post-synaptic changes occur to $GABA_A$ receptors are unknown.

Hippocampus

The hippocampus, Greek for sea horse, is a specialized area of the brain whose main function is short-term memory processing. Since memory is involved with so many different types of human diseases and disorders including Alzheimer's, epilepsy (including many pharmacologically intractable varieties), head injuries that cause amnesia and age associated memory loss, it is a very highly researched area of the brain. The basic circuitry of the hippocampus begins with the perforant pathway arising from the entorhinal cortex projecting glutamatergic synaptic boutons on the cell dendrites and soma of granule neurons lying in an area known as the dentate gyrus. These cells not only receive excitatory input from the entorhinal cortex but also GABAergic inhibitory synapses from hilar polymorophic neurons located within the hilus. The mossy fiber pathway is the next in this looping pathway also sending glutamatergic projections but this time from the dentate gyrus granule neurons to pyramidal neurons located in area CA3. These pyramidal neurons then send afferent glutamatergic projections to area CA1 pyramidal neurons through an axonal pathway called the schaeffer collaterals. Excitatory principal cells in each region are under the control of local inhibitory GABA-secreting interneurons. In area CA1,pyramidal neurons receive inhibitory input primarily from stratum radiatum and stratum oriens. Finally, these CA1 pyramidal neurons send afferent projections through the subiculum back to the entorhinal cortex, completing a loop. This looping network, once compromised, can lead to high frequency synchronized oscillations of action-potential bursting groups of neurons innervating themselves through a polysynaptic feedback loop.

Interneurons of the Hippocampus

The interneurons of the hippocampus, like the neocortex, are highly specialized and highly diverse class of neurons that provide inhibition to neural networks. Unlike their principal cell counterparts, these cells have highly diverse morphological and biochemical attributes. Interneurons are characterized by the synthesis and release of the neurotransmitter GABA and exert their inhibitory control on the activity of glutamatergic neurons through the activation of postsynaptic $GABA_A$ receptors, thereby creating inhibitory currents in their target cells. A number of interneurons of the hippocampus have been described based on numerous different criteria over the years. However, as researchers began to classify interneurons it was found that no single subtype could be definitively identified as many of the different markers used to separate them overlapped, and an adequate classification system that is universally accepted is still needed to this day (Buzsaki 2004, Baraban and Tallent 2004, Cossart 2005, Jonas 2004).

The morphological and physiological characteristics of adult hippocampal interneurons have been extensively reviewed (Freund and Buzsaki 1996, Somogyi and Klausberger 2005). GABAergic interneurons provide local feed-forward and feed-back inhibitory circuits. The axonal arborization of interneurons allows one interneuron to contact several hundreds of pyramidal neurons and provides them with the capability to synchronize the activity of glutamatergic neurons and therefore play a fundamental role in shaping the temporal pattern of various types of neural oscillations (Freund and Buzsaki 1996, Somogyi and Klausberger 2005). Hippocampal interneurons have been

classified into several subgroups according to their axonal projection pattern, rather than by cell morphology. Basket cells make synapses specifically with the cell bodies and proximal dendrites of principal cell, while chandelier or axo-axonic cells contact the axon initial segment of principal neurons (Somogyi et al 1983). Furthermore, basket cells establish synapses not only with the cell bodies and proximal dendrites of principal neurons but also with other basket cells (Fukuda and Kosaka 2000). Other interneurons target specific parts of the dendrites of pyramidal neurons. For example, oriens-lacunosum moleculare(O-LM) cells are located in the stratum oriens but contact the distal-most part of the apical dendrites of pyramidal neurons in the stratum lacunosum-moleculare. These various interneuron subtypes may differentially affect the activity of excitatory neurons. Inhibitory synapses on cell bodies or axon initial segments are ideally located to control the genesis of action potentials, while interneurons targeting the dendrites of pyramidal neurons may control dendritic calcium spikes (Miles et al 1996). Interneurons with specific projection patterns also express particular calcium-binding proteins or neuropeptides (Freund and Buzsaki 1996).

GABA Receptors

Many receptors are named after the primary activating agonist. This is mainly due to the historical context from which they were researched. For example, $GABA_A$ (and $GABA_C$) and $GABA_B$ are related only by the activating agonist GABA, gamma amino butyric acid. Both receptor types are widely distributed, but differ both in modes of action and molecular composition. While $GABA_B$ nomenclature matches $GABA_A$ and $GABA_C$, it is not part of the same superfamily. The $GABA_A$ receptor is the most widely

distributed and important inhibitory molecules in the central nervous system where it is responsible for IPSCs, inhibitory postsynaptic potential currents in many cell types from a diverse phylogenetic tree. Since the explosion of molecular techniques in the early 1990s we now know that the $GABA_A$ system is a member of a superfamily proteins coined Cys-loop ligand-gated ion channels (LGIC), named for their characteristic loop formed by a disulfide bond between two cysteine residues. Members of this superfamily include nicotinic acetylcholine receptors, glycine receptors, as well as the $GABA_A$ receptor. All members of this superfamily are composed of 5 subunits that form heteromeric proteins with an integrated ion pore. In the case of the $GABA_A$ receptor, this ion pore conducts chloride ions following the binding of GABA. This increases the chloride conductance, providing an inhibitory current (and hyperpolarization of the membrane) to the neuron, that if great enough ceases action potential firing.

$GABA_A$ Receptor Subunits

$GABA_A$ receptors are members of the ligand-gated ion channel superfamily. The $GABA_A$ receptor protein is a pentamer with an integrated ion channel pore formed by the heteromeric assembly of various $GABA_A$ receptor subunits. Several subunits can combine to form pharmacologically distinct receptor subtypes. These subunits include the following, α1-6, β1-3, γ1-3, d, ε, π, θ, ρ1-3 (Simon et al 2004). Of most interest to our study are primary synaptic $GABA_A$ receptors that are composed of 2 α subunits, 2 β subunits, and a single γ subunit. Mutations of the genes that encode $GABA_A$ receptors have delitarious effects. For example, humans that have mutations in genes for these subunits suffer from various forms of epilepsy.

Benzodiazepines

Status epilepticus is a syndrome consisting of a very prolonged seizure with continuous evolution of neurological state, worsening cerebral metabolism, a steady rise in core temperature, a rise in blood pressure, lactic acidosis, hyperglycemia and increased catecholamine levels (Simon et al 1984; Meldrum et al 1973).In humans, status epilepticus is treated with benzodiazepines, including diazepam, lorazepam, and midazolam and barbiturates, including phenobarbital and pentobarbital, all of which exert an anticonvulsant effect by acting on the GABA receptors (Macdonald and Kelly 1995).Once bound to the $GABA_A$ receptor, diazepam locks the receptor protein complex into a conformation which has a greater affinity for the endogenous neurotransmitter GABA. This increases the frequency of the opening of the associated chloride ion channel and hyperpolarizes the membrane of the associated neuron thereby inhibiting the neuron and decreasing action-potential firing resulting in decreased neuronal network excitability. The net effect being anticonvulsant, sedatory and/or anxiolytic effects depending on the chemical structure of the benzodiazepine and specific combination of GABA receptor subunits, as well as the region of the brain affected. Interestingly, both in humans and in experimental animals, benzodiazepines are efficacious in early, but not late status epilepticus (Yaffe and Lowenstein 1993, Walton and Treiman 1988). Thus, both experimental animal and human data suggest that the functional properties of hippocampal GABA receptors are rapidly modified during status epilepticus. This refractoriness to diazepam resulted from the loss of diazepam potency but not of diazepam efficacy. These studies suggested that refractoriness of seizures to diazepam

may result from altered GABA receptor function in the hippocampus (Kapur et al 1989, Kapur and Macdonald 1996, 1997, Kapur and Coulter 1995, Lothman et al 1991, Van Landingham et al 1991).

Benzodiazepines bind at the interface of α and γ $GABA_A$ receptor subunits. Diazepam sensitivity of GABA receptors requires the presence of the γ2 subtype with a β subtype and either α1, α2, α3, or α5 subtypes (Pritchett et al 1989, Macdonald and Olson 1994). Recombinant GABA receptors expressed without the γ2 subtype are highly sensitive to zinc (Zn), whereas GABA receptors expressed with the γ2 subtype are relatively insensitive to Zn (Draguhn et al 1990, Smart et al 1991). Therefore, one explanation for acute reduction of diazepam sensitivity of hippocampal dentate granule cell GABA receptors after seizures would be a loss of the γ2 subtype from the receptor; however this would not explain diminished Zn sensitivity of these receptors. Another potential explanation for diminished diazepam and Zn sensitivity would be an altered α subtype expression, because α subtypes are known to alter both Zn and diazepam sensitivity of the GABA receptors. More recently, $GABA_A$ receptor subunit specific surface trafficking has been shown in epileptic rats (Brooks-Kayal et al 1998). Seizures may alter GABA receptor function by other mechanisms such as posttranslational modification of GABA receptors or release of endogenous benzodiazepine-like substances. Modification of GABA receptors by phosphorylation is also well documented (Lin et al 1994, Macdonald and Olson 1994, MacDonald and Kelly 1995). For instance, seizures are known to modulate activity of calcineurin, which plays a role in GABA receptor function (Jope et al 1992, Perlin et al 1992).

Calcineurin

Interestingly, calcineurin is acutely up regulated following hypoxia-induced seizures in the neonatal rat. In this model, rats postnatal day 10-13 are susceptible to hypoxia-induced tonic-clonic and myoclonic seizures that later predisposes the animal to a lower seizure threshold and spontaneous seizures.. Calcium influx through GLUR2 negative AMPA receptors causes the acute activation of calcineurin, a highly conserved Ca^{2+} and calmodulin-dependent serine/threonine phosphatase. Following hypoxia-induced seizures, CA1 pyramidal neurons exhibit a down-regulation of $GABA_A$ receptor-mediated inhibition with a concomitant dephosphorylation of the β2/3 subunit that was reversed by calcineurin inhibitors. This study did not explore any other possible effects that calcineurin may have had on other ion channels. Recently, calcineurin was reported to regulate the hyperpolarization-activated current (I_H).

The pathological decrease in I_H in CA1 pyramidal neurons maybe due post-translational modification of the channel (Jung et al 2010). Acutely, calcineurin may cause dephosphorylation of the HCN channel (Jung et al 2010). However, this is unknown and the specific dephosphorylation site(s) have not been identified yet. Furthermore, the study associating calcineurin and I_H modulation used FK506, which may directly modulate other ion channels (Jung et al 2010). Finally, this study did not refute the hypothesis that FK506 may act by dephosphorylation of another HCN regulating protein.

This dissertation aims to broaden the characterization of altered GABAergic inhibition and elucidate underlying molecular mechanisms.

CHAPTER II

PERSISTENTLY DECREASED INHIBITION OF AREA CA1 OF THE HIPPOCAMPUS FOLLOWING PERINATAL HYPOXIA-INDUCING SEIZURES*

Introduction

Hypoxia is the most common cause of neonatal seizures and can lead to epilepsy, but the epileptogenic mechanisms are not yet understood. We have previously shown that hypoxia- induced seizures in the neonatal rat result in acutely decreased amplitudes and frequency of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs see appendixes A and B respectively) in hippocampal CA1 pyramidal neurons. In the current study, we asked whether such changes persist for several days following hypoxia-induced seizures. Similar to the acute findings, we observed decreased frequency and amplitudes of sIPSCs and decreased mIPSC amplitudes in CA1 pyramidal neurons at 3–5 days after hypoxia. However, in contrast to the acute findings, we observed no differences between hypoxia-treated and control groups in mIPSC frequency. Additionally, by 7 days after hypoxia, sIPSC amplitudes in the hypoxia group had recovered to control levels, but sIPSC frequency remained decreased. These data indicate that the persistently decreased sIPSC frequency result from decreased firing of presynaptic inhibitory interneurons, with only transient possible changes in postsynaptic responses to GABA release.

Cerebral hypoxia is the most common cause of neonatal seizures, and can lead to epilepsy, mental retardation, and cerebral palsy (Bergamasco et al 1984, Bernes and

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Kaplan 1994, Volpe 2001). Seizure-inducing global hypoxia in neonatal rats results in long-term increases in seizure susceptibility (Jensen et al 1991 and 1992) and can lead to learning deficits (Yang et al 2004), thus mimicking clinical aspects of neonatal hypoxia-induced seizures. Although such seizures in the neonate may be neocortical in origin, c-Fos immunocytochemistry several hours after hypoxia-induced seizures indicates involvement of the hippocampus (Jensen et al 1993), and electrophysiological studies in hippocampal slices from animals that experienced neonatal hypoxia-induced seizures have demonstrated consequent acute and chronic pathological increases in network excitability (Jensen et al 1998, Sanchez et al 2001). Thus, a single episode of hypoxia associated with seizures in the perinatal period can have adverse consequences for hippocampal function that may promote epileptogenesis, as well as disrupt cognitive function, in the long term.

The mechanisms that critically contribute to hippocampal dysfunction after perinatal hypoxia-induced seizures are yet poorly understood, but likely include pathological changes in synaptic inhibition. Inhibitory signaling mediated by γ-aminobutyric acid type A ($GABA_A$) receptors has been observed to be altered in the hippocampus and neocortex by various mechanisms in several experimental seizure models (for a review, see Cossart et al 2005). In the immature brain specifically, the developmental pattern of $GABA_A$ receptor subunit mRNA expression was shown to be disrupted throughout the hippocampus as early as 6 h after kainate-induced status epilepticus in postnatal day 9 (P9) rats (Lauren et al 2005), and pilocarpine-induced status in P10 rats was found to alter $GABA_A$ receptor subunit mRNA expression and

$GABA_A$ receptor function in dentate granule cells into adulthood (Zhang et al 2004). Additionally, hyperthermia-induced seizures in P10 rats resulted in increased evoked and miniature $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) within 1 week, changes that depend on pathological modulation of mechanisms that govern presynaptic GABA release (Chen et al 1999). Thus, although their precise contribution to possible hippocampal dysfunction and epileptogenesis remains under investigation, multiple mechanisms of GABA-mediated inhibition have been found to be pathologically altered acutely and chronically by seizures in the developing brain.

Using a rat model, we have previously demonstrated an acute decrease in basal synaptic inhibition of hippocampal area CA1 pyramidal neurons after hypoxia-induced seizures in the immature brain (Sanchez et al 2005). Specifically, we observed significantly decreased amplitudes and frequency of both spontaneous and miniature $GABA_A$ receptor-mediated IPSCs (sIPSCs and mIPSCs) in CA1 pyramidal neurons recorded in slices prepared 10 minutes after hypoxia-induced seizures at P10, and further found that these changes were associated with strong activation of the neuronal phosphatase calcineurin and dephosphorylation of $GABA_A$ receptors (Sanchez et al 2005). Notably, previous work demonstrated that hippocampal slices prepared as early as 10 minutes following hypoxia-induced seizures already exhibit hyperexcitability in area CA1 (Jensen et al 1998). Thus, perinatal hypoxia-induced seizures can dysregulate hippocampal synaptic inhibition through very rapid biochemical events, and this dysregulation may help to promote an immediate pathological increase in network excitability. Given the persistence of hippocampal hyperexcitability after hypoxia-

induced seizures, however, these early biochemical events must precede more permanent changes that are likely mediated by mechanisms other than kinase/ phosphatase signaling.

In the current study, we asked whether the acutely decreased basal synaptic inhibition of CA1 pyramidal neurons after hypoxia-induced seizures in P10 rat pups could be observed several days after hypoxia treatment, as we had previously found pathologically increased hippocampal excitability to persist during this period (Sanchez et al 2001). Our findings indicate that decreased inhibition persists for at least 1 week following hypoxia-induced seizures. However, whereas the immediate decreases may depend on both pre- and postsynaptic changes, the longer-lasting decrease appears to be mediated largely by pathologically decreased spontaneous firing of presynaptic inhibitory interneurons.

Methods

Animals

Long-Evans rat pups (Charles River), at an age of P10–P17, were used for these experiments. Litters were housed with their dam in the UTHSC (University of Texas Health Science Center at San Antonio) animal facility on a 12-hour light/12-hour dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with NIH guidelines on the ethical use of experimental animals.

Hypoxia Treatment

Seizures were induced in male Long-Evans rat pups on P10 by a 14–16-minute exposure to 5–7% O_2. Pairs of littermates were removed from their dam and each placed into a custom-made airtight chamber on heating pads to maintain a rectal temperature of 33–34° C. One chamber (control) was left uncovered and continuously exposed to room air, while the other (hypoxia) was covered and the O_2 concentration lowered by infusion of nitrogen gas into the chamber. The O_2 concentration was lowered to 6–7% for 4 minutes, then 5–6% for 8 minutes, and then lowered by 1% per minute until the animal became apneic for 30 seconds. The chamber was then uncovered and exposed to room air.

Using this protocol, spontaneous seizures typically began within 2–4 minutes of hypoxia, and occurred repeatedly throughout the hypoxia duration until apnea, and most animals continued to exhibit behavioral seizures for several minutes after returning to room air. At the end of hypoxia treatment, both animals in a pair were ear-marked with

sharp scissors and returned to their dam. Thus, control animals were handled identically to hypoxia-treated animals except for hypoxia exposure. All animals in the hypoxia-treated group used for this study exhibited a minimum of three tonic-clonic seizures.

Hippocampal Slice Preparation

Hippocampal slices were prepared 3–7 days after hypoxia treatment. Rat pups were killed by decapitation under isoflurane anesthesia. The brains were removed and immediately placed into ice-cold oxygenated artificial cerebrospinal fluid (ACSF) that contained (in mM): sucrose 201, KCl 3.2, Na_2PO_4 1.25, $MgCl_2$ 2, $CaCl_2$ 2, Na_2HCO_3 26, and D-glucose 10, bubbled with 95% O_2 /5% CO_2. The brains were blocked by making a razor cut along the coronal plane just anterior to the cerebellum, and the cut end was glued to the stage of a vibratome (Leica 1000 S). Coronal sections (350 μM) of slices were cut in cold, continuously oxygenated sucrose ACSF, and then incubated for at least 1 h in a custom-made holding chamber filled with continuously oxygenated recording ACSF that contained (in mM): NaCl 126, KCl 3.3, Na_2PO_4 1.25, $MgSO_4$ 1.3, $CaCl_2$ 2, Na_2HCO_3 26, and D-glucose 10, bubbled with 95% O_2 /5% CO_2 at room temperature.

Electrophysiological Recordings

Slices were transferred to a submersion chamber (Warner Instruments) that was superfused continuously with oxygenated ACSF at room temperature for recordings. Whole-cell patch-clamp recordings were obtained from CA1 pyramidal neurons under visual guidance using infrared differential interference contrast microscopy (Zeiss Axioskop FS2 with a Dage-MTI camera). The standard recording pipette solution

contained (in mM): Cs-gluconate 123, NaCl4, $MgCl_2$ 2, EGTA 10, HEPES 10, Na-ATP 4, GTP 0.3, pH 7.2. For experiments to study GABA-mediated currents at –70 mV, we used a pipette solution with approximately equimolar chloride to the extracellular solution that contained (in mM): CsCl 129, $MgCl_2$ 2, EGTA 10, HEPES 10, Na-ATP 4, GTP 0.3, pH 7.2. Filled recording pipettes had resistances of 1–3 megaohms, and series resistances were less than 20 megaohms.

Voltage clamp recordings were obtained using a Multiclamp 700A amplifier (Axon Instruments) and digitized with a Digidata 1322A (Axon) for acquisition to computer. Data were filtered at 2 kHz and digitized at 10 kHz. Input resistance and series resistance were monitored intermittently throughout experiments by applying –10 mV voltage steps and observing the capacitative transient of the current response. Input resistances ranged from 540 to 800 megaohms and did not differ significantly between groups.

Initial series resistances were estimated to be less than 20 megaohms, and data were discarded if series resistance changed by more than 30%. sIPSCs and mIPSCs were detected and analyzed off-line using Mini-Analysis (Synaptosoft) on a Windows-based computer, or using the event detection package with a variable amplitude template in Axograph 4.9 (Axon) on a Macintosh computer. Detected events were visually scanned and obvious artifacts were excluded. For sIPSCs, the first 500 events, and for mIPSCs, the first 150–200 events detected for each cell were used for analysis. Complex events (consisting of two or more IPSCs) detected by Mini-Analysis were included in the analyses of inter-event intervals and amplitudes (the Mini- Analysis algorithm calculates

event amplitudes from a baseline determined by the extrapolated decay of the preceding event), but were excluded from analyses of rise and decay times. Given the somewhat subjective nature of spontaneous event detection, these analyses were corroborated using Axograph 4.9 by a second experimenter blinded to the experimental condition. Exponential fits to averaged IPSCs were done using Igor Pro Carbon (Wavemetrics).

Except where noted, the numbers of cells given in statistical analyses represented one cell per animal. NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide),APV(DL-2-amino-5-phosphonovaleric acid), bicuculline and tetrodotoxin (TTX) were diluted from stock solutions to final concentrations in ACSF and applied by bath superfusion. All drugs were purchased from Sigma.

Results

sIPSC Amplitudes and Frequency Remain Decreased Days after Hypoxia-Induced Seizures

We initially examined sIPSCs in CA1 pyramidal neurons in hippocampal slices prepared 3–5 days after hypoxia treatment, and in slices from age-matched littermate control-handled animals. IPSCs were recorded at a holding potential of +10 mV, the approximate reversal potential for glutamate receptors, so that spontaneous GABAergic events could be isolated without blocking excitatory transmission in the slice. Figure 1 shows outward sIPSCs recorded under these conditions. The identification of these as $GABA_A$ receptor-mediated synaptic currents was confirmed by their complete blockade with 20 μM bicuculline at the end of experiments (not shown). As illustrated in figure 1, at 3–5 days after hypoxia, we observed a significant decrease in both the amplitudes and frequency of sIPSCs. Mean sIPSC amplitudes in the hypoxia-treated group were significantly diminished to 63.7% of control (mean amplitudes = 19.89 8 2.2 pA for the control group; 12.67 8 1.4 pA for the hypoxia group; n = 10 per group; p = 0.013, Student's t test), and the mean sIPSC frequency was decreased to 63.8% of control (mean frequency = 3.56 ± 0.26 Hz for the control group; 2.27 ± 0.32 Hz for the hypoxia group; p = 0.006). No differences in rise times were observed between groups (10– 90% rise times = 2.81 ± 0.11 ms for the control group; 2.92 ± 0.19 ms for the hypoxia group; p = 0.63). These data indicate that the acute decrease in basal synaptic inhibition persists at least for several days after hypoxia-induced seizures. We next examined possible pre- and post-synaptic mechanisms that maybe involved with this decrease in inhibition.

Decreased mIPSC Amplitudes but Not Frequency Days after Hypoxia-Induced Seizures

We next examined mIPSCs under the same conditions by adding 1 μ M TTX to the bath. Similarly to sIPSCs, the mIPSC amplitudes were significantly decreased in the hypoxia-treated group to 65.8% of control (figure2; mean mIPSC amplitudes = 18.36 ± 2 pA for the control

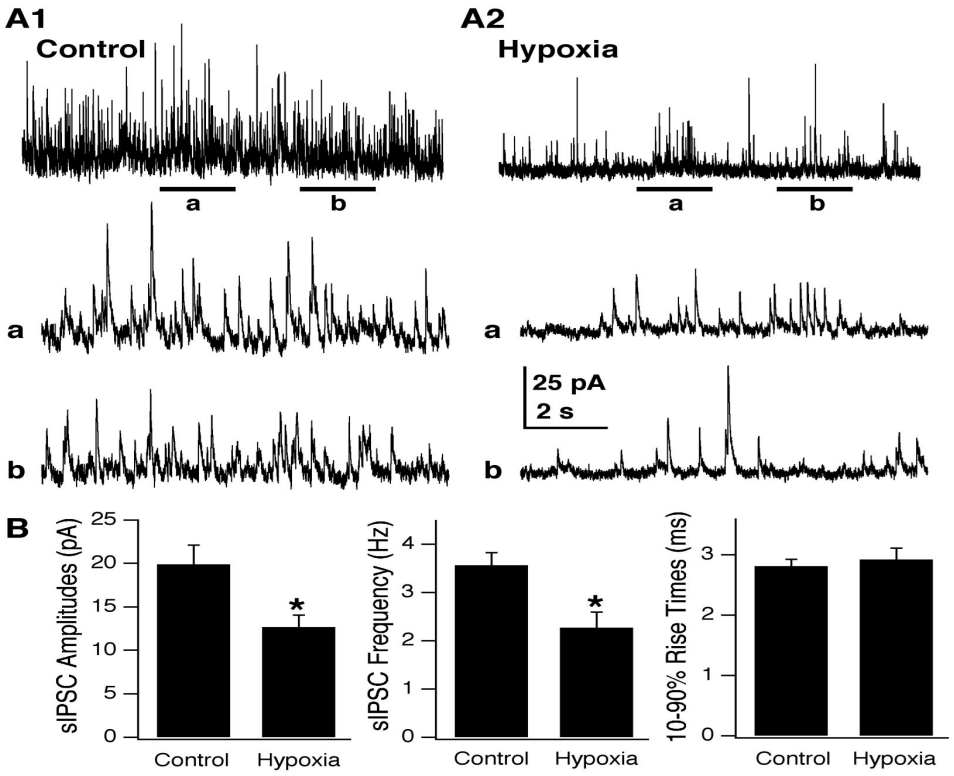

***Figure1*. sIPSCs.** Basal synaptic inhibition of CA1 pyramidal neurons is decreased at 3-5 days after hypoxia-induced seizures at P10. **A1.** An example of sIPSCs recorded by whole-cell voltage clamping of a control hippocampal area CA1 pyramidal neuron. **A2.** sIPSCs from pyramidal cell recorded from the hypoxia-treated group. Insets **a** and **b** show expanded 10- second segments from each of the longer traces. Summary data are illustrated by the bar graphs in **B**. sIPSC amplitudes and frequency were significantly decreased (*P=0.013 and 0.006 respectively).There was no difference in sIPSC rise times between groups.

group, n = 7; 12.08 ± 0.9 pA for the hypoxia group, n = 8; p = 0.01). However, in contrast to sIPSCs, the frequency of mIPSCs after hypoxia was 97.6% of control, and was not significantly different from the control group (mean mIPSC frequency = 1.02 ± 0.13 Hz for the control group; 1.0 ± 0.06 Hz for the hypoxia group; p = 0.84). The finding of unchanged mIPSC frequency suggested that at 3–5 days after hypoxia there were no persistent changes in the number of GABAergic synapses or in the probability of GABA release at inhibitory synapses.

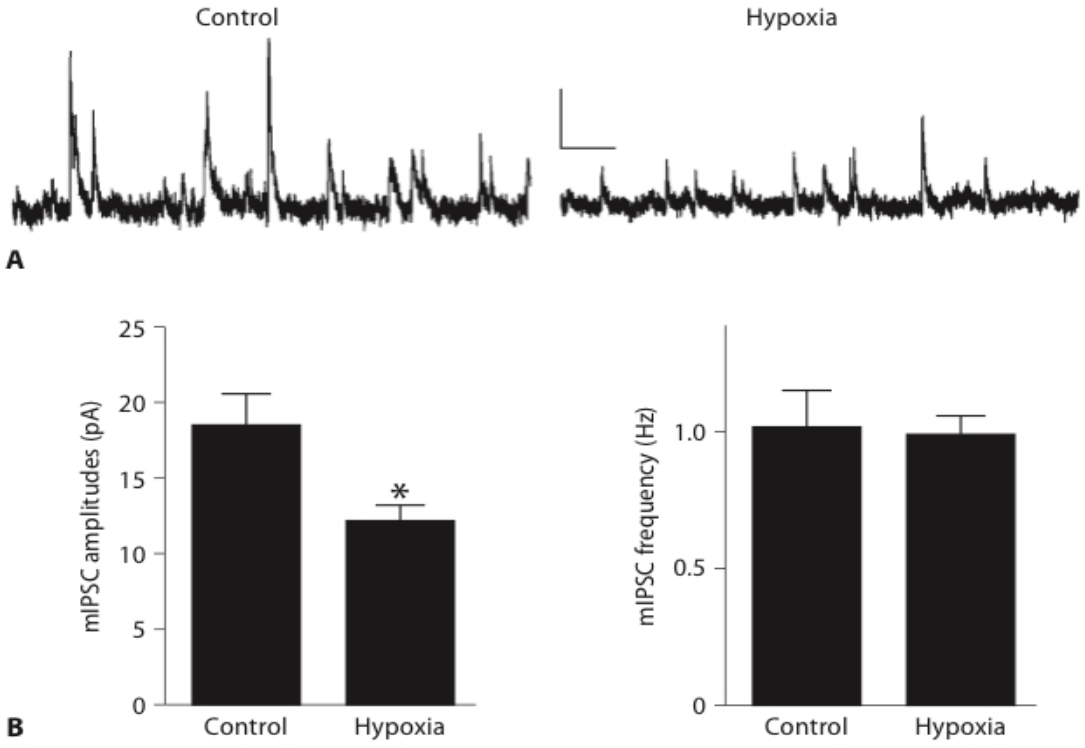

***Figure 2*: mIPSCSs.** mIPSCs recorded 3-5 days after hypoxia-induced seizures exhibited decreased amplitudes compared to sIPSCs, but there was no difference in mIPSC frequency between groups. **A.** Sample raw traces of mIPSCs. **B.** Summary data for mIPSC amplitudes (left) and frequency (right). P= 0.04.

These observations further suggested that the decreased sIPSC frequency at this phase resulted from decreased spontaneous firing of presynaptic inhibitory interneurons, and not from altered release mechanisms at the presynaptic terminals. Conversely, decreased sIPSC and mIPSC amplitudes likely resulted from altered responses of postsynaptic $GABA_A$ receptors to synaptic GABA release.

Pharmacological Sensitivity of Postsynaptic $GABA_A$ *Receptors Is Unchanged*

To further characterize possible alterations in postsynaptic $GABA_A$ receptor properties at 3–5 days after hypoxia, we next examined their kinetics and pharmacological sensitivity. Notably, previous work by others showed that kainate-induced status epilepticus in neonatal (P9) rats resulted in decreased γ2 mRNA expression throughout the hippocampus as early as 6 hours and at 3 days following kainate treatment (Lauren et al 2005). Given that the γ2 subunit confers benzodiazepine sensitivity, this suggested that decreased benzodiazepine sensitivity might be an early consequence of neonatal seizures (Sieghart et al 1999). To examine this possibility, we recorded mIPSCs 3–4 days after hypoxia treatment at a more physiological holding potential of –70 mV with glutamate receptors blocked by NBQX (20 μM) and APV (50 μM), and with 1 μM TTX in the bath. As shown in figure 3, mIPSCs recorded under these conditions again exhibited decreased amplitudes in the hypoxia-treated group to 67.2% of control (mean amplitudes = 44.1 ± 4 pA for the control group, n = 6 cells from 4 animals; 30.14 ± 4.3 pA for the hypoxia group, n = 5 cells from 4 animals; p = 0.04, Student's t test) with no differences in rise times (10–90% rise times = 0.93 ± 0.11 ms for the control group; 1.02 ± 0.14 ms for the hypoxia group; p = 0.6). mIPSC decays

were well fit by a single exponential function, and decay time constants estimated from these fits indicated no significant differences between groups despite a trend toward increased decay times in the hypoxia-treated group compared to controls (decay time = 25.6 ± 2.4 ms for the control group; 33.2 ± 4 ms for the hypoxia group; p = 0.15). As shown in figure 3, application of the positive $GABA_A$ receptor modulator diazepam (200 nM) significantly prolonged mIPSC decay times in both groups. There was no difference in the percent increase in decay times between the control and hypoxia-treated groups (percent increase = 48 ± 7.2 for the control group; 48.7 ± 8 for the hypoxia group). Though not exhaustive, these data indicate that the pharmacological and kinetic properties of postsynaptic $GABA_A$ receptors were unchanged at 3–4 days following hypoxia-induced seizures, and suggest that any changes in postsynaptic $GABA_A$ receptor properties were likely not due to altered subunit composition.

Recovery of sIPSC Amplitudes but Not Frequency at 7 Days after Hypoxia

To further characterize the time course of changes in sIPSC parameters following hypoxia-induced seizures, we again recorded sIPSCs at a holding potential of +10 mV in CA1 pyramidal neurons in slices obtained at 7 days after hypoxia-induced seizures at P10. As shown in figure 4, by 7 days sIPSC amplitudes in the hypoxia treated group had recovered to 93.7% of control (sIPSC amplitudes = 33 ± 1.8 pA for the control group; 30.9 ± 3.4 pA for the hypoxia group; n = 4 per group; p = 0.61). However, sIPSC frequency remained consistently decreased in the hypoxia-treated group at 64.4% of control (sIPSC frequency = 7.1 ± 0.64 Hz for the control group; 4.57 ± 0.79 Hz for the hypoxia group; p = 0.048). This divergence confirmed that the decrease in sIPSC

amplitude was mediated by different mechanisms than those responsible for the decreased sIPSC frequency, and further suggested that the decrease in sIPSC frequency may be a chronic consequence of perinatal hypoxia-induced seizures.

Since this article was published we have begun to explore possible mechanisms of persistently decreased sIPSC frequency following neonatal hypoxia-induced seizures. To this date, we have identified two antiepileptic drugs that counter this effect.

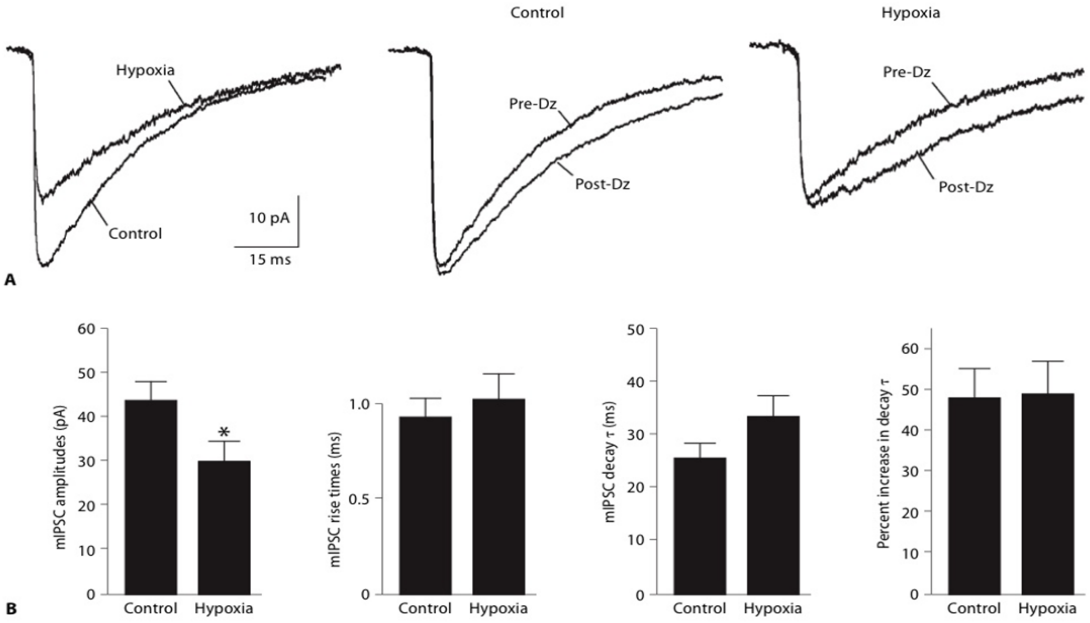

***Figure 3*: No Change in Diazepam Sensitivity.** mIPSCs recorded at a holding potential of -70mV at 3-5 days after hypoxia-induced seizures exhibited decreased amplitudes with no changes in rise and decay times and responsiveness to diazepam. A. The traces shown are averages of 50 mIPSCs from sample neurons in control and hypoxia-treated groups under each condition. B. Bar graphs showing summary data for all cells. mIPSC amplitudes again were significantly smaller in hypoxia-treated group compared to controls (*P=0.04). No differences were observed between groups in rise and decay times and in the enhancement of decay times by 200 nM diazepam.

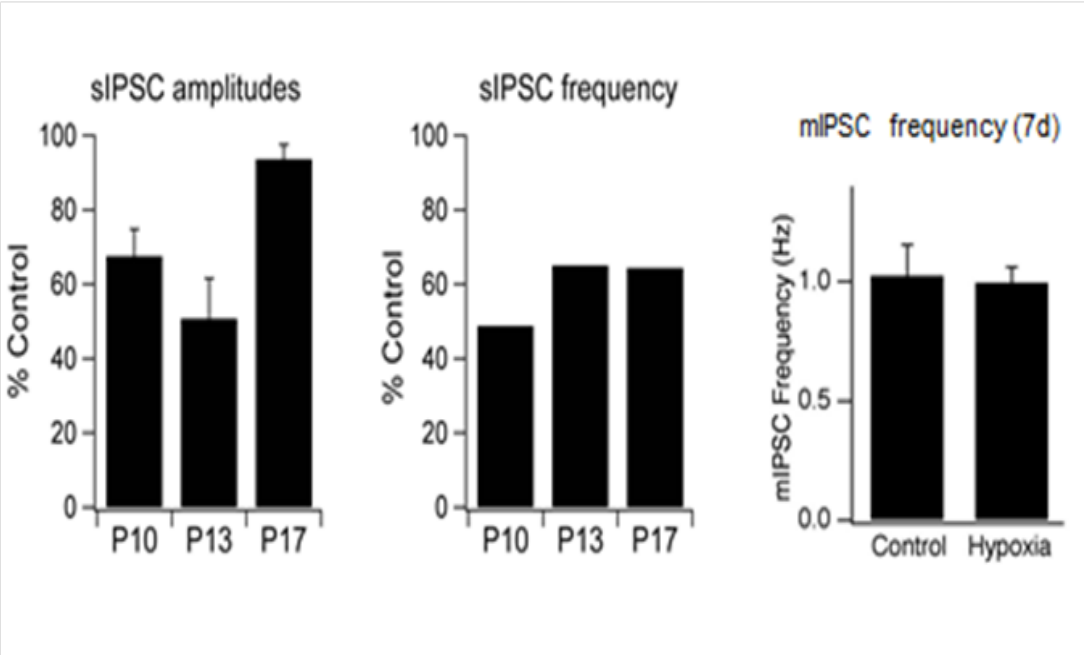

***Figure 4*: Persistently Decreased sIPSC Frequency.** sIPSC amplitude and mIPSC frequencies recover by 7 days post hypoxia-induced seizures (P10) but sIPSC frequency remains decreased to approximately 60% of control.

Conclusions

Our previous work demonstrated an acute decrease in both the amplitudes and frequency of sIPSCs and mIPSCs in CA1 pyramidal neurons recorded in slices prepared immediately following perinatal hypoxia-induced seizures in vivo (Sanchez et al 2005). In the current study, we examined further the 'subacute' time course of these changes at 3–5 and 7 days after hypoxia. We observed that the decreases in sIPSC frequency and amplitudes persisted at 3–5 days after hypoxia. Additionally, mIPSC amplitudes remained decreased at this time, comparably to the decrease in sIPSC amplitudes. Interestingly, however, mIPSC frequency recovered to control levels. This last finding is significant in that it indicates that several days following neonatal hypoxia-induced seizures, basal synaptic inhibition of CA1 pyramidal neurons is decreased despite no loss of GABAergic synapses or apparent long-lasting changes in the probability of spontaneous GABA release.

Although we did not record directly from presynaptic interneurons, this indicates that the persistent decrease in sIPSC frequency is most likely entirely due to decreased spontaneous firing of interneurons that provide synaptic inhibition to CA1 pyramidal neurons. Notably, a similar (yet not identical) scenario has been reported for layer II neurons of the entorhinal cortex at 3–7 days after pilocarpine-induced status epilepticus in adult rats (Kobayashi et al 2003) (see below).Examination of the pharmacological and kinetic properties of mIPSCs at 3–5 days after hypoxia also indicated no changes in these, although there was a trend toward longer decay times in the hypoxia-treated group. These properties of $GABA_A$ receptors can be post-translationally modulated, but

are largely determined by their specific subunit composition (Hevers and Luddens 1998, Mehta and Ticku 1999, Sieghart et al 1999), and dramatic changes in $GABA_A$ receptor subunit expression have been observed in hippocampal neurons acutely and chronically after neonatal status epilepticus in rats (Zhang et al 2004, Lauren et al 2005). The lack of significant changes at 3–5 days after hypoxia-induced seizures is thus consistent with a lack of altered $GABA_A$ receptor subunit expression in this seizure model, and suggests a posttranslational mechanism of IPSC amplitude down-modulation.

Our previous work indicated that dephosphorylation of postsynaptic $GABA_A$ receptors was at least partly responsible for decreased amplitudes and increased decay times of sIPSCs immediately following hypoxia induced seizures, and further showed that calcineurin expression was significantly increased in the hippocampus at 24 hours after hypoxia, suggesting a persistence of increased calcineurin signaling (Sanchez et al 2005). It is conceivable that the decreased amplitudes and trend toward increased decay times of mIPSCs we observed at 3–5 days after hypoxia in the current study reflected a persistent increase in basal calcineurin-mediated dephosphorylation of $GABA_A$ receptors, although we have yet to explore this as a possible mechanism during this phase. GABA-mediated synaptic inhibition of principal neurons has been reported to be pathologically altered in multiple neuron types in several experimental seizure models (Cossart et al 2005).

Whereas many studies have focused on seizure-induced changes in $GABA_A$ receptor subunit expression and function (Clark et al 1994, Gibbs et al 1997, Schwarzer et al 1997, Tsunashima et al 1997, Brooks-Kayal et al 1998) or loss of GABAergic

neurons (Andre et al 2001, Dinocourt et al 2003, Kobayashi and Buckmaster 2003, Sloviter et al 2003), recent work has underscored the importance of the regulation of intrinsic excitability and spontaneous firing of GABAergic interneurons (Kobayashi and Buckmaster 2003, Shao and Dudek 2005), in addition to mechanisms of GABA release (Chen et al 1999; Hirsch et al 1999), in controlling network excitability. For example, (Shao and Dudek 2005) reported that mIPSC frequency in dentate granule cells was significantly decreased at 4–7 days (and remained so for 3 months) after kainate-induced status epilepticus, yet they observed no differences between groups in the frequency of sIPSCs. These authors proposed that increased spontaneous firing of GABAergic interneurons may have been a compensatory response to the loss of GABAergic synapses or decreased GABA release probability indicated by the lower mIPSC frequency. In contrast, (Kobayashi et al 2003) reported that at 3–7 days after pilocarpine-induced status epilepticus, layer II entorhinal cortical neurons exhibited no changes in mIPSC frequency, but significantly decreased sIPSC frequency. These data were similar to our observations after hypoxia, and indicated a functional decrease in basal synaptic inhibition despite no apparent loss of GABAergic synapses or reduction in the probability of action potential-independent GABA release, which could be attributed to decreased spontaneous firing of presynaptic interneurons.

Although dysregulation of GABAergic transmission may be a general consequence and mechanism of seizure and epileptogenesis, it clearly is not restricted to alterations that occur at GABAergic synapses, as the intrinsic excitability and synaptic drive of GABAergic neurons are dynamically regulated and can profoundly affect

inhibition. It is worth noting that (Kobayashi and Buckmaster 2003) reported no difference between control and pilocarpine-treated groups in mIPSC amplitudes (in addition to frequency), despite decreased sIPSC amplitudes in the pilocarpine treated group. The altered amplitudes of only action potential- dependent IPSCs suggested that changes in action potential-dependent synaptic GABA release could also have impacted sIPSC frequency.

In our study, the comparable decreases in both sIPSC and mIPSC amplitudes at 3–5 days after hypoxia are more consistent with a common (as yet unidentified) postsynaptic mechanism of altered amplitudes, and the decreased sIPSC frequency in the hypoxia-treated group was most likely due entirely to decreased spontaneous firing of GABAergic interneurons. The apparent recovery of sIPSC amplitudes but not frequency by 7 days after hypoxia also argues for separate mechanisms of modulation of these two IPSC parameters. These observations further suggest that decreased interneuron excitability, due to decreased excitatory synaptic drive and/or altered intrinsic membrane properties, may be a long-term consequence of neonatal seizures induced by hypoxia, and may contribute to the chronic increase in hippocampal excitability and seizure susceptibility. Whether this effect is truly chronic has yet to be investigated. However, given that depolarizing responses to GABA during this early postnatal period contribute to activity-driven maturational processes (Ben-Ari 2002), even transient alterations in pre- and postsynaptic GABAergic transmission may have chronic consequences for hippocampal function by profoundly altering physiological maturation, and one would

expect that the longer the duration of each pathophysiological alteration, the more profound will be the chronic consequences.

In summary, we have identified persistent decreases in basal inhibitory synaptic transmission to hippocampal CA1 pyramidal neurons up to 1 week following hypoxia induced seizures in P10 rat pups. Whereas the acute decreases identified previously may be mediated by mass activity of the phosphatase calcineurin and rapid dysregulation of pre- and postsynaptic components of inhibitory synaptic transmission (Sanchez et al 2005), the persistent decrease appears gradually to depend more on decreased spontaneous firing of presynaptic inhibitory interneurons. Direct examination of the intrinsic membrane properties of GABAergic interneurons in hippocampi from animals that experienced neonatal hypoxia induced seizures may elucidate the underlying mechanism(s) of this decreased spontaneous activity, and ultimately determine if and how this contributes to epileptogenesis and other hippocampal pathologies in this setting.

CHAPTER III

$GABA_A$ RECEPTOR SUBUNIT EXPRESSION FOLLOWING PERINATAL HYPOXIA-INDUCING SEIZURES

Introduction

The $GABA_A$ receptor is the most widely distributed and important inhibitory receptor in the central nervous system where it mediates, inhibitory postsynaptic currents in many cell types. The $GABA_A$ receptor is a member of a superfamily of proteins coined Cys-loop ligand-gated ion channels (LGIC), named for their characteristic loop formed by a disulfide bond between two cysteine residues. Members of this superfamily include 5-HT3 receptors, nicotinic acetylcholine receptors, glycine receptors, as well as the $GABA_A$ receptor. All members of this superfamily are composed of 5 subunits that form heteromeric proteins with an ion pore. In the case of the $GABA_A$ receptor, this pore conducts chloride following the binding of GABA.

This increases the chloride conductance and hyperpolarizes the membrane, thereby blocking action potential firing. There are several different subunits that impart a great deal of pharmacologically distinct receptor subtypes. These subunits include the following, α1-6, β1-3, γ1-3, d, ε, π, θ, ρ1-3 (Simon et al 2004). Of most interest to our study are synaptic $GABA_A$ receptors that are composed of 2 α subunits, 2 β subunits, and a single γ subunit.

Humans that have mutations in these genes for these subunits suffer from various forms of epilepsy. A decrease in γ2 subunits has been associated with decreased benzodiazepine and zinc (Zn) sensitivity in acquired partial epilepsy models (Houser et

al 2012), and thus, decreased γ2 could suggest additional changes to the endogenous activity-dependent regulation of inhibition that are more complex. Changes in $GABA_A$ receptor subunit expression could explain our finding of transiently decreased IPSC amplitudes reported in chapter II, especially if the subunit expression follows the same time course. Such a finding would warrant further electrophysiological and pharmacological experiments to determine the functional consequences of altered $GABA_A$ receptor subunit expression. Here we examined the expression of several $GABA_A$ subunits involved with synaptic $GABA_A$ receptors, specifically α1, α2, β2/3, and γ2.

We hypothesized, based primarily on our findings of decreased IPSC amplitudes as far out as 5 days following neonatal hypoxia-induced seizures, that there would be decreased expression of the $GABA_A$ receptor subunits listed above.

Methods

Animals

Long-Evans rat pups (Charles River), at an age of P10–P16, were used for these experiments. Litters were housed with their dam in the TAMHSC animal facility in Temple, TX. Animals were kept on a 12-hour light/12-hour dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with NIH guidelines on the ethical use of experimental animals.

Hypoxia Treatment

Seizures were induced in male Long-Evans rat pups on P10 by a 14–16-minute exposure to 5–7% O_2. Pairs of littermates were removed from their dam and each placed into a custom-made airtight chamber on heating pads to maintain a rectal temperature of 33–34° C. One chamber (control) was left uncovered and continuously exposed to room air, while the other (hypoxia) was covered and the O_2 concentration lowered by infusion of N_2 gas into the chamber. The O_2concentration was lowered to 6–7% for 4 minutes, then 5–6% for 8 minutes, and then lowered by 1% per minute until the animal became apneic for 30 seconds. The chamber was then uncovered and exposed to room air. Using this protocol, spontaneous seizures typically began within 2–4 minutes of hypoxia, and occurred repeatedly throughout the hypoxia duration until apnea, and most animals continued to exhibit behavioral seizures for several minutes after returning to room air. At the end of hypoxia treatment, both animals in a pair were ear-marked with sharp scissors and returned to their dam. Thus, control animals were handled identically to

hypoxia-treated animals except for hypoxia exposure. All animals in the hypoxia-treated group used for this study exhibited a minimum of five tonic-clonic seizures.

Tissue and Sample Preparation

Animals from 2 groups (hypoxia-vs.-control) at 3, 6 and 7 days post-hypoxia for a total of 6 groups were sacrificed at the same time each day. Rat pups were killed by decapitation under isoflurane anesthesia. The brains were removed and immediately placed into ice-cold oxygenated (with 95% O_2 / 5% CO_2) artificial cerebrospinal fluid (ACSF) that contained (in mM): NaCl 126, KCl 3.3, Na_2PO_4 1.25, $MgSO_4$ 1.3, $CaCl_2$2, Na_2HCO_3 26, and D-glucose 10.The hippocampi were quickly dissected and placed into ice cold 1x RIPA buffer complete with protease inhibitors. The hippocampi were rapidly homogenized by sonification with brief quick pulses while on ice. A Bradford colorometric assay was performed on all samples. The samples were aliquoted and stored at -80°C until Western blots were performed.

Western Blot

Antibodies were acquired from various vendors but most were acquired from the Ticku laboratory in San Antonio at UTHSCSA. We thank Dr. Ticku's laboratory for kindly providing us with access to his antibody supplies. Western blot analysis of α1, α2, β2/3 and γ2 $GABA_A$ receptor subunit expression were conducted on whole hippocampal homogenates from 2 groups at 3 different time points: 24 hours, 48 hours and 6 days post-hypoxia (6 group's total). Samples were run on ready-made gels (BIORAD anyKD) at 200 Volts for 40 minutes. Transfer to PVDF membrane was performed at 90 volts for 90 minutes in an ice bath.

The blots were then blocked with Licor blocking solution 1x for a minimum of 2 hours at room temperature. Blots were incubated overnight with primary antibodies to $GABA_A$ receptor subunits (SOURCE)at 1:500-1:1000 dilutions and 1:10000 dilution anti-GAPDH (SOURCE). We used enhanced chemiluminescence for detecting most of the $GABA_A$ subunits, but detection of the β2/3 subunit was performed using both ECL and Licor. The quality of Licor Western blots is much greater than that of ECL and we performed separate β2/3 Western blots for both technologies and found no difference in the result. Optical density for each band was then measured by NIH software ImageJ. Finally, each band was background subtracted and normalized to GAPDH.

Normalized data were analyzed by student's t-test or ANOVA where appropriate. We found no significant changes in the expression of any of the $GABA_A$ receptor subunits at any of the time points studied by either statistical method.

Results

To characterize possible alterations in postsynaptic $GABA_A$ receptor subunit expression we examined several $GABA_A$ receptor subunits known to mediate most postsynaptic GABA mediated currents called IPSCs.

$GABA_A$ α1 Receptor Subunit Expression

We measured at 3 days post hypoxia-induced seizures (P13) the $GABA_A$ subunit α1. As can be seen in figure 5, there are no statistically significant changes in the expression of α1 following neonatal hypoxia-induced seizures at P13. $p = 0.40$, Student's t test. Since GABA receptors are composed of several different types of subunits, we further examined the most common subunits found in area CA1 of the hippocampus.

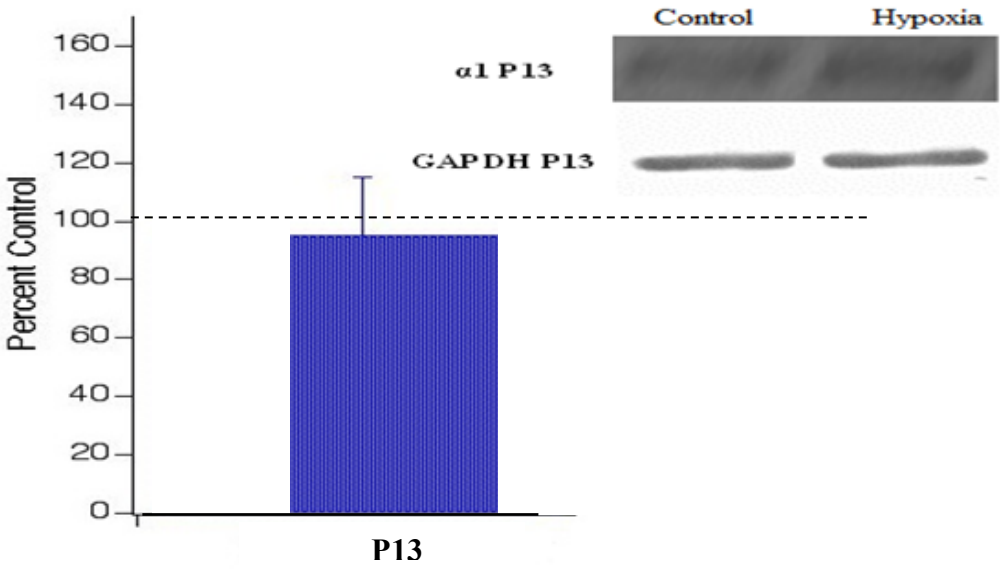

Figure 5: **Hippocampal $GABA_A$ α1 Subunit Expression.** Samples were normalized to GAPDH. No significant change in α1 expression was found at P13. Student's T-test P>0.05 N=5-7 animals.

We measured at 3, 6 and 7 days post hypoxia-induced seizures (P13, P16, P17) the $GABA_A$ subunit α2. As can be seen in figure 6, there are no statistically significant changes in the expression of α2 following neonatal hypoxia-induced seizures at P13. p = 0.40, ANOVA. We next looked at the *β2/3* subunit known to be responsible for binding GABA to the receptor itself.

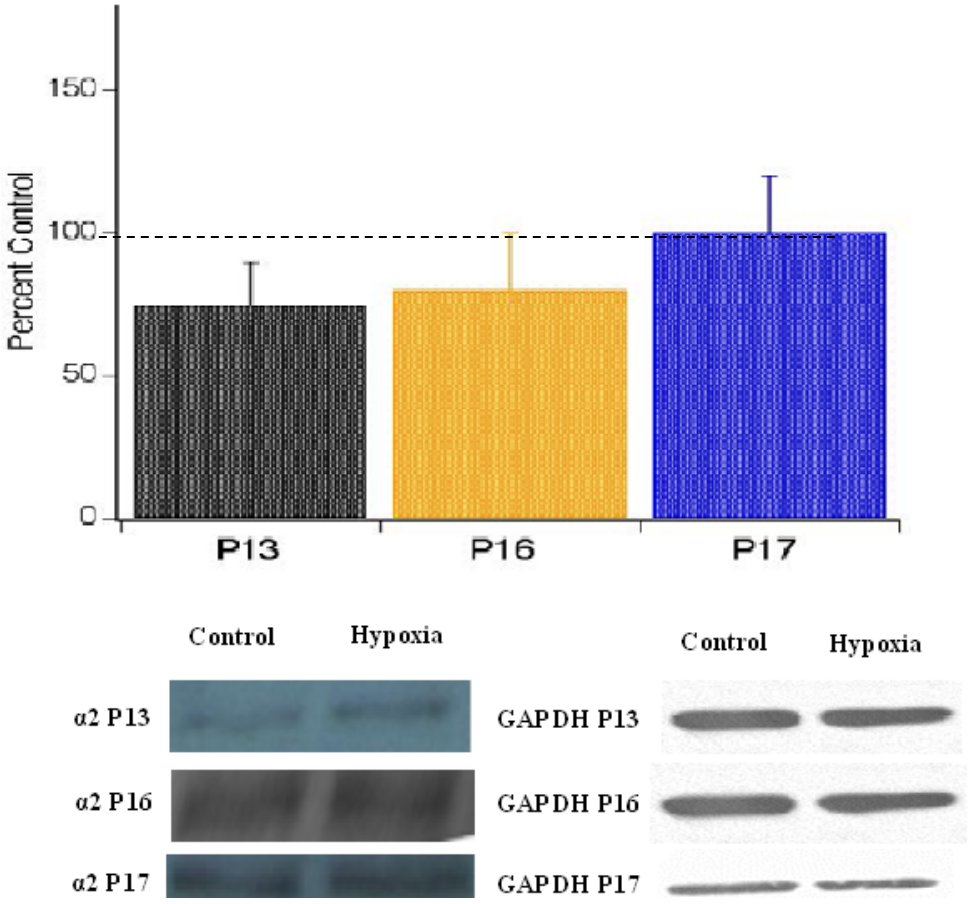

Figure 6: **Hippocampal $GABA_A$ α2 Subunit Expression.** Samples were normalized to GAPDH. No significant change in α2 expression was found at P13. ANOVA P>0.05 N=5-7 animals.

$GABA_A$ Receptor β2/3 Subunit Expression

We measured at 3 and 7 days post hypoxia-induced seizures (P13 and P17) the $GABA_A$ subunit β2/3. As can be seen in figure 7, there are no statistically significant changes in the expression of the $GABA_A$ receptor subunit β2/3 following neonatal hypoxia-induced seizures at P13, P16, P17. Student's t test p=0.38. We next looked at the *γ2* subunit known to play a key role in benzodiazepine regulation of the $GABA_A$ receptor.

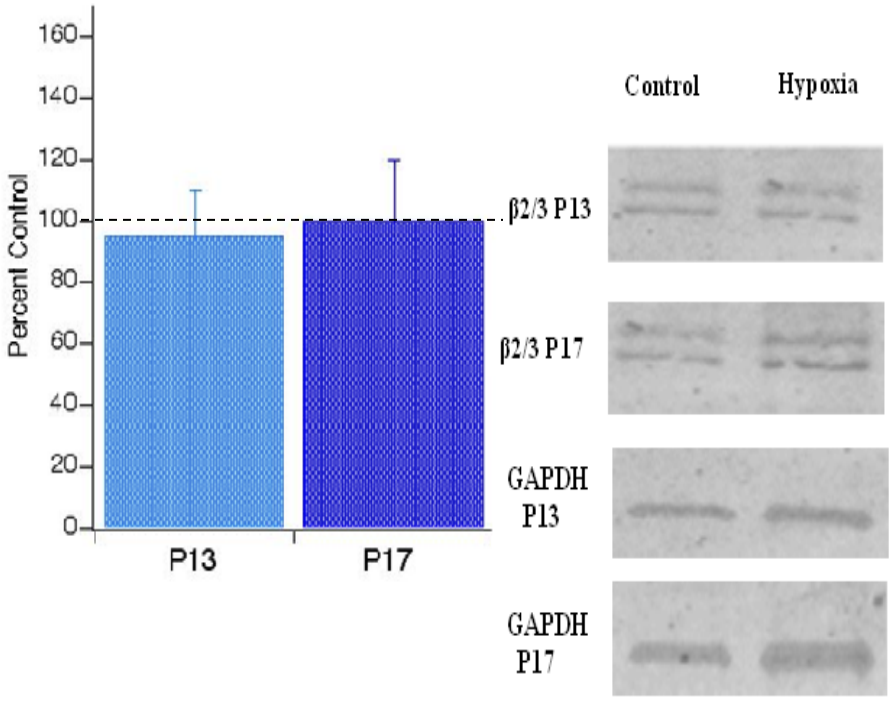

Figure 7: **Hippocampal $GABA_A$ β2/3 Subunit Expression.** Samples were normalized to GAPDH. No significant change in β2/3 expression was found at P13. ANOVA P>0.05 N=5-7 animals.

$GABA_A$ Receptor γ2 Subunit Expression

We measured at 3 days post hypoxia-induced seizures (P13) the $GABA_A$ subunit γ2. As can be seen in figure 8, there are no statistically significant changes in the expression of the $GABA_A$ receptor subunitγ2 following neonatal hypoxia-induced seizures at P13 and P17. p=0.58 Student's t test.

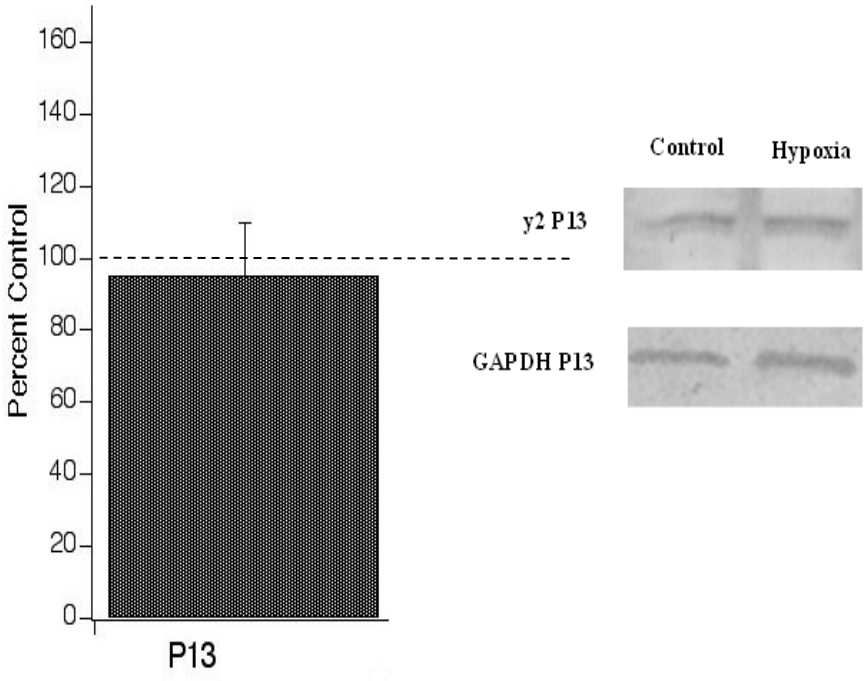

Figure 8: **Hippocampal $GABA_A$ γ2 Subunit Expression**. Samples were normalized to GAPDH. No significant change in γ2 expression was found at P13. Students t-test P>0.05 N=5-7 animals.

Summary

The combined Western blot results from all $GABA_A$ subunits suggest that there are no significant changes to the protein expression of the $GABA_A$ receptor (see figure 9). Since, in chapter 2 we measured possible changes in diazepam sensitivity and found none, the data support the same conclusion, that there are no changes in subunit composition of the $GABA_A$ receptors in area CA1 of the hippocampus following neonatal hypoxia-induced seizures.

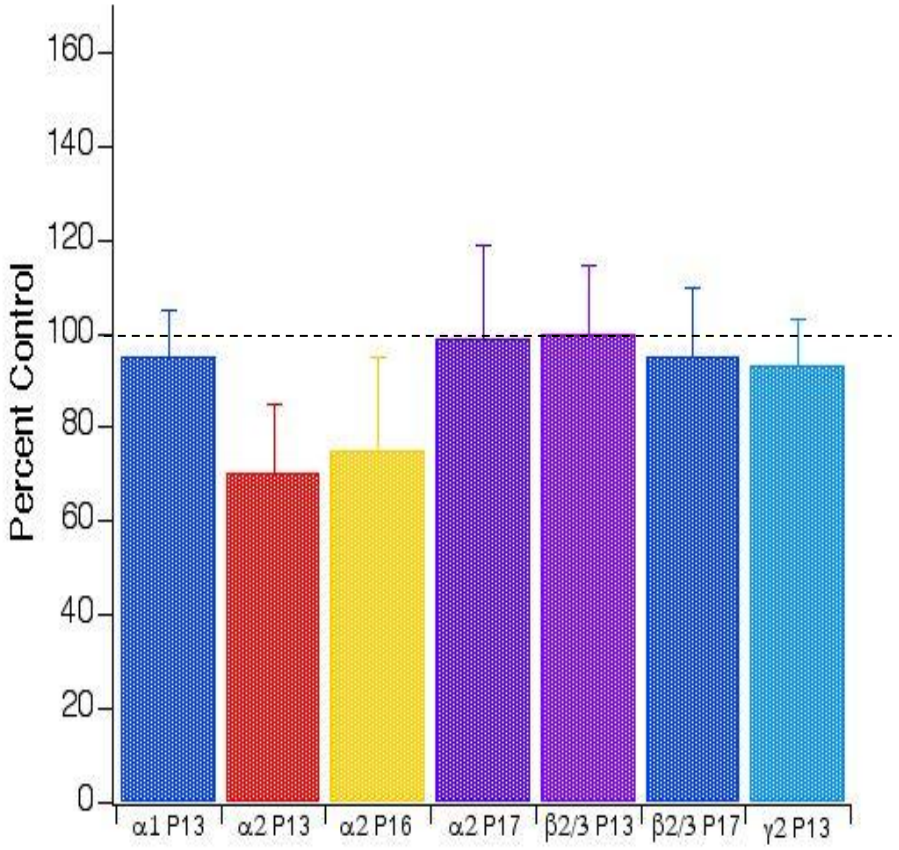

***Figure 9*: GABA$_A$ SUBUNIT EXPRESSION.** Summary of hippocampal $GABA_A$ subunit expression. Samples were normalized to GAPDH. No significant change in subunit at any time point studied. ANOVA $P>0.05$ N=5-7 animals.

Conclusions

Altered $GABA_A$ receptor subunit expression and pharmacological sensitivity have been reported in neonatal as well as adult rodent models of seizures and human epileptic hippocampi (Zhang et al 2007, Schwarzer et al 1997, Bouilleret et al 2000, Raol et al 2006, Loup et al 2000). Pyramidal neurons in the rat hippocampus express phasic $GABA_A$ receptors composed of 2αs 2βs and one γ subunit(s). Human patients with temporal lobe epilepsy display decreased $GABA_A$ receptor subunit expression in hippocampal neurons (Loup et al 2000). Although there is no substantial loss of neurons in the neonatal hypoxia rat model as in other models of acquired epilepsies there still may be changes in the expression of various $GABA_A$ receptor subunits, especially considering the decrease in IPSC amplitudes reported in chapter II.

We aimed to determine whether decreased inhibition after neonatal hypoxia-induced seizures is associated with changes in expression of postsynaptic $GABA_A$ receptors. Based on acute changes in IPSC amplitudes, we hypothesized that α1, α2, β2/3, and γ2 subunit expression would be decreased after hypoxia-induced seizures, compared to age matched control littermates. Previously, we had shown that the chronic decreases of sIPSC frequency after hypoxia-induced seizures were likely due to decreased firing of presynaptic interneurons. However, we also observed that s- and mIPSC amplitudes were transiently decreased during this critical maturational window after hypoxia-induced seizures. Changes in $GABA_A$ subunit expression could explain our finding of transiently decreased s- and mIPSC amplitudes, especially if the subunit expression follows the same time course, decreasing initially but recovering by 7 days

post hypoxia-induced seizures. For example, a decrease in the γ2 subunit of the $GABA_A$ receptor has been associated with decreased benzodiazepine and zinc sensitivity in acquired partial epilepsy models (Houser et al 2012), and thus, decreased γ2 could suggest additional changes to the endogenous activity-dependent regulation of inhibition that are more complex. Such a finding would warrant further electrophysiological and pharmacological experiments to determine the functional consequences of altered $GABA_A$ receptor subunit expression.

Western blot analysis of key $GABA_A$ subunits is an important experiment to conduct and seizure-dependent changes in subunit expression have been reported in animal models of acquired epilepsy and epileptic humans. For example, GABA-mediated synaptic inhibition of principal neurons has been reported to be pathologically altered in multiple neuron types in several experimental seizure models (Cossart et al 2005). Importantly, although numerous studies have focused on seizure-induced changes in $GABA_A$ receptor subunit expression and function or loss of GABAergic neurons, much of the evidence is not from actual protein expression but rather changes in mRNA. Changes in $GABA_A$ subunit expression could explain our finding of transiently decreased IPSC amplitudes, especially if the subunit expression follows the same time course, decreasing initially but recovering by 7 days post hypoxia-induced seizures. We found no changes in the expression of α1, α2, β2/3, and γ2 $GABA_A$ receptor subunits up to a week following hypoxia induced seizures compared to age-matched littermate controls (See figure 9). Although this contradicts our hypothesis, the Western blot data confirms our finding of no change in diazepam sensitivity following hypoxia-induced

seizures and supports our conclusion that no change in $GABA_A$ subunit expression occurs following hypoxia-induced seizures. Finally, combined with our electrophysiology data, the Western blot data tends to support the concept that no significant changes in composition of the post synaptic $GABA_A$ receptor within the first few days following hypoxia-induced seizures. This strongly suggests a post-translational mechanism, such as enzymatic modification of $GABA_A$ channel function. For example, calcineurin was shown to be acutely up regulated and when blocked can overcome the loss of both IPSC amplitudes and frequency we report in chapter one. We will address calcineurin mediated regulation of $GABA_A$ receptor inhibition as reported in Chapter 4.

Although these findings are consistent with electrophysiology data reported in chapter II, it is possible that by using whole hippocampus homogenates, we lost the ability to detect regionally specific changes in protein expression. Another way to perform this experiment is by micro-dissecting out area CA1, since this is the area we have been recording and found our effects on $GABA_A$ mediated inhibition. Also, utilizing other methodological approaches such as FACS or the Nano-pro system could be performed in a cell specific manner. Thus, future experiments using more sensitive technologies could identify more subtle cell-specific mechanisms. Nonetheless, the combined electrophysiology and Western blot results suggest that persistently altered $GABA_A$ receptor expression is not a mechanism of decreased GABAergic inhibition in this setting.

CHAPTER IV

THE ROLE OF CALCINEURIN IN DECREASED INHIBITION FOLLOWING NEONATAL HYPOXIA-INDUCING SEIZURES

Introduction

During postnatal day 10-13 rats are susceptible to hypoxia-induced tonic-clonic and myoclonic seizures that later predisposes the animal to a lower seizure threshold and spontaneous seizures. These hypoxia-induced seizures appear to depend on AMPA receptor activation, since pharmacological blockade prevents them. Interestingly during this time period, many AMPA receptors lack the GLUR2 subunit that confers calcium impermeability. Calcium influx through GLUR2 negative AMPA receptors causes the acute activation of calcineurin, a highly conserved Ca^{2+} and calmodulin-dependent serine/threonine phosphatase, which has been shown to be acutely up-regulated after pilocarpine- and hypoxia-induced seizures. Emphasizing the importance of the calcineurin pathway, a 30 minute pre-application of FK-506 blocked hypoxia-induced seizures (Sanchez et al 2005). Interestingly, following hypoxia-induced seizures, CA1 pyramidal neurons exhibited a down-regulation of $GABA_A$ receptor mediated inhibition with a concomitant dephosphorylation of the β2/3 subunit that was reversed by the calcineurin inhibitor FK-506, suggesting a possible proepileptogenic mechanism. Furthermore, application of FK-506 increases both spontaneous IPSC amplitudes and frequency following hypoxia-induced seizures.

Calcineurin activity has been shown to be increased following other models of epilepsy. For example, in the rat pilocarpine model, calcineurin activity was found to be

increased as measured by the phosphorylation status of the protein DARPP-32, a specific substrate of calcineurin's phosphatase activity (Jung et al 2010). Another pilocarpine study reported a significant status epilepticus-dependent increase in hippocampal calcineurin activity, as measured by dephosphorylation of p-nitrophenol phosphate. However, the increase in calcineurin activity was not associated with an increase in calcineurin enzyme levels (Kurz et al 2001). This suggests that the increase in enzyme activity may be due to another mechanism, such as post-translational modification of the enzyme, or increased Ca^{2+}/CaM binding to the enzyme following SE (Kurz et al 2001, 2008). This provides yet another possible mechanism of decreased inhibition of CA1 pyramidal neurons by calcineurin and demonstrates the need to determine whether calcineurin activity increases following neonatal hypoxia-induced seizures. A persistent increase in calcineurin expression and/or activity following neonatal hypoxia-induced seizures may provide long lasting effects on inhibition of area CA1 of the hippocampus, as well as other areas of the brain, and may promote the development of epilepsy in susceptible individuals.

In this study we aimed to determine whether or not calcineurin activity remained increased past the 24 hours reported previously (Sanchez et al 2005). Based on our finding of persistently decreased GABAergic inhibition reported in Chapter One above, we hypothesized that calcineurin expression and or activity remained increased beyond 24 hours post hypoxia-induced seizures. Therefore, in this study we measured both calcineurin expression as well as activity following neonatal hypoxia-induced seizures. This was accomplished by western blot analysis of calcineurin and DARPP-32.

Methods

Animals

Long-Evans rat pups (Charles River), at an age of P10–P16, were used for these experiments. Litters were housed with their dam in the TAMHSC animal care facility Temple, Texas on a 12-hour light/12-hour dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with NIH guidelines on the ethical use of experimental animals.

Hypoxia Treatment

Seizures were induced in male Long-Evans rat pups on P10 by a 14–16-min exposure to 5–7% O_2. Pairs of littermates were removed from their dam and each placed into a custom-made airtight chamber on heating pads to maintain a rectal temperature of 33–34° C. One chamber (control) was left uncovered and continuously exposed to room air, while the other (hypoxia) was covered and the O_2 concentration lowered by infusion of N_2 gas into the chamber. The O_2 concentration was lowered to 6–7% for 4 min, then 5–6% for 8 min, and then lowered by 1% per minute until the animal became apneic for 30 s. The chamber was then uncovered and exposed to room air. Using this protocol, spontaneous seizures typically began within 2–4 min of hypoxia, and occurred repeatedly throughout the hypoxia duration until apnea, and most animals continued to exhibit behavioral seizures for several minutes after returning to room air. At the end of hypoxia treatment, both animals in the pair were ear-marked with sharp scissors and returned to their dam. Thus, control animals were handled identically to hypoxia-treated

animals except for hypoxia exposure. Animals in the hypoxia-treated group used for this study exhibited an average of eight tonic-clonic seizures.

Tissue and Sample Preparation

Animals from 2 groups (hypoxia-vs.-control) at 3 different time points – 24 hours, 48 hours and 6 days post-hypoxia for a total of 6 groups were sacrificed at the same time each day. Rat pups were sacrificed by decapitation under isoflurane anesthesia. The brains were removed and immediately placed into ice-cold oxygenated (with 95% O_2 / 5% CO_2) artificial cerebrospinal fluid (ACSF) that contained (in m M): NaCl 126, KCl 3.3, Na_2PO_4 1.25, $MgSO_4$ 1.3, $CaCl_2$ 2, Na_2HCO_3 26, and D-glucose 10 .The hippocampi were quickly dissected and placed into ice cold 1x RIPA buffer complete with protease inhibitors. Both hippocampi were rapidly homogenized by sonification with brief quick pulses while on ice. A Bradford colorometric assay was performed on all samples. The samples were aliquoted and stored at -80C until Western blots were performed.

Western Blots (calcineurin)

Western Blot analysis of calcineurin expression was conducted on whole hippocampal homogenates from 2 groups at 3 different time points: 24 hours, 48 hours and 6 days post-hypoxia (6 group's total). Samples were run on ready-made gels (BIORAD anyKD) at 200 Volts for 40 minutes. Transfer to PVDF membrane was performed at 90 volts for 90 minutes in an ice bath. The blots were then blocked with Licor blocking solution 1x for a minimum of 2 hours at room temperature. Primary anti-rabbit Calcineurin antibody (obtained from Sigma) 1:1000 and mouse anti-GAPDH

antibody 1:10,000 was then used and the blot was allowed to incubate overnight at 4 Degrees Celsius with constant movement. Optical density for each band was then measured by NIH software ImageJ. Finally, each band was background subtracted and normalized to GAPDH. Normalized data were then analyzed by ANOVA.

Western Blots (DARPP-32)

Western Blot analyses of total DARPP-32 to phosphorylated DARPP-32 THR34 and SER97 were conducted on whole hippocampal homogenates from 2 groups at 3 different time points – 24 hours, 48 hours and 6 days post-hypoxia (6 groups total). Samples were run on ready-made gels (BIORAD anyKD) at 200 Volts for 40 minutes. Transfer to nitrocellulose membrane was performed using the BIORAD Trans-Blot Turbo Transfer System. The blots were then blocked with Licor blocking solution 1x for a minimum of 2 hours at room temperature. Primary anti-rabbit DARPP-32 antibody (obtained from Cell Signaling Technologies) at 1:1000 dilution, primary anti-rabbit phospho (THR34) DARPP-32 (obtained from Cell Signaling Technologies)1:1000 dilution, primary anti-rabbit phospho (SER97)DARPP-32 (obtained from Cell Signaling Technologies) 1:1000 dilution and anti-mouse α-tubulin (Sigma) antibody 1:15,000 dilution was then used and the blot was allowed to incubate overnight at 4 Degrees Celsius with constant movement. Secondary antibodies were used at room temperature for 30 minutes anti-rabbit 680 CW (Licor) and anti-mouse 800 CW (Licor) 1:20,000. Optical density for each band was then measured by NIH software ImageJ. Finally, each band was then background subtracted and normalized to α-tubulin. Normalized data was then analyzed by 2-way ANOVA.

Results

Calcineurin Expression

We measured the protein expression of calcineurin by Western blot analysis. As illustrated by figure 10, only the first 24 hours post hypoxia-induced seizures did calcineurin expression remain increased (P11 n= 17 control group and hypoxia group, p=0.0007; P12 n=12 control group and hypoxia group, p=0.63; P17 n=10 control group and hypoxia group p=0.91). This result matches the results published previously by the Jensen laboratory (Sanchez 2005) Since an increase in the expression of calcineurin does not necessarily result in increased activity, we also measured the phosphorylation status of Threonine 34 (THR34) of DARPP-32 which is directly dephosphorylated by calcineurin and used as a measure of calcineurin enzymatic activity in previously published studies (Jung 2010).

Calcineurin Activity (Threonine 34 Phosphorylation Status of DARPP-32 [THR34])

The Western blot results for THR 34 phosphorylation failed to support our hypothesis of increased calcineurin activity. Interestingly we found no calcineurin specific dephosphorylation of THR34 ofDARPP-32, but instead we found a mild increase (1.5-1.8 fold) in THR 34 PHOSPHO-DARPP-32 in the hypoxia group (See figure 11). The number of animals used in this study were; P11 n= 7 control group and n=7 hypoxia group, P12 n=4 control group and n=5 hypoxia group. A 2-way analysis of variance (2 Way-ANOVA) was used to analyze statistical significance between control and hypoxia as well as days post hypoxia-inducing seizures on P10 (hypoxia treatment p=0.012, no age-dependence).

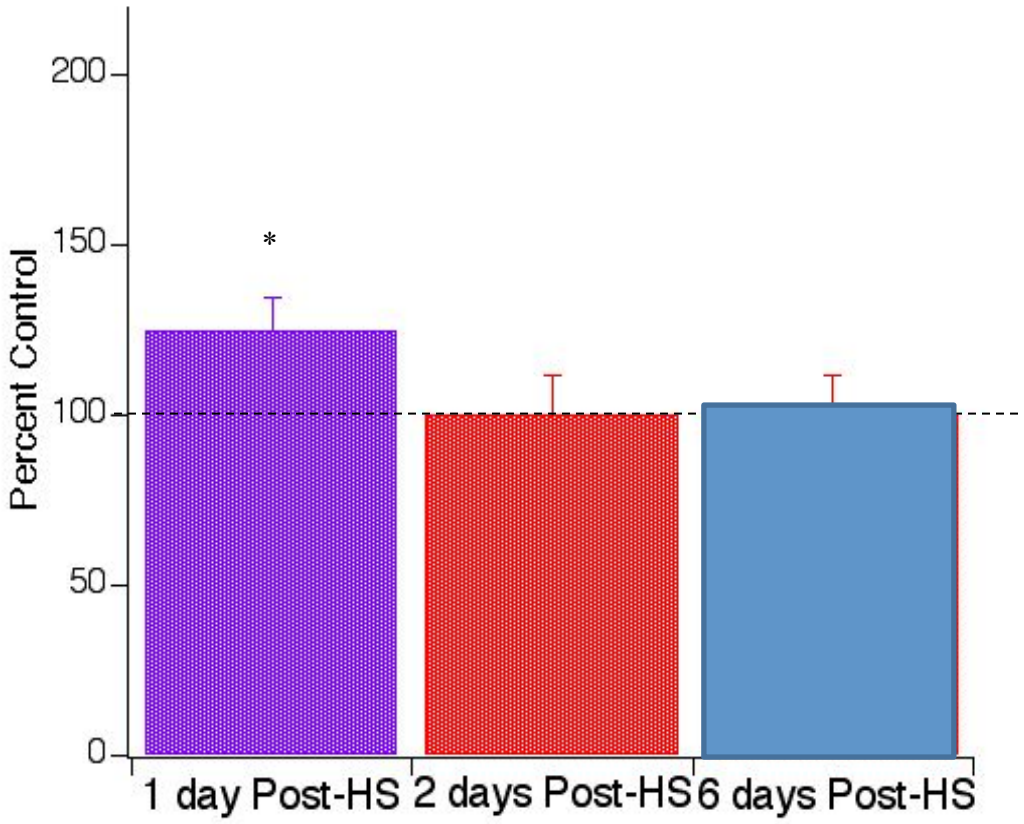

Figure 10: **Calcineurin Expression.** Summary of calcineurin expression. ANOVA was used to analyze results. Calcineurin expression increased at 24 hours post hypoxia-induced seizures and recovered by 48 hours post hypoxia-induced seizures and remained so at 6 days post hypoxia-induced seizures. $P < 0.01$ (N=17-18 each group).

DARPP-32 (Regulation of Threonine 34 Phosphorylation via Serine 97 Phosphorylation Status [SER 97])

Phosphorylation of DARPP-32 at SER97 indirectly increases the phosphorylation of THR34 by protein kinase A (Hamada et al 2005). Therefore, we also measured SER97 phosphorylation using Western blot analysis. At the same time points, we found a mild but significant increase in SER97 phosphorylation in the hypoxia group as well (see

figure 12). The number of animals used in this study were; P11 n=8 control group and n=5 hypoxia group, P12 n=4 control group and n=4 hypoxia group. A 2-way analysis of variance (ANOVA) was used to analyze statistical significance (hypoxia treatment p=0.006, no age-dependence).

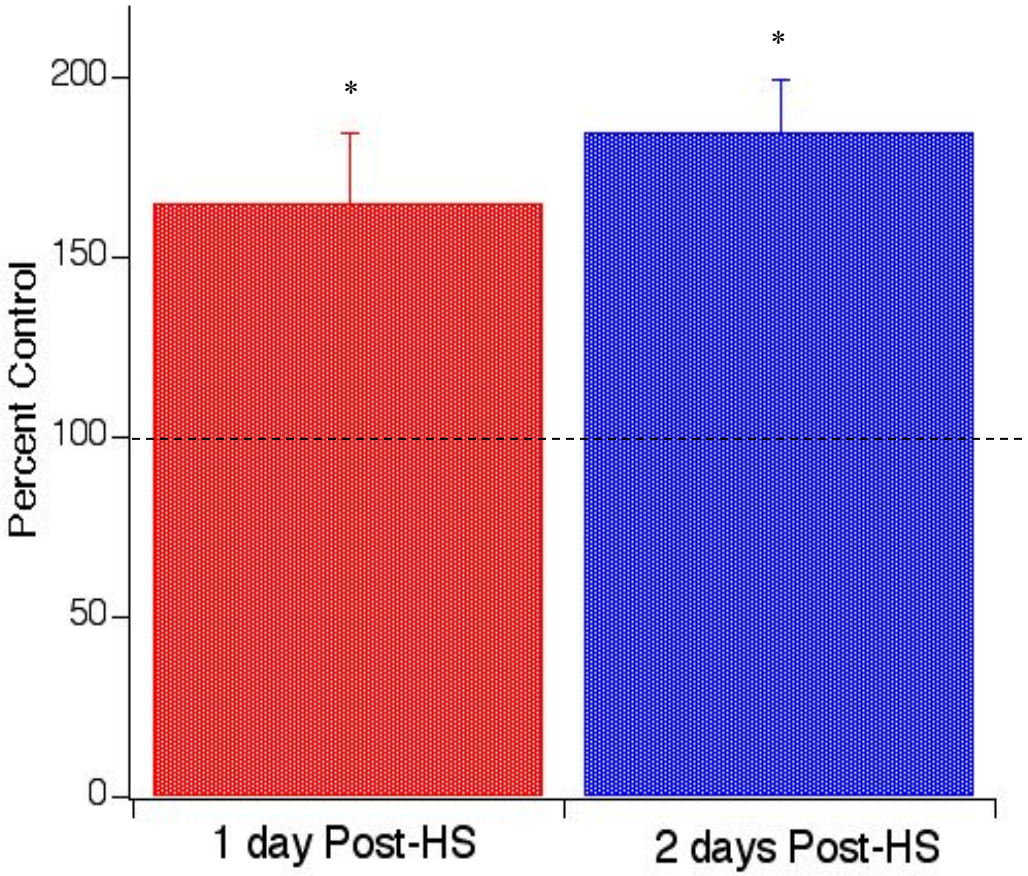

Figure 11: **Threonine-34 DARPP-32.** The ratio of THR34 phospho-DARPP-32 to total DARPP-32 within each sample is significantly increased following hypoxia-induced seizures at 24 hours and up to 48 hours post hypoxia-induced seizures. * P<0.05 (N=5-6 each group).

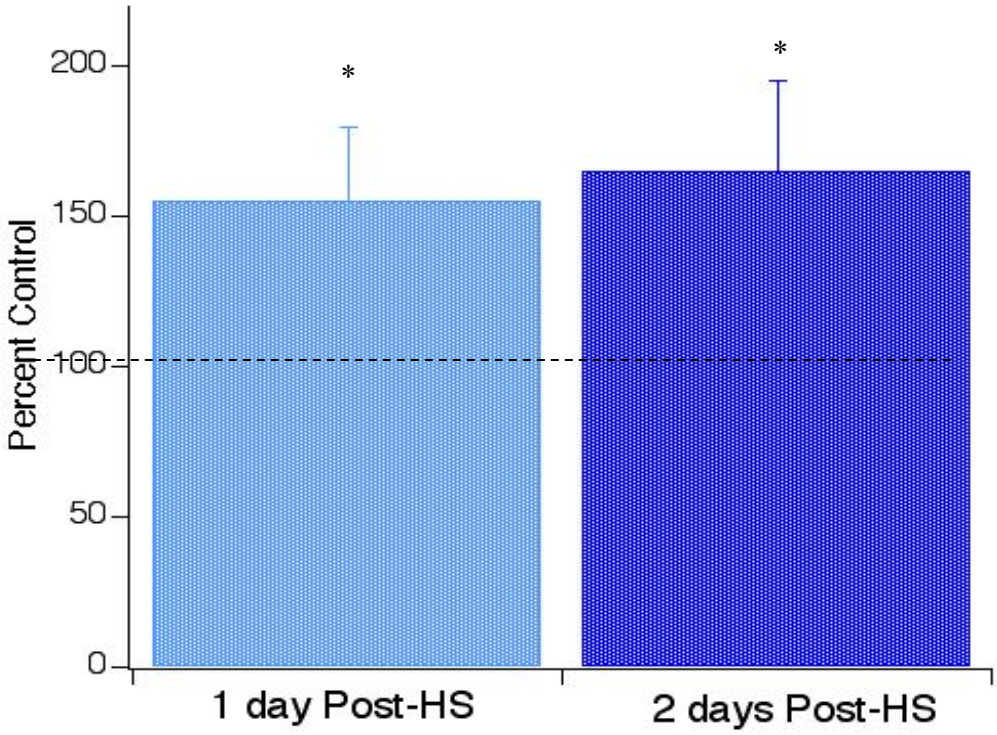

Figure 12: **Serine-97 DARPP-32.** Summary of average ratio of phosphorylated Serine 97 of DARPP-32 to total DARPP-32 within each sample. The ratio of Serine 97 of phospho-DARPP-32 to total DARPP-32 within each sample is significantly increased following hypoxia-induced seizures at 24 hours and up to 48 hours post hypoxia-induced seizures. * $P<0.05$ (N=5-6 each group).

Conclusions

An acute increase in calcineurin expression has been reported to coincide with a decrease in the phosphorylated form of the β2/3 subunit of the $GABA_A$ receptor (Sanchez et al 2005). This was interpreted as a decrease in the functional membrane associated $GABA_A$ receptor and maybe responsible for the acutely decreased IPSCs we recorded from P10-P11 animals. It was not known whether or not calcineurin expression remained increased beyond P11. Since we reported changes in GABAergic inhibition up to a week post-hypoxia, we focused on measuring calcineurin expression from P11-P17. The mechanisms by which hypoxia may increase calcineurin expression and or activity most likely differs short term (24 hours) versus long term (7 days). Since it would require translation of a significant amount of enzyme in a relatively short amount of time, it is unlikely that the short term effects are due to increased calcineurin protein expression. For example, no increase in total calcineurin protein concentration was detected by Western blot analysis of cerebellum, cortex or hippocampus homogenates taken from rats 60 minutes post pilocarpine-induced seizures compared to age-matched controls from rats 60 minutes post pilocarpine-induced seizures, compared to age-matched controls (Kurz et al 2001). Short-term increases in calcineurin activity may occur due to pathological loss of Ca^{2+} homeostasis and chronic stimulation of converging Ca^{2+} enzymatic pathways that either directly or indirectly activates the catalytic and or regulatory domains of the calcineurin molecule.

Calcineurin is a Ca^{2+} and calmodulin-stimulated serine/threonine phosphatase. The enzyme consists of two subunits, a catalytic subunit and a regulatory subunit. The

catalytic subunit, calcineurin A, contains two auto-inhibitory domains. Calcineurin B is the regulatory subunit that binds Ca^{2+} (Klee et al 1998). Calcineurin is inhibited by phosphorylation at the same location by both protein kinase C and CaM kinase II (Hashimoto and Soderling 1989, Martensen et al. 1989). Both calcineurin and CaM kinase II share a similar distribution in the brain supporting the notion of CaM kinase II regulation (Sola et al. 1999). Calcineurin regulates NFAT-mediated and CREB-mediated gene transcription important in modulating the function of both the GABA and the NMDA receptors, affects cytoskeletal architecture through dephosphorylation of microtubules and associated proteins, regulates neurotransmitter release, and may be involved in the initiation of apoptosis (Ankarcrona et al 1996, Huang and Dillon 1998, Krupp et al 2002, Goto et al 1985, Cordeiro et al 2000). All of these processes may play a role in the pathology of SE.

More recently, calcineurin has been implicated in modulating HCN channel function (Jung et al 2010). Calcineurin expression has been shown to increase following pilocarpine and hypoxia-induced seizures. Furthermore, FK-506 a calcineurin antagonist, was demonstrated to reversibly inhibit seizures in neonatal hypoxia and amygdaloid kindled rats and ascomycin prevented seizures at higher dosages (Kurz et al 2001; Sanchez et al 2005, Vazquez-Lopez et al 2006). It has also been demonstrated calcineurin activity is enhanced in hippocampal area CA1 from chronically epileptic animals, and that pharmacological reversal of this abnormal phosphorylation signaling restores pyramidal neuron excitability to control levels (Jung et al 2010). Recently, calcineurin was implicated in the regulation of dendritic spines via dephosphorylation of

cytoskeletal actin (Kurz et al 2008). Dendritic spines are small projections of the membrane located on the dendrites of neurons, which are thought to be a critical modulatory compartments of neurotransmission that provide increased surface area for synaptic connectivity and a relatively isolated compartment for modulation, especially Ca^{2+}-dependent enzymatic pathways. Dendritic spine loss has been shown to occur in both human epilepsy and animal models of epilepsy (Muller et al 1993, Isokawa 1998, Isokawa and Levesque 1991, Multani et al 1994). Interestingly, both our report of decreased GABA currents, but also our decrease in I_H following hypoxia-induced seizures can be explained by increased calcineurin expression/activity. One thing to note is that I_H is functionally decreased after kainate-induced status epilepticus in adult rats but is increased after hyperthermia-induced seizures in immature rat pups, suggesting a possible age-dependence and/or conditional response. It is unknown whether this up-regulation of calcineurin expression and or activity persists beyond 24 hours, thus promoting persistent changes in inhibition.

We hypothesized that calcineurin expression and/or activity would increase persistently in the hypoxia-induced seizures group as compared to age-matched control littermates. However, we found that calcineurin expression did not increase beyond 24 hours post hypoxia-induced seizures. Furthermore, calcineurin activity did not increase, as measured by specific dephosphorylation of THR 34 of DARPP-32. Although this does not support our hypothesis, it does open up the possibility of other key players previously not reported in this model of epilepsy.

By further exploring DARPP-32 regulation via Serine 97 (SER97)

phosphorylation, we found that the possibility of a protein kinase A (PKA)/casein kinase II (CKII) dependent phosphorylation of SER97 existed at 24 hours to 48 hours post hypoxia-induced seizures. Since, it was shown that SER97 phosphorylation increases protein kinase A-dependent phosphorylation of THR34, our finding of increased phosphor-THR34 at the 24 and 48 hour time point coincided with that of phosphor-SER97 and suggests that the increase in THR34 phosphorylation, and hence increased inhibition of protein phosphatase-1 (PP1), may not be due to an increase in protein kinase A activity (or decrease in calcineurin) but rather through an alternative pathway that may involve Interleukin-1 (IL-1), receptor activation and casein kinase II regulation of the SER97 site of DARPP-32. Although, it is unclear what is mediating the increase in THR34 phosphorylation, the functional consequence of increased phosphor-THR34 DARPP-32 is increased inhibition of PP1 which regulates various receptors and channels such as voltage-gated Ca^{2+} and NMDA.

Although increased calcineurin activity was reported in our neonatal model of hypoxia-induced seizures it was not done so by measuring phosphorylation changes on THR34 of DARPP-32 as used by Poolos and others, but rather by enzymatic assay (Jung et al 2010, Sanchez et al 2005). Furthermore, the increased activity of calcineurin was interpreted as direct (or DARPP-32 independent) dephosphorylation of β2/3 of $GABA_A$ receptors as reported previously and not by a DARPP-32 dependent regulation of PP1, which is known to be an important regulator of numerous ion channels that affect excitation and inhibition, possibly causing an imbalance when regulation becomes dysregulated following an insult such as hypoxia-induced seizures. Furthermore, we

have data to suggest that IL-1β, the major endogenous ligand of the IL-1 receptor, increases at 24 hours remaining so for several days following hypoxia-induced seizures compared to age-matched control animals. This is significant because we show that, when IL-1β is applied to hippocampal slices from control animals, both spontaneous IPSC frequency and amplitude decrease. Although speculative, combined with the data presented here, suggest that, increased IL-1 receptor activation by key players such as IL-1β could cause a decrease in inhibition as measured by sIPSCs in area CA1 of the hippocampus causing an imbalance of excitation and inhibition. Also, since this occurs over a period of days, this abnormal signaling, through several different pathways, including the IL-1 receptor and casein kinase II pathways, could contribute to the development of spontaneous seizures and epilepsy in the neonatal hypoxia model.

CHAPTER V

SUMMARY AND CONCLUSIONS

Introduction

An imbalance between excitation and inhibition has long been thought to underlie epilepsy. Previously, our laboratory and the laboratory of Frances Jensen reported an acute decrease in both the amplitudes and frequency of mIPSCs and sIPSCs in CA1 pyramidal neurons recorded in slices prepared immediately following perinatal hypoxia-induced seizures in vivo (Sanchez et al 2005). Decreases of GABergic inhibitory neurotransmission (IPSCs) could be due to several different mechanisms including: (**1)** Changes in the release of GABA from presynaptic stores (**2)** The composition/expression of the $GABA_A$ receptor and (**3)**. The state of the $GABA_A$ receptor modulation through various post-translational processes, especially calcineurin, a highly conserved Ca^{2+} and calmodulin-dependent serine/threonine phosphatase, which has been shown to be acutely up-regulated following neonatal hypoxia-induced seizures (Sanchez et al 2005). Persistently decreased GABAergic inhibition could promote the development of epilepsy, epileptogenesis.

In this study, we tested several hypotheses. One, does the acute (P10) decrease in GABA mediated inhibitory currents, IPSCs, persist? We hypothesized based on several lines of evidence, including the fact that rats exposed to hypoxia-induced seizures exhibit chronic increases in excitability in area CA1 of the hippocampus which is the final hippocampal region in a 3 circuit pathway that begins in the entorhinal cortex, that there would be a persistent increase in GABAergic inhibition. Two, are there persistent

changes to the composition/expression of the $GABA_A$ receptor? We hypothesized that there would be persistent decreases in the overall expression and or composition of the $GABA_A$ receptor, based primarily on our report of acutely decreased IPSC amplitudes, which could be due to changes in composition or expression of $GABA_A$ receptor subunits (mechanism 2 above). Three, are there persistent changes in calcineurin expression/activity following neonatal hypoxia-induced seizures? We hypothesized that there would be a persistent increase in calcineurin expression and or activity following neonatal hypoxia-induced seizures. This last hypothesis was based mainly on the observation that when calcineurin is blocked with FK-506 acutely (P10) sIPSC frequency and amplitudes increase providing yet another mechanism by which GABAergic inhibition maybe down regulated (mechanism 3 above).

Hypotheses

Hypothesis 1

In the current study, we hypothesized that there would be persistent changes in $GABA_A$ mediated inhibition in the hippocampus. We studied specifically, the fast phasic inhibition provided by synaptically-located $GABA_A$ receptors, those containing 2 αs 2βs and a γ subunit(s), and not δ subunit containing extra-synaptic $GABA_A$ receptors responsible for tonic inhibition. Although it is possible that tonic GABA currents are compromised and contribute to increased excitability of area CA1 pyramidal neurons following hypoxia-induced seizures, tonic GABAA receptor currents do not contribute to the decrease in IPSCs studied in chapter 2. The data supported our hypothesis, revealing a persistent decrease in $GABA_A$ mediated currents, IPSCs, which are regulated by both pre- and post-synaptic mechanisms. We found decreases in: action-potential dependent, sIPSC frequency and amplitudes that persisted at 3–5 days after hypoxia. This finding could be due to several different possible mechanisms. The decrease in sIPSC frequency could be due to differences in firing rates of presynaptic interneurons and the sIPSC amplitudes could be due to changes in the state of modulation by post-translation processes, especially calcineurin activity/expression that has been shown to be pathologically increased following hypoxia-induced seizures (and modulates both sIPSC frequency but also sIPSC amplitude) (Sanchez et al 2005). The fact that there is no change in the mIPSC frequency but only a change in sIPSC frequency supports the hypothesis that during P13-P15 there are significantly less action-potential firing of interneurons and release of GABA.

Changes in sIPSC amplitude could be due to presynaptic changes in the amount of GABA released, the efficacy of GABA at the $GABA_A$ receptor, and possibly changes in either composition or expression of $GABA_A$ receptors. We found that action-potential independent mIPSCs were decreased in amplitude, but not frequency. 3-5 days post-hypoxia, mIPSC amplitudes were decreased and comparable to the decrease in sIPSC amplitudes. This supports the hypothesis that there are post- synaptic changes to the $GABA_A$ receptor, perhaps either by expression and/or composition of the $GABA_A$ receptor itself. We continued to look at this decrease in GABA mediated inhibition out to 7 days post-hypoxia-induced seizures and we found a recovery of action-potential independent frequency and amplitude, as well as action-potential dependent amplitudes. Interestingly, since mIPSC frequency recovered but not sIPSC frequency, only the action-potential driven IPSC frequency changed persistently out to 7 days post-hypoxia-induced seizures and possibly longer. Combined with the recovery of both sIPSC and mIPSC amplitudes, this indicated that several days following neonatal hypoxia-induced seizures, basal synaptic inhibition of CA1 pyramidal neurons is decreased despite no apparent long-lasting changes in the probability of spontaneous GABA release, indicating that the persistent decrease in sIPSC frequency is most likely due to decreased spontaneous firing of interneurons that provide synaptic inhibition to CA1 pyramidal neurons. Still, since we do find changes in sIPSC and mIPSC amplitudes 3-5 days post hypoxia there could be changes in the composition/expression of the $GABA_A$ receptor itself which provided us with further rationale to analyze this possibility. For hypothesis 2 we used a combination of techniques.

Hypothesis 2

We utilized two methodological approaches in order to explore the possibility that there were changes in the composition and or expression of $GABA_A$ receptors following neonatal hypoxia-induced seizures. We looked both at the functionality of the $GABA_A$ receptor by electrophysiology and by measuring total protein of various $GABA_A$ receptor subunits that are known to be synaptically located in area CA1 of the hippocampus. Altered $GABA_A$ receptor subunit expression and pharmacological sensitivity have been reported in neonatal as well as adult rodent models of seizures and human epileptic hippocampi (Zhang et al 2007, Schwarzer et al 1997, Bouilleret et al 2000, Raol et al 2006, Loup et al 2000). For example, research studies of both humans with temporal lobe epilepsy (TLE) and in rodent models of TLE found reduced expression of GABA receptor α1 subunits and increased expression of $GABA_A$ receptor α4 subunits in the dentate gyrus of epileptic individuals (Brooks-Kayal et al 1998 and 1999). In the adult rat model of pilocarpine-induced status epilepticus these subunit alterations are associated with diminished benzodiazepine sensitivity (Gibbs et al 1997; Brooks-Kayal et al 1998). When similar studies were performed at an earlier developmental time point (postnatal day 10), rat pups that experienced pilocarpine-induced SE had significantly increased $GABA_A$ receptor α1 expression in dentate gyrus and enhanced benzodiazepine sensitivity (Zhang et al 2004). However, it was not known if there are changes in expression of $GABA_A$ receptor subunits following neonatal hypoxia-induced seizures.

Since $GABA_A$ channels sensitive to diazepam require an α , β and a γ to function

correctly, any hypoxia-associated change in the prolongation of the decay time of GABA currents following diazepam application would suggest a change in the $GABA_A$ receptor subunit composition. We found no statistical difference in diazepam sensitivity between control and hypoxia groups at 3-5 days post-hypoxia. Furthermore, by Western blot, we found no significant changes in the expression of α1, α2, β2/3, and γ2 subunits of the $GABA_A$ receptor at numerous time points. The combined electrophysiological and Western blot data strongly suggest that no significant changes in the post-synaptic composition of the $GABA_A$ receptor during P13-P15. Other mechanisms of decreased GABAergic inhibition following neonatal hypoxia-induced seizures likely exist, such as post-translational modification of the $GABA_A$ receptor.

Hypothesis 3

In the neonatal hypoxia-induced seizure model, calcineurin was shown to have increased activity and blocking calcineurin with FK-506 increased sIPSC frequency and sIPSC amplitudes reversing the losses associated hypoxia-induced seizures at P10 (Sanchez et al 2005). Furthermore, blocking calcineurin activity with FK-506 prevented hypoxia-induced seizures at P10. Persistent changes in calcineurin expression/activity have not been reported. Persistently increased expression/activity of calcineurin following hypoxia-induced seizures could persistently decrease GABAergic inhibition. Calcineurin expression/activity is known to modulate both IPSC frequency and amplitude, possibly by dephosphorylating the β2/3 subunit of the $GABA_A$ receptor and causing internalization of the receptor complex thereby decreasing the frequency and amplitudes of GABA mediated IPSCs. To explore the possibility of a persistent increase

in calcineurin activity/expression we used Western blot analysis of calcineurin expression and THR34 phosphorylation status of DARPP-32. DARPP-32 is specifically dephosphorylated on THR34 by calcineurin and has been used as a measure of calcineurin activity (Jung et al 2010). Since there were no apparent changes in $GABA_A$ subunit composition 3-5 days following hypoxia, we hypothesized that the $GABA_A$ receptors were being post-translationally modified by increased calcineurin activity/expression. As previously reported, we found that calcineurin expression was increased up to 24 hours following hypoxia (Sanchez et al 2005). However, we report that there were no significant changes in expression beyond the initial 24 hours (P10-P11) and as far out as 6 days (P16) following hypoxia-induced seizures.

Since, expression isn't the only way calcineurin can increase its enzymatic activity, we measured THR34 phosphorylation of DARPP-32 which is a specific substrate for calcineurin. Increases in calcineurin activity should be measurable as a decrease in the ratio of phosphoTHR34 DARPP-32 to total DARPP-32. If our hypothesis was correct we should have found decreased phosphoTHR34 DARPP-32 to total DARPP-32. However, our Western blot analysis demonstrated that THR34 phosphorylation was mildly increased (~ 150% control) following hypoxia-induced seizures, raising the question of whether protein kinase A or some other unknown mechanism may overcompensate for any calcineurin specific activity. Since THR34 is a direct and specific substrate of phosphorylation by protein kinase A, it is possible that protein kinase A activity expression may have increased. This may very well be the case, but a relatively recent paper reported that protein kinase A activity among other enzymes

were acutely increased but recovered by 12 hours following neonatal hypoxia-induced seizures (Rakhade et al 2008). We then asked, "Could there be another pathway involved in THR34 phosphorylation status?"

Alternative Pathways

DARPP-32 is regulated by numerous phosphatases and kinases that specifically and directly act at various phosphorylation sites and some of these sites can interact and affect the phosphorylation of others. Specifically, residue SER97 is directly phosphorylated by casein kinase II (Girault et al 1989). Furthermore, when residue SER97 is phosphorylated, phosphorylation of THR34 by protein kinase A increases several fold (Girault et al 1989). So it is possible that THR34 phosphorylation can be increased, independent of changes of both protein kinase A and calcineurin. We next explored the question of whether SER97 may also have increased in phosphorylation.

We found that SER97 phosphorylation status was also increased following neonatal hypoxia-induced seizures (~ 160%). Together with the calcineurin Western blot data, it is unlikely that our hypothesis of post-translational modification of the $GABA_A$ receptor by calcineurin exists beyond the initial 24 hours following hypoxia-induced seizures (P10-P11). These findings question the validity of using THR34 phosphorylation status of DARPP-32 as a measure of calcineurin activity as previously reported (Jung et al 2010).

However, it's important to note that since the phosphorylation status of a number of residues and the enzymatic pathways that are involved in the regulation of DARPP-32 are highly characterized it permits us to explore alternate pathways that may

be compromised as a consequence of neonatal hypoxia induced seizures. Although we do not know yet which particular enzymatic pathways are being activated to increase the phosphorylation of THR34 and SER97, the physiological consequence of this is that the phosphorylation of THR34 of DARPP-32 increases inhibition of protein phosphatase 1 (PP1) (Svenningsson et al 2004). PP1 interacts with numerous receptors and channels in the brain where it is expressed in neurons. PP1 is highly abundant in neurons, and following NMDA receptor activation, it is recruited to the receptor and associates with it by binding to specific targeting partners. There, it decreases NMDA receptor activity and synaptic strength by reducing the receptors open probability (Wang et al 1994). Furthermore, postsynaptic $GABA_A$ receptor currents in neostriatal medium spiny neurons are decreased in a D1 dopamine receptor/PKA/DARPP-32/PP1 signaling cascade targeting the phosphorylation of $GABA_A$ receptor β1subunits (Flores-Hernandez et al 2000). It is not known whether PP1 function is decreased following hypoxia-induced seizures but our Western blot data of THR34 raises the possibility of decreased inhibition of PP1 via DARPP-32. Furthermore, whether NMDA receptor function maybe increased via decreased PP1 activity following hypoxia-induced seizures is yet to be determined. The implications of increased NMDA activity following neonatal hypoxia-induced seizures include increased excitability and increased calcium signaling, both of which contribute to increased acute as well as persistent hyperexcitability of the hippocampal network and possibly epileptogenesis, in susceptible individuals.

We identified SER97 as a specific and direct substrate of casein kinase II, raising the possibility of this pathway being involved. Casein kinase II is known to be activated

as a consequence of inflammation (Gaestel et al 2009). It is unknown whether casein kinase II is up-regulated following hypoxia-induced seizures but our Western blot data of SER97 suggests this as a possibility. Casein kinase II has also been shown to regulate NMDA receptor function (Lieberman and Mody 1999, Kimura and Matsuki 2008). Although casein kinase II is not u-pregulated in the kainite model of epilepsy (Wyneken et al 2001) an increase in either casein kinase II protein expression or casein kinase II enzymatic activity may increase following hypoxia-induced seizures since as discussed earlier each model of epilepsy have unique characteristics that depend on the age and area of the brain involved as two examples.

Casein kinase II plays a key role in regulating interleukin-1, or IL-1, biosynthesis. Interestingly, our laboratory has found increases in the endogenous ligand of the IL-1 receptor, IL-1β, following perinatal hypoxia-induced seizures. Furthermore, by applying IL-1β to the bath and recording IPSCs from hippocampal slices in area CA1 by whole-cell voltage clamp, an increase in both amplitude and frequency of IPSCs occurs, providing an alternative mechanism for decreased IPSC frequency and amplitudes following hypoxia-induced seizures. Much work must be done to further characterize the pathways that lead to increased phosphorylation of THR34 and SER97, as well as the downstream sequence of events that occur as a response to phosphorylation of THR34 and SER97. It is important to note that the phosphatase for SER97 is unknown and may also be involved. The hypothetical pathways of DARPP-32 regulation following neonatal hypoxia induced seizures are summarized in figure 13.

Finally, by 7 days following hypoxia only sIPSC frequency remained decreased,

and this strongly suggests when combined with the rest of our data, that only the presynaptic firing of interneurons remained suppressed. Interestingly, our laboratory reported decreased I_H in CA1 pyramidal neurons (Zhang et al 2006). Since I_H is known to regulate the intrinsic firing rate of spontaneously active interneurons (Maccaferri and McBain 1996) we measured I_H in interneurons of area CA1 of the hippocampus in studies done outside the scope of this dissertation. We found that, of the cells recorded, only 85 percent expressed I_H. Furthermore, of the cells recorded we found no change in I_H.

We still have much work to determine whether or not there are specific subpopulations of interneurons affected by hypoxia-induced seizures that respond by decreased firing. This experimental question is confounded by the fact that numerous different subtypes of interneurons exist in the hippocampus (See Introduction Chapter).

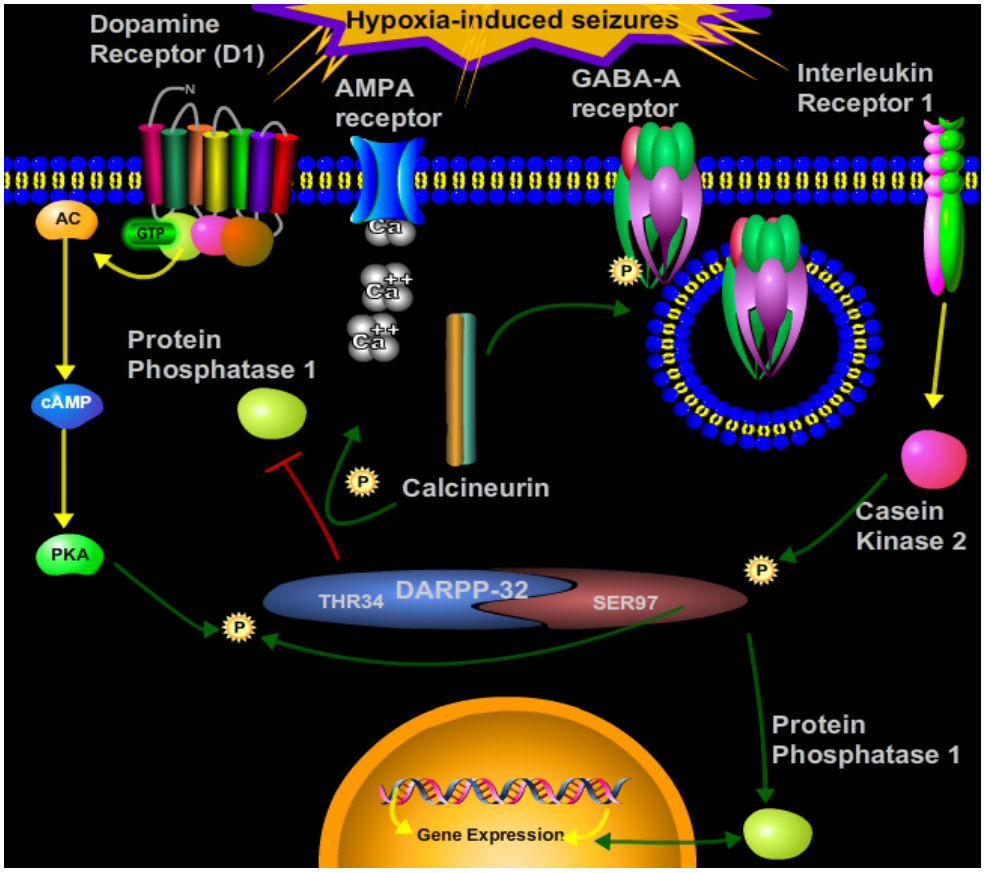

***Figure 13*: Hypothetical Model of DARPP-32 Regulation.** Hypothetical model of DARPP-32 regulation following hypoxia-induced seizures. Green and yellow arrows: activation of an enzyme or in the case of SER97 to protein kinase A phosphorylation increase in activity of protein kinase A phosphorylation of THR34. Red line with blunt end: inhibition of Protein phosphatase 1.

Conclusions and Future Directions

We have characterized altered inhibition after neonatal hypoxia as a likely pro-epileptogenic consequence and have begun to elucidate underlying mechanisms. Future directions of our laboratory include: determining the functional consequences of THR34 phosphorylation of DARPP-32 in the rat model of neonatal hypoxia-induced seizures and determining how action potential-dependent GABAergic inhibition is altered following hypoxia-induced seizures and whether this decrease persists beyond 7 days following hypoxia, and determining how from 0 –5 days following hypoxia GABA mediated IPSC amplitudes decrease. Even though our data seem to suggest that the decrease in GABA IPSC amplitudes are not due to changes in the $GABA_A$ receptor expression/composition and that calcineurin expression/activity does not persist beyond the initial 24 hours post-hypoxia-induced seizures it is possible that internalization of the $GABA_A$ receptor is still occurring through another enzymatic pathway (perhaps CK2 or PP1 for example).

The next, and simplest, experiment is to use an assay for surface expression of $GABA_A$ receptors. This can be accomplished by using biotin to label proteins that are located on the extracellular side of the cell membrane and then pulling down these proteins with, for example, Avidin UltraLink Resin by Thermoscientific. The samples can then be used for Western blot analysis, thereby allowing us to quantify changes to the $GABA_A$ receptor surface expression. Since we only measured changes out to 7 days following hypoxia and sIPSC frequency alone remained decreased it is possible and probable that this remains decreased. If sIPSC frequency remains decreased persistently

especially into adulthood it could provide novel pharmacotherapy.

For example, drugs that are known to increase the firing rate of interneurons, i.e. through interacting with HCN channels and effecting I_H, could provide an alternative pharmacological therapy. Interestingly, we recently reported that that the anticonvulsant drugs Lamotrigine and Gabapentin increased the excitability of presumed inhibitory s.o. non-pyramidal neurons via enhancement of I_H, and that this effect was associated with increased spontaneous synaptic inhibition of CA1 pyramidal neurons, possibly identifying an additional anti-convulsant mechanism that was previously unknown (Peng et al 2010, 2011).

Much work must be done to begin to understand the mechanisms underlying decreased GABAergic inhibition following perinatal hypoxia-inducing seizures. We have just discovered that persistently decreased GABAergic inhibition may be a proepileptogenic mechanism providing us with further rationale to explore this area in this animal model of acquired epilepsy.

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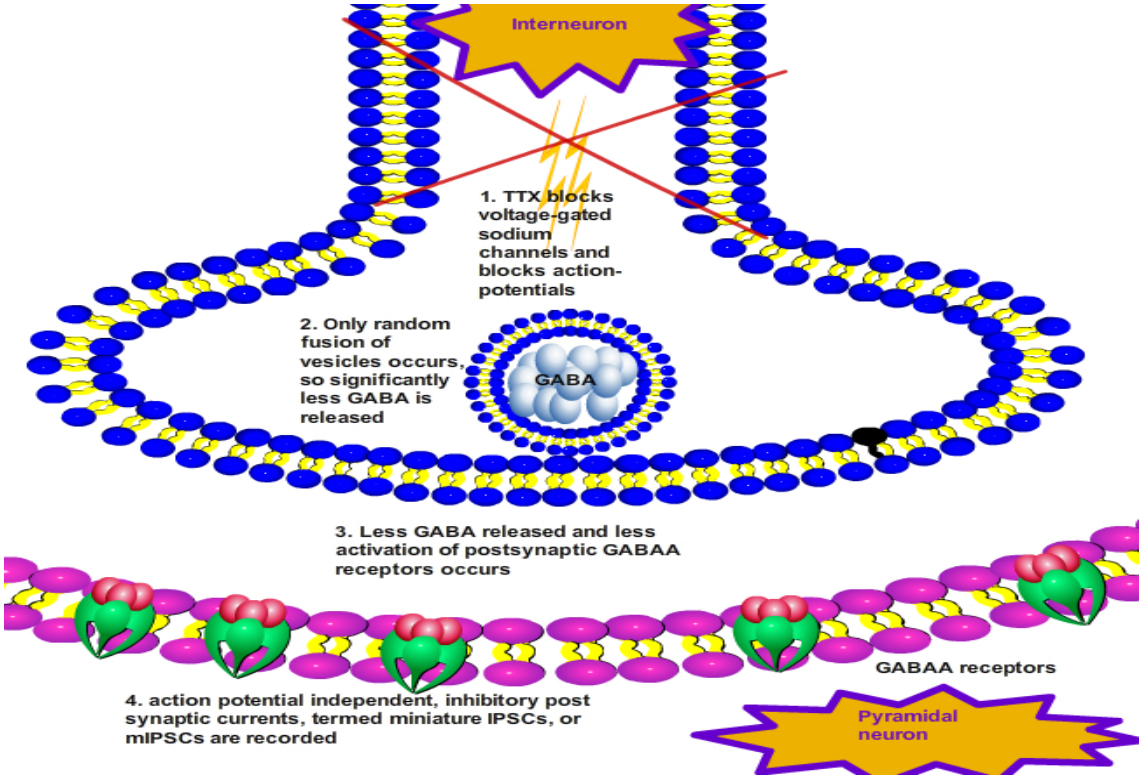
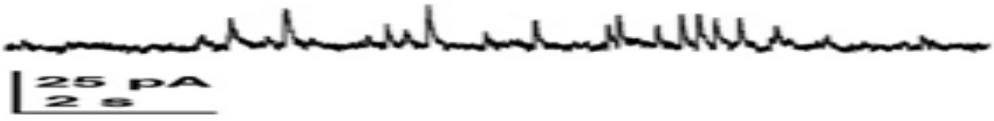
APPENDIX A

Appendix A: Illustration of Miniature Postsynaptic Currents (mIPSCs)

A.



B.

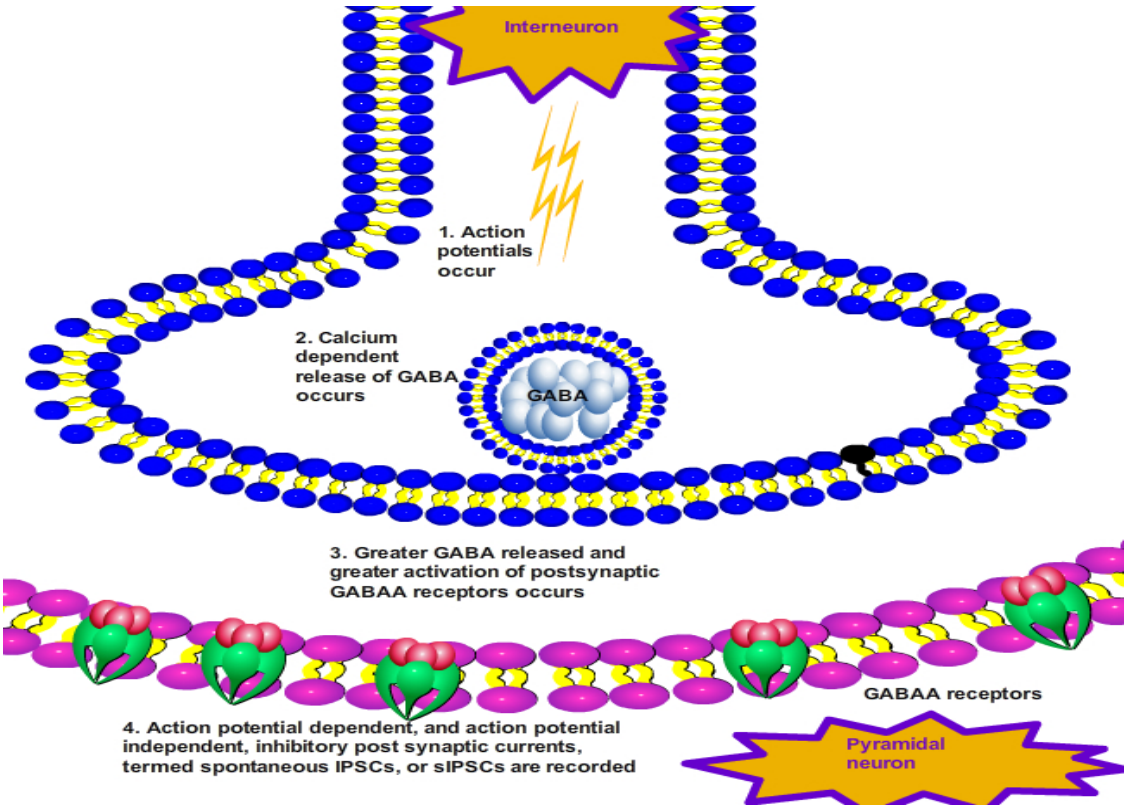


Appendix A: A. Miniature Inhibitory Post Synaptic Currents. A basic set of events occur that result in miniature inhibitory post synaptic currents that are mediated by GABA and synaptically located $GABA_A$ receptors. 1) Tetrodotoxin blocks action potential firing of interneurons and allows 2) only random fusion of GABA containing vesicles releasing significantly less GABA into the synapse where 3) less $GABA_A$ receptors are activated post-synaptically leading to 4) miniature inhibitory post-synaptic currents called mIPSCs. B. example whole-cell voltage clamp trace of mIPSCs recorded from hippocampal area CA1 pyramidal neurons.

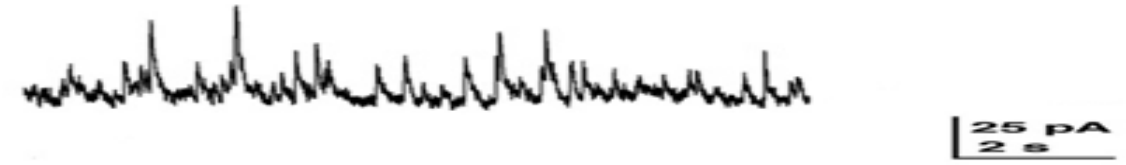
APPENDIX B

Appendix B: Illustration of Spontaneous Postsynaptic Currents (sIPSCs)

A.



B.



Appendix B: A. Spontaneous Inhibitory Post Synaptic Currents. 1) Action potential firing of interneurons occur in the absence of TTX and allows 2)greater release of GABA by calcium dependent mechanisms where 3) greater activation of $GABA_A$ receptors occur post-synaptically leading to 4)spontaneous inhibitory post-synaptic currents called sIPSCs. B. Example whole-cell voltage clamp trace of sIPSCs.