EXTRINSIC INHIBITION OF THE INFERIOR COLLICULUS DURING AUDIOGENIC SEIZURES: EFFECTS OF UNILATERAL DORSAL NUCLEUS OF THE LATERAL LEMNISCUS LESIONS IN YOUNG RATS

A Thesis
By
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FOREWORD

This thesis is written in accordance with the style of the *Publication Manual of the American Psychological Association (5th Edition)* as required by the Department of Psychology at Appalachian State University

Running head: EXTRINSIC INHIBITION DURING AUDIOGENIC SEIZURES IN RATS

Extrinsic Inhibition of the Inferior Colliculus during Audiogenic Seizures: Effects of Unilateral Dorsal Nucleus of the Lateral Lemniscus Lesions in Young Rats

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Abstract

In this study, a model of generalized epilepsy was used to investigate the role of extrinsic inhibition of the inferior colliculus (IC) during audiogenic seizures (AGS). There is evidence to suggest that a deficiency of inhibition within the IC plays a major role in AGS; however, the importance of extrinsic inhibition of the IC is not as well understood. To investigate this, neurotoxin lesions of dorsal nucleus of the lateral lemniscus (DNLL) were made, therefore eliminating the main source of extrinsic inhibition to the IC. Long-Evans rats (N = 18) were primed for AGS susceptibility via acoustic insult on post-natal day (pnd) 18, tested for susceptibility on pnd 32, and then subjected to a series of inductions on seven occasions over 14 days beginning on pnd 35. Seizure-susceptible rats were divided into three groups (n = 6). One group received a neurotoxic injection in the DNLL, a second group was prepared for surgery, but did not receive the injection, and a third group experienced no aspects of the surgical procedure. Subjects then followed a post-surgery induction schedule to evaluate differences across three measures: latency to enter a wild-running phase, latency to enter clonus, and duration of the seizure. The hypotheses that subjects in the lesion group would have a shorter latency to enter a wild running phase in post-surgery inductions (p = .5289) and would exhibit a shorter latency to enter clonus in post inductions (p = .1713) were not supported by the data. The hypothesis that subjects in the lesion group would exhibit longer AGS post-surgery approached statistical significance (p = 0.0584); however, rats in the lesion group exhibited shorter AGS than controls. While a lack of intrinsic inhibition of the IC plays an important role in AGS, the data from this study suggest that a lack of extrinsic inhibition to the IC does not have a significant role in inhibiting the initiation and propagation of AGS activity in rats, but may aid in decreasing the duration of clonic seizures.

Extrinsic Inhibition of the Inferior Colliculus during Audiogenic Seizures: Effects of Unilateral Dorsal Nucleus of the Lateral Lemniscus Lesions in Young Rats

Epilepsy is a neurological disorder characterized by temporary abnormal neuronal activity which causes seizures ranging in severity from temporary lapses in consciousness to muscular convulsions. Over three million Americans of all ages are affected by epilepsy or seizures, with approximately 200,000 new cases occurring each year (Epilepsy Foundation, 2010). While there are many seizure disorders, the seizure itself always involves abnormal excitatory and inhibitory activity in the brain. Seizure disorders can be characterized as either focal (also referred to as partial) or generalized. In focal seizures, the abnormal neuronal activity is confined to one area of the brain. Symptoms of this type of seizure are categorized as either simple or complex. Simple focal seizures do not result in unconsciousness, but rather an altered state of consciousness including exaggerated emotions or hallucinations. Complex focal seizures can result in either consciousness or unconsciousness (Epilepsy Foundation; Freeman, Vining, & Pillas, 2002). Abnormal neuronal activity that begins in one structure and spreads throughout the entire brain is known as a generalized seizure (Ross & Coleman, 2000). Like focal seizures, generalized seizures are also categorized by symptoms exhibited. Individuals experiencing absence seizures (referred to as petit mal seizures) may exhibit blank stares and mild muscle twitches. Tonic seizures result in stiff muscles and immobility. Conversely, atonic seizures exhibit loss of muscle tone. During a clonic seizure, the muscles contract and relax, resulting in full body jerks. Myoclonic seizures are similar to clonic seizures, with the exception that muscle jerks are confined to the limbs and upper body. Tonic-clonic seizures, also known as grand mal seizures, display stiffening and repeated jerking of the limbs (Epilepsy Foundation; Freeman et al.).

Epilepsy, particularly with generalized seizures, is a heritable disorder; however, seizures can also be acquired in response to an environmental insult such as an intense or unexpected acoustic, visual, or tactile stimulus, a condition referred to as reflex epilepsy. Most reflex epilepsy in humans is the result of exposure to a visual stimulus such as sunlight, intense bright or flashing light, or even some video games (Epilepsy Foundation, 2010; Freeman et al., 2002). Rodent models can be used to study seizure disorders, and generally rodents will express reflex seizure activity in response to intense acoustic stimulation (Pierson & Swann, 1991). In this thesis, a rodent model of acquired reflex epilepsy was used to investigate neural mechanisms underlying reflex seizures.

While the role of runaway excitation within the auditory midbrain during audiogenic, or sound-induced seizures has been investigated extensively (Garcia-Cairasco,Terra, & Doretto, 1993; Pierson & Swann, 1991; Pollack, Burger, & Klug, 2003; Ross & Coleman, 2000), the importance of normal inhibition, or lack thereof, during seizures is not as well understood. This thesis examined the relationship between over-excitation of the inferior colliculus (IC), the initiation site of audiogenic seizure (AGS) activity, and the lack of extrinsic inhibition, which is supplied by the dorsal nucleus of the lateral lemniscus (DNLL; Garcia-Cairasco et al.; Pierson & Swann; Pollack et al.; Raisinghani & Faingold, 2002; Ross & Coleman).

Audiogenic Seizure Model of Epilepsy

There are multiple models used to investigate generalized seizures in rats, including electric and chemical kindling models, genetically epilepsy-prone rat models (GEPRs), and developmentally-primed audiogenic seizure models (Pierson & Swann, 1991; Ross & Coleman, 2000). In the present study, a developmentally-primed model in which rats

acquired susceptibility to AGS through a procedure in which the animal is subjected to intense acoustic insult at specific times during development was used (Ross & Coleman, 1999).

Priming and testing. According to the developmentally primed model, seizure-resistant strains of rats can be made seizure prone at a young age through exposure to intense acoustic stimulation around the time the ear canal opens, a procedure referred to as priming. Priming causes a disruption of nerve connections of the normal auditory pathway, damage that the brain will attempt to repair by reorganizing nerve connections which allow for over-excitation of neurons if acoustic insult is presented later (Pierson & Swann, 1991; Ross & Coleman, 1999). Subjects must then be tested via exposure to loud noise approximately two weeks after priming to determine seizure susceptibility. The parameters for both priming and testing, such as intensity of noise and days to perform the procedures, are fairly specific and strain-dependent. For the Long-Evan strain, the rat stain used in this study, the optimum day to prime is postnatal day (pnd) 18, and the optimum day to test is pnd 32 (Ross & Coleman, 1999; Ross & Coleman, 2000).

Inductions. A series of seizure inductions are necessary after priming and testing in order to evaluate AGS severity. The number of inductions varies depending upon the investigation and research design. There is a reliable progression of AGS behavior exhibited during each trial (Garcia-Cairasco, 2001; Ross & Coleman, 1999). In the first phase of AGS activity, referred to as the wild running phase, the subject runs uncontrollably at a high rate of speed around the walls of the cylindrical testing chamber some time after acoustic stimulation begins. Wild running gives way to one of four possible outcomes: a period of inactivity, a second run, a clonic seizure, or tonic-clonic seizure (Pierson & Swann, 1991;

Ross & Coleman). In the Audiogenic Response Score (ARS), a scale for measuring AGS response to sound stimulation in rats, AGS inductions with only one phase of wild running before entering a seizure state are given a higher rating on the scale than episodes with two phases of wild running. It is suggested that a temporary pause between runs is a demonstration of control provided by inhibition within the brain, indicating that a subject may have more control over the runaway excitation than subjects who are unable to stop running (Jobe, Picchioni, & Chin, 1973).

A wild running phase is often followed by either a tonic, clonic, or tonic-clonic seizure, the only types of seizures exhibited by the many rat strains, including Long-Evans. Tonic seizures, the most severe seizure displayed in the rat, are given the highest ARS (Jobe, et al., 1973). These seizures are characterized by muscle rigidity of the limbs, neck, and back, and can sometimes lead to death. Clonic seizures in rats are characterized by an arched back and neck, stiffness of the limbs, and full-body muscle spasms accompanied by rocking motions (Ross & Coleman, 2000). Because it is the least severe type of seizure exhibited by the rat, it is given the lowest ARS of the seizures. If the clonic convulsions are separated by moments of muscle rigidity, the seizure is characterized as tonic-clonic. While latency to enter a seizure state is strain-dependent, the average latency for the Long-Evans rat is approximately 30 s, and the duration of clonus averages approximately 20 s. The final phase of AGS activity is referred to as the post-ictal period, the period after the seizure when the subject exhibits behaviors such as immobility and vocalization, and is typically unresponsive to acoustic stimulation (Ross & Coleman, 1999; Ross & Coleman, 2000).

The Auditory Midbrain and the DNLL

Auditory processing begins within the cochlea of the inner ear, which tranduces

acoustic information into neural signals. Potentials from hair cells of the cochlea activate the auditory nerve, which is the source of innervation for the cochlear nucleus (CN). The CN projects to multiple ascending pathways. The major projection from the CN is to the contralateral IC, a major processing, integration, and relay center for all auditory input (Coleman & Clerici, 1987; Pollack et al., 2003). CN also projects to the medial and lateral superior olive (MSO, LSO), which are responsible for the detection of interaural time differences, aiding in sound localization. Once excitatory signals leave MSO or LSO, they can project either directly to the IC, or a second route starting in the LSO, which goes then to the ventral nucleus of the lateral lemniscus (VNLL), and finally to the IC (Faye-Lund & Osen, 1985).

The DNLL, located just ventral to the IC, lies within the fibers of the lateral lemniscus (LL) and is a part of the ascending pathway (Bajo, Merchán, Lopez, & Rouiller, 1993; Pollack, et al., 2003; Schneiderman, Chase, Rockwood, Benson, & Potashner, 1993; Wu & Kelly, 1996), and is the primary source of extrinsic inhibition to the IC (Kelly, Li, & van Adel, 1996; Wu & Kelly; Zhang, Li, Kelly, & Wu, 1998). The main source of excitation to the DNLL ipsilateral to a sound source comes from the contralateral CN and superior olivary complex (SOC). This DNLL in turn projects to the contralateral DNLL, which then projects to the IC (Bajo et al., 1993; Pollack et al.). Thus, hearing a sound involves excitation of the IC contralateral to the sound source and inhibition of the IC ipsilateral to the source via the DNLL.

Most excitatory neurotransmission within the auditory system, including the DNLL, is modulated by neurons with glutamate receptors. There are three types of receptors that may be present on glutamate neurons, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-

5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid (KA). Furthermore, each receptor is broken down into four or five protein subunits. NMDA receptor subunits are labeled NR1 and NR2A-D. AMPA receptors contain four subunits, GluR1-4, and KA receptors are labeled GluR5-7 and KA1 and 2, dependent upon gene family (Hermit, Greenwood, & Brauner-Osborne, 2004; Johansen, Greenwood, Frydenvang, Madsen, & Krogsgaard-Larson, 2003; Parks, 2000). Most if not all DNLL neurons also contain the enzyme glutamate decarboxylase (GAD_{67}), a catalyst that aids in the production of γ-aminobutyric acid (GABA), the most common inhibitory neurotransmitter in the nervous system (Garcia-Cairasco et al., 1993). Therefore, GABA-producing neurons are activated through glutamate synapses, which is why much of the DNLL's projection to the IC is inhibitory in nature (Merchán, Saldaña, & Plaza, 1994; Schneiderman et al., 1993; Wu & Kelly, 1996). Because AMPA receptors are essential to the activation of glutamate neurons, an AMPA agonist, such as quisqualic acid, can target and kill GluR1 and GluR4 subunits, ultimately preventing activation of glutamate neurons within the DNLL (Hermit et al.; Johansen, et al.). If glutamate neurons cannot be activated, GABA cannot be released to provide extrinsic inhibition to the IC.

Neural network for AGS. The neural network is identical for all models of AGS, including the developmentally primed model used in this thesis as well as in GEPRs, and electrical and chemical kindling models (Ross & Coleman, 2000; see Figure 1). Once altered by acoustic insult, the complex connections within the auditory pathway reorganize, beginning with those from hair cells to the auditory nerve. If very intense sound is presented later, neurons within the pathway become over-excited due to the new organization of excitatory and inhibitory connections, which culminates in a seizure. Specifically, AGS

induction begins with the over-stimulation of the cochlea within the inner ear. From the cochlea, acoustic information is sent to the SOC, which in turn relays the information via the LL. The LL is responsible for sending acoustic information to the IC where abnormal excitation becomes sufficient to produce a seizure (Garcia-Cairasco et al., 1993; Raisinghani & Faingold, 2002).

The IC relays information to higher areas of the forebrain for interpretation, and is also the initiation sight for AGS activity (Garcia-Cairasco et al.1993; Pierson, & Swann 1991; Pollack et al., 2003; Raisinghani & Faingold, 2002; Ross & Coleman, 2000). There is ample evidence to suggest that when the efficacy of GABAergic cells is compromised within this structure, an animal is more susceptible to AGS (Faingold & Anderson, 1991). The central nucleus of the IC (CNIC) relays information from the LL to the medial geniculate body (MG), which in turn sends information to the acoustic cortex, as well as areas of the forebrain including the lateral amygdala and the hippocampus (Garcia-Cairasco et al.; Raisinghani & Faingold). The amygdala projects to the frontal motor cortex via the perirhinal cortex, which could explain some of the motor activity involved in AGS (Hirsch et al 1997). The external cortex of the IC (ECIC), which research implicates in the sensorimotor aspect of AGS activity, including wild running, is the source of efferent projections to multiple areas of the reticular formation (RF) as well as to the superior colliculus (SC; Faingold, 2002; Garcia-Cairasco et al.; Raisinghani & Faingold; Ribak & Morin, 1995; Ross & Coleman; Zrull, Mengle, Hall, Hairston, & Sedivec, 1999). Data from lesion studies suggest that while the SC can propagate AGS activity, its role is not obligatory like the IC (Ribak, Khurana, & Lien, 1994; Ross & Coleman, 2000). Parts of the substantia nigra (SN) project to both the IC and the SC, regulating efferent seizure pathways. Each structure in the neural network plays a

role in the propagation of AGS activity; however, the purpose of this thesis was to investigate the relationship between two particular structures within the network, the DNLL and the IC. Although there is already substantial evidence to suggest that the IC is the initiation site of AGS, and that abnormalities associated with the inhibition within this structure contributes to this activity, further investigation is necessary to determine the importance of extrinsic inhibition within the DNLL.

Statement of Purpose

In this study, the developmentally primed model was used to investigate sound-induced seizures in the Long-Evans rat. Ample research has been conducted to determine the role of the IC in AGS activity. There is evidence to suggest that the IC is the initiation site of AGS, and that runaway excitation initiated within this structure is the cause of generalized seizures exhibited when loud sound is presented (Garcia-Cairasco et al., 1993; Garcia-Cairasco, 2001; Pollack et al., 2003; Raisinghani & Faingold, 2002; Ross & Coleman, 2000). The role of normal inhibition in AGS, however, is not as clear as the role of excitation. A review of the literature failed to find any prior investigation specifically of the role of extrinsic inhibition of the IC during these seizures. The purpose of this experiment was to determine the importance of this inhibition during sound-induced seizures, an investigation which required a chemical lesion of the DNLL to eliminate GABAergic neurons within the structure. To conduct this experiment, three groups of animals were used. An experimental group received a neurotoxin that created a chemical lesion of the area. Two control groups, one that was prepared for surgery and received identical post-operative care, but did not receive any injection, and one that did not experience any aspects of the surgery were used for comparison. All groups were made seizure-prone prior to surgery by following the

procedures of the developmentally primed model. Because the chosen neurotoxin purportedly eliminated the IC's main source of extrinsic inhibition in the brains of experimental animals, it was hypothesized that there would be a shorter latency to enter the wild running phase, a shorter latency to enter clonus, and longer seizures in the group who received the chemical lesion than either control group. This hypothesis was tested with both pre- and post-surgery series of AGS inductions identical in procedure.

Method

Subjects and Experimental Groups

Three litters of Long-Evans hooded rats ($N = 36$), born in the Arts and Sciences Animal Facility at Appalachian State University, were bred for this study. Rat pups were weaned from their dams between pnd 21 and 24, and thereafter were housed in same-sex groups of three or four in plastic shoebox cages. Subjects were given free access to food and water. The housing room within the facility maintains a 14 h light/10 h dark illumination schedule with a constant temperature of 22 ^{o}C and 50% relative humidity. All animal care and use procedures were approved by the Institutional Animal Care and Use Committee at Appalachian State University prior to being performed (protocol #09-07, approved on 5/13/2009, see Appendix).

All subjects were primed on pnd 18 and tested for AGS susceptibility on pnd 32. To be included in this study, subjects must have exhibited an audiogenic response during testing on pnd 32. Pups in one litter ($n = 11$) did not meet this requirement, and therefore were eliminated from the experiment. Prior to group assignment, all remaining subjects ($N = 25$) were subjected to seven AGS inductions across 12 days beginning on pnd 35. Subjects must have responded appropriately to hearing tests, indicating no signs of hearing loss, prior to

each induction. Group membership was determined based upon matching similar audiogenic responses in pre-surgery inductions across all dependent measures. One group (*n* = 9*)* received a neurotoxin injection of quisqualic acid into the DNLL (lesion group). This procedure was expected to result in a chemical lesion of the DNLL, thereby eliminating the IC's primary source of extrinsic inhibition. The number of subjects in this group was reduced *(n* = 6) during histological procedures either due to the inability to verify lesion sites, or the inability to assess damage due to the nature of the tissue. A second group (*n* = 7*)* served as a sham operated control (sham control), undergoing all steps of the surgical procedure with the exception of the chemical injection. One brain was damaged during tissue processing and could not be used thus necessitating removal of the rat from the study, bringing the number of subjects in this group to six as well *(n* = 6*)*. The final group (*n* = 9*)*, which also served as a control group (unoperated control), did not experience any aspect of the surgical procedure. Three subjects were removed from this group either due to injuries sustained during seizure inductions that prevented the completion of behavioral testing, or because the brain was damaged, and therefore histological data could not be provided, which left six in this group as well *(n* = 6*)*. After surgery was performed on assigned subjects, all rats were again subjected to a series of AGS inductions identical to the pre-surgery schedule, beginning on pnd 63 and lasting 14 days.

Priming

All pups were primed for AGS on pnd 18. Using a standard protocol for the laboratory (e.g., Adams, Glenn, Richardson, & Zrull, 2008; Hodgin, 2006), subjects were placed individually into ½-in. hardware cloth cages, 9.5 cm x 9.5 cm x 9.5 cm, and exposed to high intensity (120 dB re 0.0002 dyne/cm^2, A scale) 10 kHz tone pips (8/s, 75 ms on, 50

ms off, 5 ms rise and fall times) for 8 min. Cages were equidistant from a paper cone speaker (Realistic 40-1270D) and situated on a wooden platform. A digital wave form generator (TDT, Inc WG-1) created, shaped, and gated a 10 kHz sine wave at the desired rate, and then the signal was amplified (Realistic MPA-101) before it was switched to the tweeter. Stimulus production and presentation was controlled by an Apple 2E computer interfaced with the auditory signal generating apparatus (A-D/D-A board, Applied Engineering). After the priming procedure, pups were monitored for 20 min and then returned to the appropriate cages with their dams.

Audiogenic Response Induction and Testing

Following the priming procedure, all pups were assessed for susceptibility to AGS on pnd 32, and subsequently subjected to inductions on seven occasions over 12 days beginning on pnd 35. After surgery, rats were subjected to eight AGS inductions beginning on pnd 63 and that were continued every other day until pnd 77. Testing and inductions were identical in procedure, which is standard for the lab (e.g., Hodgin, 2006). To begin, subjects were placed individually into a cylindrical ¼-in. hardware cloth chamber (36 cm tall with a diameter of 30 cm) with a wooden roof and floor. White noise (100 Hz to 20 kHz) generated by a Coulbourn Noise Generator (S81-02) was used to elicit AGS activity. This noise was amplified to at least 120 dB (re 0.0002 dyne/cm^2, A scale) using a Realistic amplifier (MPA-101) and delivered through broad range speakers (Realistic 40-1354A). Each trial lasted for 2 min, or until a clonic seizure began. All trials were immediately evaluated by multiple raters, and videotaped for a second analysis by one individual in order to increase reliability of audiogenic response measures. Severity of audiogenic response was evaluated based on three

measures, latency to enter the first wild running phase, latency to enter clonus, and the duration of the seizure.

Chemical Lesion Surgery

After pnd 50, each rat in the lesion or sham control group was anesthetized (ketamine 70 mg/kg b.w., ip and xylazine, 8 mg/kg b.w., ip) and prepared for surgery. Once anesthetized, the rat's head was positioned in a stereotaxic instrument, the scalp was cleaned and incised, and a burr hole was drilled at AP 0.0 to 0.5 mm, relative to interaural zero, lateral 2.8 to 3.0 mm, relative to midsagittal suture, and DV -5.0 to -5.4, relative to dura mater. Animals in the sham operated control group experienced all steps of the surgical procedure with the exception of receiving the neurotoxic injection that produced the chemical lesion. Lesion group rats received a 0.5 µL pressure injection of 100 mM quisqualic acid into one DNLL over 1 min, and 5 min was allowed for diffusion. After the injection was complete, the burr hole was filled with bone wax and the skin was sutured. Prior to removal from the stereotaxic instrument, each rat receives a prophylactic injection of diazepam (5 mg/kg b.w., sc) to prevent an excitotoxin-induced seizure. Each animal was kept in a warm place and monitored closely for the first 24 h after surgery. They were returned to their home cages once awake and monitored for an additional 72 h after surgery. After a recovery period of 10 to 13 days, the post-surgery induction schedule began.

Histology

After post-surgical AGS inductions, subjects in all groups were sacrificed via an overdose of sodium pentobarbital (100 mg/kg b.w., ip) and perfused intracardially with 100 mL of pH 7.2, 10 mM phosphate buffered saline (PBS) solution followed by 500 mL of 4% paraformaldehyde in phosphate buffer (PB). Brains were removed and postfixed for at least

24 h in 100 mL of a 10% sucrose-4% paraformaldehyde solution at 4 ^{o}C. Brain tissue was cut into 50 µm sections using a Vibrotome®. Tissue sections which included the DNLL were placed into individual wells of 24-well Falcon tissue culture plates with approximately 1 mL of PB (7.2 pH, 10mM). These sections were used to quantify the GABAergic neurons within the DNLL by staining for GAD_{67} positive cells. The procedure was performed using a standard laboratory protocol (Hodgin, 2006).

Immunohistochemistry (IHC) sections were exposed to a number of solutions while in the wells on a rotator operating 45-50 rpm. Sections were incubated in 0.1% hydrogen peroxidase for 30 min then rinsed 3 times in PBS. After rinsing, 10% goat serum in PBS was applied for 1 to 1.5 h, followed by the application of the primary antibody to GAD_{67} (Millipore, made in mouse). After 12 to 18 h incubation at room temperature, the tissue was again rinsed three times for 10 min and then exposed to a biotinylated secondary antibody to mouse made in goat (Vector Laboratories) for 1.5 h. The tissue was again rinsed three times for 10 min in PBS before being exposed to a solution containing a peroxidase labeled avidin-biotin complex (ABC, Vector Laboratories) that binds to the secondary antibody. After 1 h, tissue sections were rinsed (3 x 10 min) with PBS. Sections were then exposed to VIP (Vector laboratories), a peroxidase substrate which forms a purple precipitate following an enzymatic reaction, in order to visualize immunoreacted GAD_{67}-containing neurons. Next, tissue was again rinsed in PBS for 10 min before being mounted onto gelatin-coated slides from distilled water, where they were allowed to air dry. Each section was then dehydrated in graded ethanols, cleared with toluene, and cover-slipped with Permount (Fisher Scientific). This process allowed for the visualization of GABAergic neurons, which in turn yielded cell densities necessary for comparison. The remaining tissue sections containing DNLL were

also mounted, air dried, dehydrated in graded ethanols, and then rehydrated, stained with Thionin, differentiated in acid-alcohol, again dehydrated, cleared in toluene cover-slipped with Permount. Thionin stained sections were used to visualize the cytoarchitecture of the midbrain, and verify the location of the DNLL, as well as to compute neuron densities.

Microscopy

To locate the DNLL and assess mechanical damage, slides were viewed using a Nikon Eclipse light microscope and Plan achromatic 4 and 10 objectives. The Plan 10 objective and a 1.2 megapixel digital firewire camera (PixeLink) were used to project images onto a computer screen (Dell). A transparency with eight 200 x 200 μm scale boxes was placed over the computer screen, and cells within the both the right and left DNLL of each tissue section were counted on separate sides of the same transparency. In Nissl sections, only large cell bodies with visible nuclei were counted as neurons (Zrull & Coleman, 1991). Because the GAD_{67} enzyme is only present in neurons, all cell bodies were counted in the IHC tissue sections. Cells were marked using three different colored markers, each representing a different plane through the depth of each tissue section. The first plane of each section was excluded from the final count due to the possibility of these cells being in that are the adjacent section. For each brain, four to six sections of GAD_{67}, Nissl, or a combination of both, were used.

Data Analysis

The lesion study fits a 3 x 2 mixed design, and an analysis of variance (ANOVA) was used to partition the variance. The hypothesis was tested with a series of contrasts. For each dependent measure, it was expected that all three groups would exhibit similar AGS responses prior to surgery. After this procedure, however, it was expected that subjects in the

lesion group would have a shorter latency to enter the first wild running phase, a shorter latency to enter clonus, and longer seizures than subjects in either of the control groups. Because this experiment fits a mixed design, and measures of AGS severity were tested prior to and after surgery, each rat essentially served as its own control. The average of each dependent measure across the last four inductions prior to surgery served as a baseline for comparison to the average times for each measure across the last four inductions after surgery. Because the means and standard deviations of the latency to enter clonus and the duration of clonus dependent measures were somewhat proportional and the variances were heterogeneous, a logarithmic transformation was used to equalize the variability across groups as much as possible. This transformation was deemed most appropriate based upon criteria for transformations found in Kirk (1982).

The histology part of this thesis fits a somewhat different 3 x 2 mixed design (Group x Brain Side), and an ANOVA was again used to partition the variance. The density of Nissl or GAD_{67} positive cells was computed for both sides of all brains to evaluate differences between DNLL of individual subjects as well as across groups. A gain-loss ratio of neurons in Nissl sections, and GABAergic neurons in IHC sections, as indicated by GAD_{67} densities, was computed for each animal, and then average ratios among groups were compared. Finally, a one-factor ANOVA was used to compare gain-loss ratios of GABAergic neurons among the groups. Individual differences, as indicated by GAD_{67} positive neurons, were also taken into account with this design.

Results

Behavior

To test the hypothesis that unilateral DNLL lesions would have an effect on the first dependent measure, latency to enter a sound-induced wild running phase, an a priori contrast on the group by surgery interaction was assessed, and was not statistically significant $F(1,15) = 0.42$, $p > .05$, $\eta^2 = .01$. The contrast compared the combined unoperated and sham controls to DNLL lesion rats across pre- and post-surgery AGS inductions for this measure. The lesion group exhibited a 31% decrease in latency to enter a wild run from pre- to post surgery inductions. The sham control group exhibited a 7% decrease on the same measure, and the unoperated control group exhibited a 33% decrease in the latency (see Figure 2).

Because the assumption of homogeneity of variance was violated for the second dependent measure, latency to enter clonus, a logarithmic transformation was used prior to inferential data analysis. To test the hypothesis that unilateral DNLL lesions would have an effect on the latency to enter clonus, an a priori contrast was tested on the group by surgery interaction effect for this measure, $F(1,15) = 2.08$, $p > .05$, $\eta^2 = .06$. The contrast assessed differences between the combined unoperated and sham control groups and the lesion group across pre- and post-surgery AGS inductions for this measure. The lesion group exhibited an 8% decrease in latency to enter clonus from pre- to post surgery inductions. The sham control group exhibited a 20% decrease on the same measure, and the unoperated control group exhibited a 46% decrease in latency to enter clonus (see Figure 3).

The between-group variability for the third dependent measure, duration of the clonic seizure, again warranted the use of a logarithmic transformation (see Figure 4). To test the hypothesis that unilateral DNLL lesions would have an effect on the duration of clonic

seizures, an a priori contrast was tested on the group by surgery interaction effect for this measure $F(1,15) = 4.20$, $p = .0584$, $\eta^2 = .10$. The contrast again compared the two control groups and the lesion group across pre- and post-surgery AGS inductions for the duration of clonus measure. Contrary to the hypothesis, the lesion group exhibited only a 4%, rather than a greater, increase in clonic seizure duration from pre- to post surgery inductions. In contrast, the sham control group exhibited a 76% increase on the same measure, and the unoperated control group exhibited a 21% increase in duration.

Histology

In order to assess the extent of the damage created by lesions, tissue was processed to visualize DNLL neurons and cell densities were calculated. An a priori contrast was tested to compare the lesion group and the sham control and unoperated control groups in neuron density changes in the DNLL of subjects, which was marginally statistically significant $F(1,15) = 4.48$, $p = .0514$, $\eta^2 = 0.23$. Another contrast was tested to determine if there was a difference between the sham control and unoperated control groups in neuron density changes, which was not statistically significant $F(1,15) = 0.15$, $p > .05$, $\eta^2 = .01$. Overall, the sham and control groups exhibited minimal or no change in neural density, while the lesion group exhibited substantial difference in cell density of DNLL within subjects (see Table 1).

Discussion

The purpose of this study was to investigate the importance of the role of extrinsic inhibition during sound-induced seizures, specifically the role of the DNLL in the neural network that subserves audiogenic responses. While the IC is a key processing, integration, and relay center of all auditory input (Coleman & Clerici, 1987; Faingold, 2002; Faingold & Anderson, 1991; Garcia-Cairasco, 2001; Garcia-Cairasco et al 1993; Pierson & Swann, 1991;

Pollack et al., 2003), the main source of extrinsic inhibition to this structure is supplied by the DNLL (Bajo et al., 1993; Kelly et al 1996; Merchán et al., 1994; Schneiderman et al., 1993; Zhang et al., 1998). There is ample evidence to implicate the IC as the initiation site of the abnormal neural excitation implicated in AGS activity (i.e., Faingold; Faingold & Anderson; Garcia-Cairasco; Garcia-Cairasco et al.; Raisinghani & Faingold, 2002; Ross & Coleman, 2000). While the lack of intrinsic inhibition of the IC, in conjunction with abnormal excitation, may play a role in the propagation of AGS (Faingold; Faingold & Anderson; Garcia-Cairasco; Ross & Coleman), the role of extrinsic inhibition of the IC, specifically the role of the DNLL, during AGS had not been investigated prior to this study.

It was expected that a reduction of extrinsic inhibition of the IC via unilateral DNLL lesions would negatively impact the severity of AGS seizures due to a reduction of the inhibition available to control runaway excitation originating in the IC. This hypothesis was only marginally supported by data for one of the three dependent measures, the duration of sound-induced seizures. The other dependent measures of seizure activity, latency to enter a wild running phase and latency to enter clonus, were not affected by a lack of extrinsic inhibition.

The hypotheses that unilateral DNLL lesions would have an effect on both the latency to enter a wild running phase and the latency to enter clonus measures were not supported by the data of this study, suggesting that a lack of extrinsic inhibition of the IC does not play a significant role in the initiation or propagation of audiogenic responses. The data implies that extrinsic inhibition of the IC does not provide significant inhibition to aid in slowing or abolishing the abnormal excitation initiated in the IC that leads to a clonic seizure (Garcia-Cairasco, 2001; Garcia-Cairasco et al., 1993; Raisinghani & Faingold, 2002; Ribak et al.,

1994; Ribak, & Morin, 1995; Ross & Coleman, 2000). Thus, seizure-prone rats with or without the normal inhibitory connections of the auditory midbrain were equally likely to exhibit audiogenic responses (see Figure 2 and Figure 3).

In contrast, the converse of the third hypothesis of this study, that DNLL lesions would negatively impact clonic duration, was marginally supported by the data of this study. While the group by surgery interaction contrast for this measure accounted for a relatively small proportion of variability in seizure duration ($\eta^2 = .10$), rats with DNLL lesions exhibited shorter or similar duration clonic AGS than those in control groups (see Figure 4) suggesting that a lack of the integrity of one DNLL in the ascending auditory pathway may prevent the gradual increase in duration often seen with repeated seizure activity (e.g., Ross & Coleman, 2000). Perhaps alterations in the circuitry of the auditory midbrain in the AGS-prone rat may provide some control to the animal, shortening the duration of the seizure (cf. Pierson & Swann, 1991; Ribak et al., 1994; Zrull et al., 1999).

The behavioral data from this experiment seem to suggest that lack of extrinsic inhibition of the IC does not play as significant a role in the either the initiation or propagation of audiogenic seizure activity as does the lack of intrinsic inhibition of the structure (Faingold & Anderson, 1991). The histology data of this thesis provides further support for this conclusion because the average loss of neurons in the lesion group was dramatically higher than in either control group (see Table 1 and Figure 5), indicating that the quisqualic acid injection did cause significant cell loss in the DNLL of lesioned animals. However, results also yielded an approaching-significant effect of the group by surgery interaction contrast for the third dependent measure, duration of clonus. These results indicate that, while reduced extrinsic inhibition of the IC is not a key factor in seizure

initiation and propagation, it does decrease the duration of clonus. This suggests that this inhibition can aid the animal in regaining control after an AGS has begun, a proposal in contrast to that of previous AGS studies where a temporary pause between wild runs is presumed to be a demonstration of control provided by inhibition within the brain (Jobe et al., 1973; Ross & Coleman, 1999).

Evidence from previous AGS research implicates the external cortex of the IC (ECIC), not the central nucleus of the IC (CNIC), as the area responsible for the sensorimotor aspect of AGS activity, including wild running (i.e., Garcia-Cairasco et al., 1993; Ribak & Morin, 1995; Zrull et al., 1999). Furthermore, bilateral lesions of the IC that damage the ECIC have effectively eliminated sensorimotor activity exhibited during AGS (Ribak et al., 1994; Ross & Coleman, 2000). Therefore, it is possible that lesions of the DNLL, a structure which projects to the CNIC, do not actually eliminate any extrinsic inhibition to the area critical for sensorimotor activity (Garcia-Cairasco et al.; Zrull et al.). Another important aspect to consider is the binaural nature of the auditory pathway. Only bilateral lesions of the IC have effectively abolished AGS activity (Faingold, 2002; Ribak et al; Ross & Coleman). It is possible that reducing projection from one DNLL did not effectively reduce available extrinsic inhibition during AGS. In this study, unilateral DNLL lesions did not abolish AGS activity; subjects in the lesion group did not exhibit shorter latencies to enter a wild running phase or shorter latencies to enter clonus, as was expected from previous studies (e.g. Faingold, 2002; Ribak et al; Ross & Coleman). Similarly, lesions did not increase seizure duration, suggesting that disruption of midbrain circuitry or reduction of extrinsic inhibition may aid in controlling a seizure once it has begun.

Although interesting conclusions can be drawn from the results of this study, further investigation is necessary to make proper implications about the importance of the relationship between the role of the DNLL and the IC during sound-induced seizures. Only bilateral lesions of the IC can effectively eliminate AGS activity (Faingold, 2002; Ribak et al., 1995; Ribak & Morin, 1994; Ross & Coleman, 2000), suggesting that due to the binaural nature of the auditory pathway, damage to one structure could have little impact if the contralateral structure remains intact. Subjects with bilateral DNLL lesions should be examined to evaluate the significance of projection from both DNLL. Furthermore, all three groups in this experiment were primed and tested for AGS susceptibility, and all followed an identical pre- and post-surgery induction schedule. An unoperated control group (one that is not exposed to any aspect of the AGS process) should be included in further studies to understand the relationship between the DNLL and the IC in normal, properly functioning brains. It is also possible that younger rats have the ability to cope with detrimental cells loss within this structure better than adult rats, thus lesions of the DNLL may have less of an impact on the age group of animals used for this study (i.e., Adams et al., 2008; Minton & Zrull, 2010). Further investigation across various age groups is necessary to determine if cell loss within this structure has more of an impact at particular stages in life.

Additionally, although quisqualic acid was an adequate choice to create lesions in the DNLL, creating a larger lesion of the structure in further studies may provide more insight into the importance of extrinsic inhibition in AGS. Combining quisqualic acid and AMPA would enhance binding affinity at the receptors, creating more cell death (Johansen et al., 2003; Parks, 2000). While a 30% neuron loss in this structure in the lesion group subjects is

substantial, it is possible that more cell death within the structure, and therefore even less projection to the IC, could produce different results than those of this study.

One final limitation of this study could be the number of subjects used. Due to unavoidable circumstances such as injuries sustained during seizure inductions, loss of sections during tissue processing, and inability to assess tissue due to staining anomalies, the sample size for this study was ultimately relatively small. Just as there is variation in audiogenic responses across strains (Ross & Coleman, 1999), there are also individual differences within strains. Thus, there was an extensive amount of variability within groups for all three dependent measures, which could have significantly impacted the results. Individual differences across subjects within groups used in this study may also explain why the severity of unoperated control subjects increased so dramatically in post-surgery inductions. However, it is possible that a larger sample size would simply allow room for more individual differences within groups.

Summary and Conclusion

The results of this thesis provided interesting information about the importance, or perhaps lack thereof, of the role of the DNLL during AGS. The data suggest that significant cell loss within one DNLL does not impact the latency to enter a wild running phase or the latency to enter clonus components of AGS activity; therefore, lack of extrinsic inhibition of the IC, the initiation site of AGS (Faingold, 2002; Faingold & Anderson, 1991; Garcia-Cairasco, 2001; Raisinghani & Faingold, 2002; Ross & Coleman, 2000), as provided by the DNLL (Bajo et al., 2003; Coleman & Clerici, 1987; Merchán et al., 1994; Schneiderman et al., 1993; Zhang et al., 1998), may not be a key component in the initiation and propagation of sound-induced seizures. However, the data also suggest that a lack of extrinsic inhibition

to the IC may play a somewhat important role in AGS duration, by aiding in decreasing duration of clonic seizures. It is possible that creating bilateral lesions of this structure, therefore reducing extrinsic inhibition from both structures could have a significant effect on AGS activity.

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Appendix

Appalachian
STATE UNIVERSITY
Research and Sponsored Programs
ASU Box 32068
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(828) 262-2130
Fax: (828) 262-2709
www.graduate.appstate.edu/orsp

TO: Dr. Mark Zrull
~~Department of Biology~~ Psychology

FROM: James C. Denniston, Chair
Institutional Animal Care and Use Committee

DATE: May 13, 2009

SUBJECT: Institutional Animal Care and Use Committee
Request for Animal Subjects Research

REFERENCE: The role of inhibition in the audiogenic seizure network of young, developmentally primed Long-Evans rats

IACUC Reference #09-7

Initial Approval Date – May 13, 2009
End of Approval Period – May 12, 2012

The above reverenced protocol has been approved by the IACUC for a period of three (3) years.

Best wishes with your research.

JCD/lab

Author Note

First and foremost I would like to thank my thesis chair, Dr. Mark Zrull, whose extraordinary guidance, patience, and advice made this thesis possible. I would also like to thank the undergraduate research assistants who dedicated a great deal of their time to assist with this project, particularly Benjamin Minton, Chase Francis, Joshua Smith, Alexandra Fuller, Alexandria Squires, Jamie Milton, and Zachary Riemenschneider. Additional thanks are warranted to my committee members, Dr. James Denniston and Dr. Kurt Michael for their advice and assistance with this project.

Finally, I wish to dedicate this thesis to my parents, Robert and Sharon Morgan, for their love and support throughout my graduate experience.

Table 1

Means (M) and Standard Deviations (SD) for Group Ratios of Loss of GABAergic Neurons in the Dorsal Nucleus of the Lateral Lemniscus

Group	n	*M*	*SD*
Lesion	6	-.30	.08
Sham Control	6	-.06	.06
Unop Control	6	0	.06

Figure Captions

Figure 1. The neural network for audiogenic seizures in rat. This figure shows the propagation of the abnormal excitation, which culminates in a generalized seizure, as well as the projection from the dorsal nucleus of the lateral lemniscus (DNLL) to the inferior colliculus (IC; adapted from Garcia-Cairasco, 2002).

Figure 2. The group by surgery interaction effect on latency to enter wild running measure is shown. The graph shows the mean latency to wild running with standard deviations from the last four pre- and post-surgery inductions for the lesion (*n = 6*), sham (*n = 6*), and unoperated control (*n = 6*) groups.

Figure 3. The group by surgery interaction effect on latency to enter clonus measure is shown. The graph shows the mean latency to clonus with standard deviations from the last four pre- and post-surgery inductions for the lesion (*n = 6*), sham (*n = 6*), and unoperated control (*n = 6*) groups.

Figure 4. The group by surgery interaction effect on latency to enter wild running measure is shown. The graph shows the mean duration of clonus with standard deviations from the last four pre- and post-surgery inductions for the lesion (*n = 6*), sham (*n = 6*), and unoperated control (*n = 6*) groups.

Figure 5. Example photomicrographs of GABAergic neurons in the dorsal nucleus of the lateral lemniscus (DNLL) containing GAD_{67}. Images (800 x 600 pixel) were made using a Pixelink camera (1.3 MB) attached to a Nikon Eclipse light microscope (Plan 10 infinity objective). The images show far more neurons in the DNLL of control rats (*A* and *B*) than with neurotoxin injections. *A.* Section 22 from the brain of unoperated control Rat 0920. *B.*

Section 17 from the brain of sham operated control Rat 0909. *C.* Section 14 from the brain of Rat 0914 with a quisqualic acid lesion of the DNLL.

Figure 1.

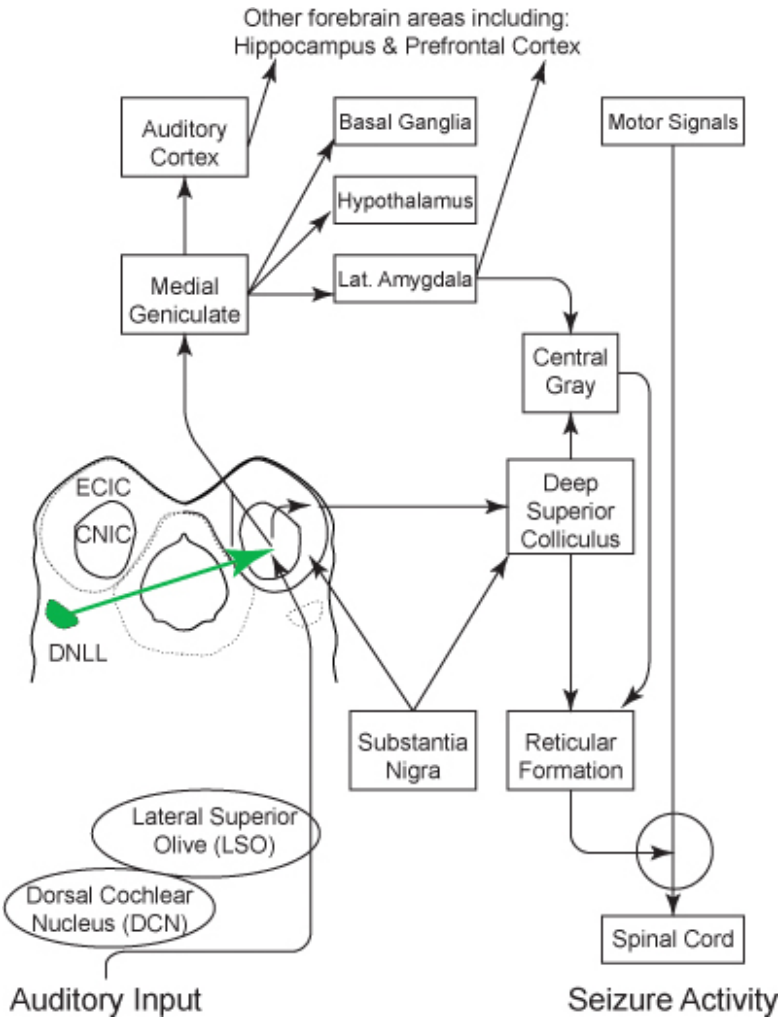
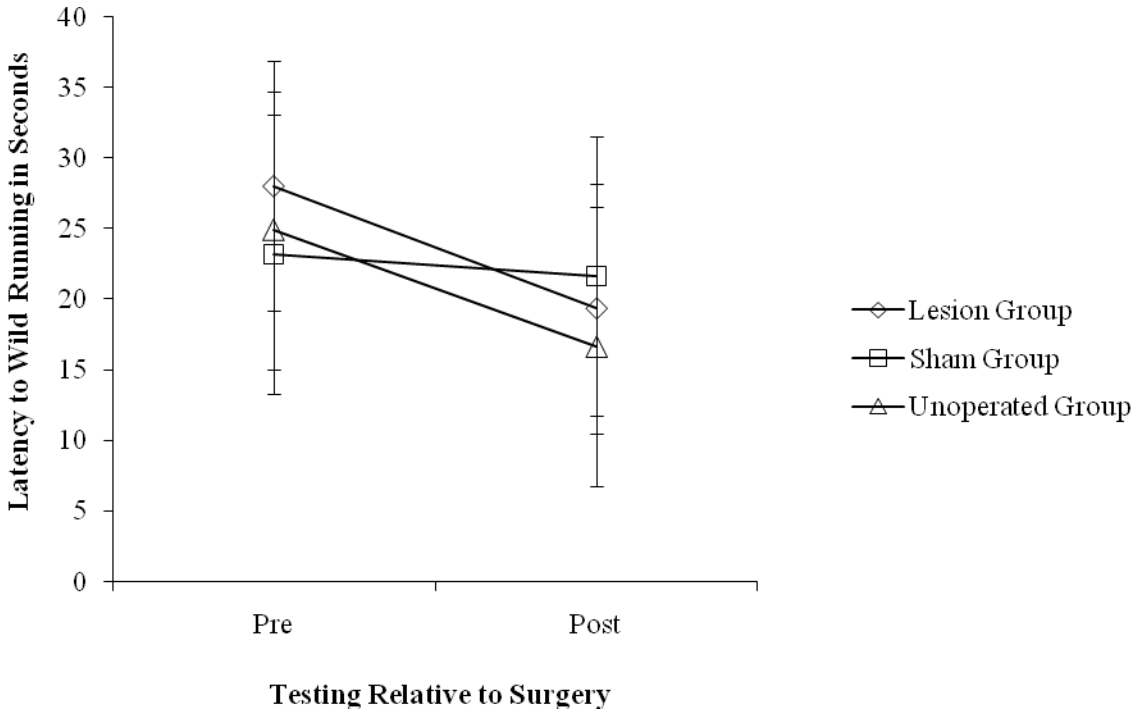

Other forebrain areas including:
Hippocampus & Prefrontal Cortex
Auditory Cortex
Basal Ganglia
Motor Signals
Hypothalamus
Medial Geniculate
Lat. Amygdala
Central Gray
ECIC
CNIC
DNLL
Deep Superior Colliculus
Substantia Nigra
Reticular Formation
Lateral Superior Olive (LSO)
Dorsal Cochlear Nucleus (DCN)
Spinal Cord
Auditory Input
Seizure Activity

Figure 2.


Latency to Wild Running in Seconds
40
35
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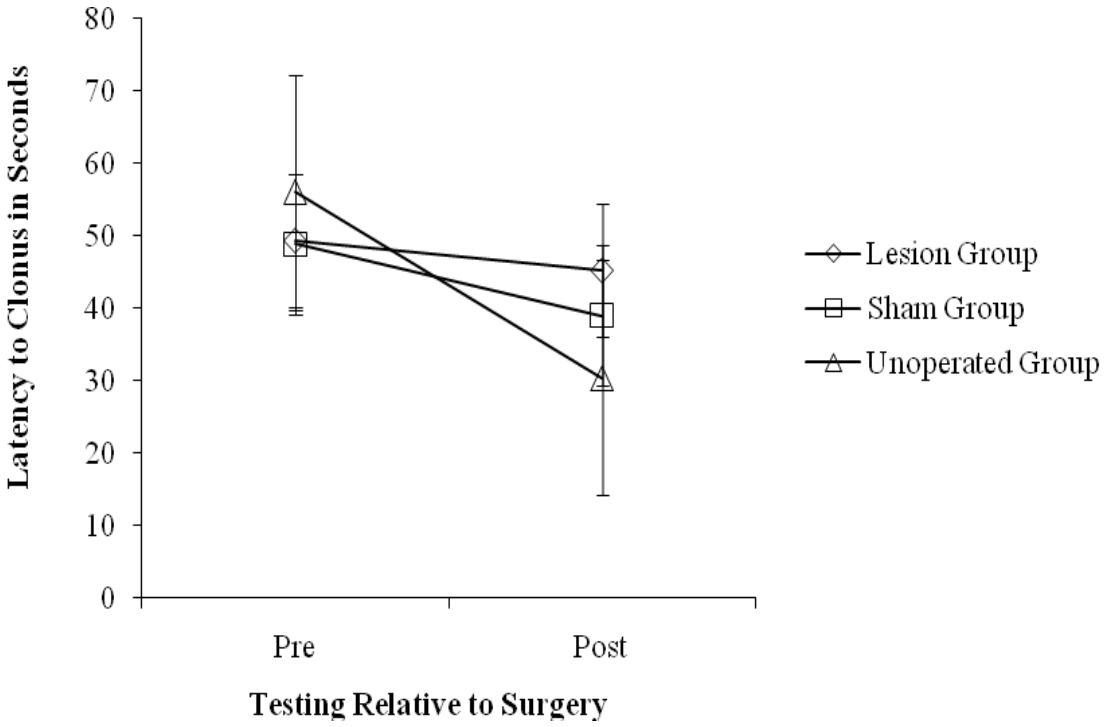
15
10
5
0
Pre
Post
Testing Relative to Surgery
Lesion Group
Sham Group
Unoperated Group

Figure 3.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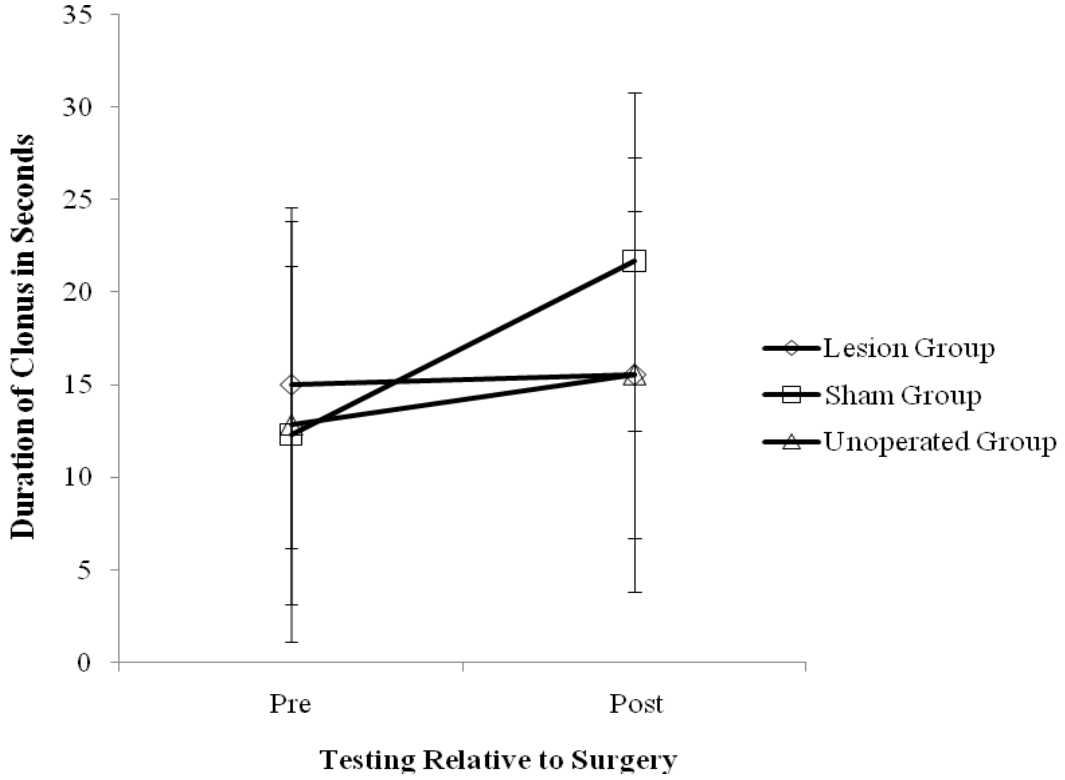
Latency to Clonus in Seconds
Pre
Post
Testing Relative to Surgery
Lesion Group
Sham Group
Unoperated Group

Figure 4.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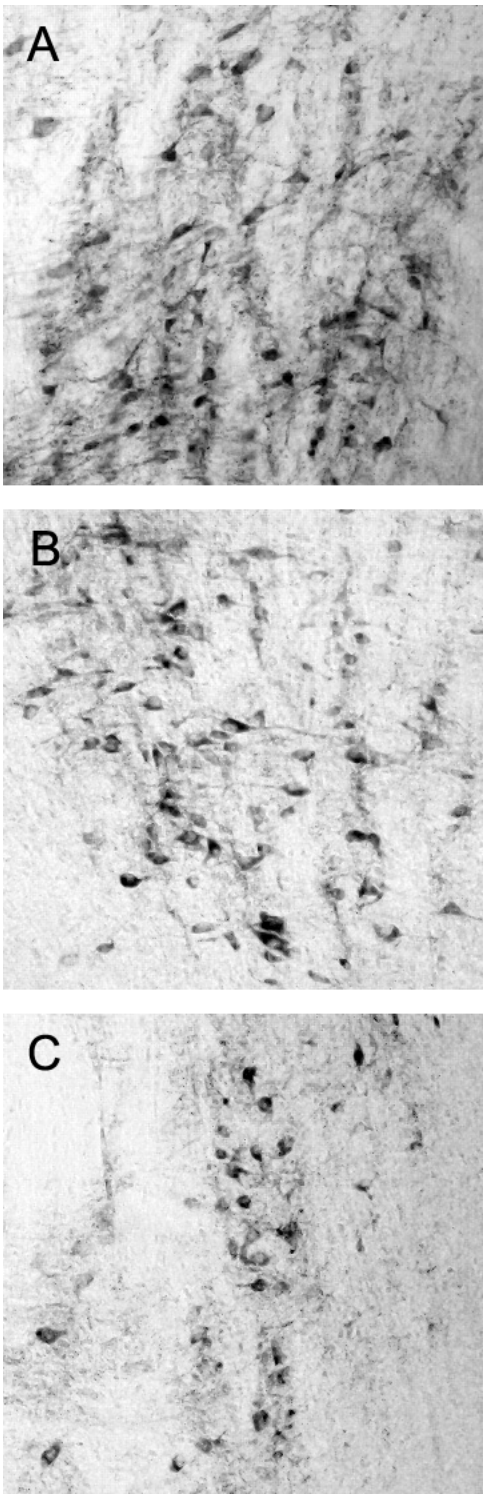
Duration of Clonus in Seconds
Pre
Post
Testing Relative to Surgery
Lesion Group
Sham Group
Unoperated Group

Figure 5.



Vita

Amy Marie Morgan was born in Ruston, Louisiana on February 12, 1986. She attended elementary school in that city, and graduated from Choudrant High School in May 2004. In the fall of 2004 she entered Louisiana Tech University, where she majored in psychology. In May 2008, she was awarded the Bachelor of Arts degree. In August 2008, she accepted a research assistantship in experimental psychology at Appalachian State University and began study toward a Master of Arts degree, which was awarded in August 2010. Ms. Morgan is a member of Phi Gamma Mu, the Society for Neuroscience, and was the president of the Psychology Graduate Student Organization of Appalachian State University from 2009-2010. Her home address is 1947 Florida St, Arcadia, LA, 71001. Her parents are Robert and Sharon Morgan of Arcadia and Choudrant Louisiana.