NEURAL ACTIVITY IN THE AMYGDALA OF YOUNG RATS EVOKED BY ONE OR MORE SEIZURES

A Thesis
by
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Abstract

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Generalized seizures that involve clonic convulsions of the entire body are often a feature of epilepsy and result from abnormal neural firing that spreads throughout the forebrain. When neuronal activity leading to such a seizure begins in the brain stem, the amygdala is often required to relay abnormal excitatory activity to the forebrain. In a model of acquired reflex epilepsy, rats are made susceptible to generalized sound-induced or audiogenic seizures (AGS) and exhibit generalized clonic convulsions. In this thesis, AGS-induced activation in the amygdala was examined using the immediate early gene (IEG) c-fos as a neural marker. A group of Long-Evans rats were used to model reflex epilepsy and primed for AGS on post-natal day (PND) 18. Rats were divided into three seizure induction conditions: AGS 1, 6, or 12. AGS was induced with 120 dB noise for 120 s or until seizure onset. Brains were collected, cut into sagittal sections, and stained for the c-fos protein or cell bodies. Cell counts of c-fos located in lateral (LA) and basolateral amygdala (BLA) neurons were made using a light microscope and digital camera system. Statistical analyses of
differences between seizure and control groups and within the AGS group revealed a
significant increase in LA neuron activation between 1 and 6 seizures but no additional
activation with 12 AGS. No changes in neuronal activation of BLA dependent on number of
AGS were observed. Repeated AGS affects LA neurons more than BLA neurons in this
model of acquired reflex epilepsy with activation of LA neurons stabilizing after a number of
seizures. Overall, the results from this model of an acquired seizure disorder are comparable
to models of inherited epilepsy.
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Dedication

To my family: I am because of you and for that, I am grateful.
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Neural Activity in the Amygdala of Young Rats Evoked by One or More Seizures

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Abstract

Generalized seizures that involve clonic convulsions of the entire body are often a feature of epilepsy and result from abnormal neural firing that spreads throughout the forebrain. When neuronal activity leading to such a seizure begins in the brain stem, the amygdala is often required to relay abnormal excitatory activity to the forebrain. In a model of acquired reflex epilepsy, rats are made susceptible to generalized sound-induced or audiogenic seizures (AGS) and exhibit generalized clonic convulsions. In this thesis, AGS-induced activation in the amygdala was examined using the immediate early gene (IEG) c-fos as a neural marker. A group of Long-Evans rats were used to model reflex epilepsy and primed for AGS on postnatal day (PND) 18. Rats were divided into three seizure induction conditions: AGS 1, 6, or 12 inductions. AGS was induced with 120 dB noise for 120 s or until seizure onset. Brains were collected, cut into sagittal sections, and stained for the c-fos protein or cell bodies. Cell counts of c-fos located in lateral (LA) and basolateral amygdala (BLA) neurons were made using a light microscope and digital camera system. Statistical analyses of differences between seizure and control groups and within the AGS group revealed a significant increase in LA neuron activation between 1 and 6 seizures but no additional activation with 12 AGS. No changes in neuronal activation of BLA dependent on number of AGS were observed. Repeated AGS affects LA neurons more than BLA neurons in this model of acquired reflex epilepsy with activation of LA neurons stabilizing after a number of seizures. Overall, the results from this model of an acquired seizure disorder are comparable to models of inherited epilepsy.

Keywords: Audiogenic seizure, Amygdala, c-fos, Neuronal activity
Neural Activity in the Amygdala of Young Rats Evoked by One or More Seizures

Epilepsy is a family of neurological disorders that affects about 2.2 million Americans (Epilepsy Foundation, 2013). While the occurrence of seizures is not the sole diagnosing criterion for the disorder, it is a defining characteristic of epilepsy. Physiologically, a seizure is an abnormal neural event that is restricted to one area or spread throughout the entire brain. Seizure type is defined by how much of the brain exhibits abnormal neural activity. When abnormal neural activity is restricted to a particular area of the brain, often a specific area of one hemisphere, the event is called a partial seizure; in contrast, a generalized seizure is when the abnormal neural activity affects both hemispheres of the brain (Epilepsy Foundation, 2013). Generalized seizures are further divided by the expression of symptoms. An absence, or petit mal, seizure is a brief abnormal neural event that results in a person experiencing a lapse in consciousness and mild twitches. A clonic seizure is when the body’s muscles contract and relax at a rapid rate: this is typically known as a convulsion. In contrast to a clonic seizure, a tonic seizure during which the muscles contract and remain rigid. A clonic-tonic, or grand mal, seizure starts with muscle rigidity and then proceeds to the convulsing of the muscles (Epilepsy Foundation, 2013). Regardless of type, if a seizure is elicited by an environmental stimulus, such as flashing light, the event is a reflex seizure; it is theorized 5% of people with epilepsy suffer from reflex seizures (Panayiotopoulos, 2005). This study focuses on an acquired reflex seizures by using a rodent model.

Like many human diseases, seizure disorders are modeled using nonhuman animals. The rodent model is often used for several reasons: 1) the genome is similar to humans, 2) they are easily obtainable, 3) and the rodent’s environment is easy to manipulate which is
vital in creating scientifically sound experiments (Simmons, 2008). Seizures can be induced in rodents in a variety of ways including exposure to loud sound, introduction of toxic and nontoxic chemicals, and electrical stimulation (Eells, Clough, Browning, & Jobe, 2004; Hirsch et al., 1997; Pierson & Swann, 1991). Genetically epilepsy prone rats can express seizures spontaneously or with presentation of an inducing stimulus (Tupal & Faingold, 2012). In this study, sound is used to induce audiogenic seizures (AGS) in a rat model of acquired reflex epilepsy (e.g. Pierson & Swann, 1991; Ross & Coleman, 1999). Specifically, Long-Evans rats are used to model reflex epilepsy because this strain displays little susceptibility to seizures outside of those induced by a particular stimulus; this allows the researcher to control the number of seizure responses (Ross & Coleman, 1999, 2000). The ability to control seizures in the Long-Evans model is of particular importance to this study because the neural network changes as an animal has an increasing number of seizures.

The Long-Evans rat, like other strains, can be shaped into a model of acquired AGS through a series of steps. Initially, rat pups are exposed to loud noise at a specific post-natal day (PND) which coincides with ear canal opening resulting in susceptibility to sound-induced seizures; this is called priming. The priming portion of Pierson and Swann’s (1991) AGS model exposed Wistar rat pups with newly opened ear canals on PND 14 to the sound produced by a loud (at least 120dB re 0.0002 dyne/cm²) alarm bell for a number of minutes. Priming a rat pup produced cochlea trauma and at a later date, the rat was exposed to a loud (i.e., 120 dB) sound causing the auditory neurons to become over-excited which resulted in a seizure (Pierson & Swann, 1991; Ross & Coleman, 1999). Using the previously stated knowledge, Ross and Coleman (1999) found that similar cochlear trauma could be produced in Long-Evans rats on PND 18; however, the effectiveness of the tone as a priming stimulus
for inducing seizure susceptibility decreased steadily after that day. In both Wistar and Long-Evans models, the first AGS must be induced 14 days after priming using loud broadband noise (Pierson & Swann, 1991; Ross & Coleman, 1999). The primed Long-Evans rat has a lower auditory threshold that makes the auditory receptors more sensitive to sound and exposure to a loud, broadband noise becomes the necessary event to initiate or induce an audiogenic response that often includes an AGS (Coleman, Ross, Mullaney, & Cooper, 1999). Interestingly, the broadband noise used in seizure induction does not have to be increased in intensity over time to accommodate for increased hearing loss that occurs with each seizure induction (Ross & Coleman, 1999); however, noise intensities of 100 dB or less decrease the likelihood of successful seizure induction substantially (Simler et al., 1994; Ross & Coleman, 1999).

Audiogenic responses (AGR) are induced by a loud sound, occur in a particular sequence, and may or may not end in an AGS. During induction, noise onset usually generates a startle response after which the rat will then freeze before AGR progresses (Ferreria-Netto, Borelli, & Brandão, 2007). The marked start of an AGR occurs when the rat begins a phase of wild running which is when the rat runs rapidly and uncontrollably around the induction chamber. The wild running phase can occur in one of two ways: uniphasic and biphasic. Uniphasic running is when the rat exhibits one wild running cycle before a clonic seizure, or clonus, and biphasic is when the rat exhibits two wild running phases before entering clonus. There must be at least 5 seconds of inactivity between the first and second running phase for the event to be considered biphasic (Ross & Coleman, 1999). If an AGS is to occur, it will happen after the wild-running phase, or in other words, an AGS will not occur without an AGR occurring first. The nature of an AGS depends upon the location of
the abnormal activity in the brain. Unlike tonic seizures which involve anomalous neural activity in the brainstem, clonus requires the activation of the forebrain structures (Raisinghani & Faingold, 2005). During clonus, the hindlimbs of the rat extend and retract because the muscles are contracting and releasing at a rapid rate (Eells, Clough, Browning, & Jobe, 2004). Research suggests that as a rat experiences more inductions and clonus occurs, the duration of the clonic seizure becomes longer (Ross & Coleman, 1999) due to growing neuronal damage from each audiogenic event (Feng & Faingold, 2002a; Ross & Coleman, 1999).

AGSs are initiated by sound and abnormal processing in the auditory pathway. The auditory pathway begins with the cochlea of the inner ear where sound waves are transduced into neural signals by activation of hair cells (see Figure 1). The neural signal produced by the hair cells is propagated down the auditory nerve to the cochlear nucleus (CN). Three pathways leave the CN and travel to the superior olivary complex; the pathways involved in AGR are the lateral and medial superior olive with projections then leading to the inferior colliculus (IC) (Coleman & Clerici, 1987; Pollak, Burger, & Klug, 2003). It is in the IC that excitatory neural activity starts the initiation of a seizure (Garcia-Cairasco, 2002; Simler et al., 1994). The abnormal neural signal is propagated from the IC to the medial geniculate nucleus (MGN) via brachium of the IC (Coleman & Clerici, 1987; Garcia-Cairasco, 1993). From the MGN, the over-excitation spreads within the auditory pathway to the cerebral cortex (Pollak et al., 2003) and out of the auditory pathway to the amygdala. The amygdala relays the abnormal activity to brainstem structures as well as other cortical structures and results in generalization of the seizure (Browning, 1986, Feng & Faingold, 2002b, Hirsch et al., 1997, Merrill, Clough, Jobe, & Browning, 2005). In addition to playing roles in multiple
sensory processes, fear, and arousal (Kolb & Whishaw, 2009), the amygdala in an important area of the brain with regard to seizures (Raisinghani & Faingold, 2005). The amygdala enhances and relays the neural activity to forebrain areas such as the hippocampus and cerebral cortex that underlies generalized seizure behavior (Garcia-Cairasco, 1993; Raisinghani & Faingold, 2003, 2005). Overall, the neural processes and the structures of the AGS network underlying seizure initiation and propagation are well known with the amygdala playing a critical role in AGS generalization.

The importance of many of the structures in the AGS network to the initiation and propagation of seizure activity has been examined. Anomalous excitatory neural activity in the central nucleus of the IC initiates abnormal activity throughout the AGS network and seizure behavior (Browning, 1986; Coleman, Ross, Mullaney, & Cooper, 1999; Simler et al., 1994; Raisinghani & Faingold, 2003). When Browning (1986) removed the IC bilaterally in a seizure susceptible rat, he found that AGSs could not be initiated. In addition, neurons of the IC innervate the MGN where intense neural activation is evident after an AGS (Synder-Keller & Pierson, 1992). In the AGS network (Garcia-Cairasco, 2002), the MGN sends efferents to the lateral and basolateral amygdala (BLA) that allow for the spread of neuronal activation to this structure (Hirsch et al., 1997; Synzdler et al., 2009). Feng and Faingold (2002b) noted changes in the pathway leading to the lateral amygdala (LA) after AGS kindling, and Raisinghani and Faingold (2003) looked specifically at the LA and its role in propagating abnormal neural activity during AGS. Subsequently, Raisinghani and Faingold (2005) microinjected a toxin into the LA and successfully blocked AGS activity by blocking the N-methyl-d-aspartate (NMDA) receptors in the amygdala.
A great deal of the research specifically implicating amygdala as the critical relay from brain stem to forebrain in the AGS network comes from models with an inherited (e.g., Garcia-Cairasco, 2002; Merrill, Clough, Jobe, & Browning, 2005; Raisinghani & Faingold, 2005) or invasively kindled (e.g., Simler et al., 1994; Synzdler et al., 2009) rather than environmentally acquired foundation. Previous studies utilizing environmentally acquired seizures have found that the amygdala is known as the last single place that seizure activity can be controlled before it is dispersed throughout the brain because the structure is a relay center between brain stem and forebrain areas (see Figure 1) (Garcia-Cairasco, 2002). Each seizure, and possibly each AGR, alters the amygdala (Merrill, Clough, Jobe, & Browning, 2005), but this effect only occurs until a certain number of AGS or AGRs have occurred; afterwards, alteration in the amygdala stabilizes, which is evident by the activation of certain neural markers (Hirsch et al., 1997).

An AGR occurs when neurons within the AGS network contribute to the response by becoming active. As these neurons become excited through depolarizing membrane potentials, intracellular changes also occur and include the activation of immediate early genes (IEGs) (Simler et al., 1994; Snyder-Kelly & Pierson, 1992). Activity and products of IEGs are precursors to a variety of cellular activity including protein synthesis and neuronal plasticity (Perez-Cadahla, Drobic & Davie, 2011). The activation of IEGs starts with the binding of a neurotransmitter or neuromodulator to a receptor and the subsequent activation of messaging systems within a particular neuron which ultimately results in transcription of IEGs. These transcription factors are specialized for a specific IEG and are activated after exposure to a stimulus (Szyndler et al., 2009). With exposure to a sound stimulus capable of producing an AGR, the proto-oncogene c-fos, an IEG, is activated and its protein expressed
throughout the structures in the AGS network as well as in forebrain areas of the seizure-prone rat (Dragunow & Robertson, 1987; Simler et al., 1994; Snyder-Kelly & Pierson, 1992); however, there is a time limit to the duration of c-fos protein expression following stimulation (Sagar, Sharp, & Curran, 1988). Synder-Keller and Pierson (1992) found peak c-fos expression occurred in a rat model within 2 hours after an AGS. After 4 hours, the c-fos activation had greatly diminished, which suggests that c-fos expression in neurons is time limited. In the current research, the time frame used to capture peak c-fos expression after an AGR induction was between 1 and 2 hours (Dragunow & Robertson, 1987; Simler et al., 1994; Szyndler et al., 2009).

Another important factor in the level of c-fos expression following any one AGR induction is the number of AGSs that the particular rat has experienced (Hirsch et al., 1997). While a single AGS resulted in c-fos expression in the amygdala, Szyndler and colleagues (2009) suggested that it took four clonic seizures to express c-fos in the BLA which implies that activation in other areas of the amygdala occur before activation in the BLA. Importantly, Hirsch et al. (1997) found no activation of the c-fos IEG in rats not prone to AGS following exposure to sound capable of eliciting seizures in susceptible rats which they supported by providing evidence of a lack of protein in any neurons of the AGS neural pathway. As for a marker of neuronal activation following various forms of stimulation (Sagar et al., 1988), a body of research (e.g., Hirsch et al., 1997; Simler et al., 1994; Szyndler et al., 2009) supports the use of c-fos as a reliable activity marker for neurons involved in mediating seizures and thus, useful in the identification of the areas of the rodent brain affected by seizure activity.
**Statement of Purpose**

In this study, a model of acquired reflex epilepsy was used to investigate the impact of the number of prior seizures on activation of with LA and BLA following a seizure induction. Naturally seizure resistant Long-Evans rats (Ross & Coleman, 1999) were primed for AGR susceptibility on PND 18 and tested for AGRs and AGSs in response to a sound stimulus 1, 6 or 12 times. AGSs occur in these rats because of the abnormal neurological cascading effect elicited by a loud noise and due to cochlea trauma from the priming event (Ross & Coleman, 1999) at which then the abnormal neural signals propagate through the auditory network until it reaches the amygdala and then generalizes throughout the forebrain (Garcia-Cairasco, 2002; Pierson & Swan, 1991; Raisinghani & Faingold, 2003). This research focused on two specific areas of the amygdala in rats with acquired epilepsy, the LA and BLA, because of their relationship to the seizure network in rats with inherited epilepsy as supported by previous research (Feng & Faingold, 2002b; Raisinghani & Faingold, 2005). To investigate the activation of BLA and LA after an AGR, the presence of c-fos protein was used as a marker of activated neurons in these specific amygdala areas (e.g., Dragunow & Robertson, 1987; Sagar et al., 1988; Synder-Keller & Pierson, 1992, Szyndler et al., 2009). Because each AGR or AGS should result in increasing LA and BLA activation, it was hypothesized that there should be an initial increase of neural activation in the LA and BLA between animals having 1 and 6 seizures but that neural activation should remain constant between animals having 6 and 12 seizures. To assess the hypothesis, groups of seizure susceptible and age-matched controls were used. Brain tissue was collected on AGS 1, 6, and 12 from rats in the control and seizure susceptible group. The brain tissue from both control
and seizure groups was stained for c-fos and then examined under a microscope to determine the number of stained neurons in the LA and BLA for both the control and seizure groups.

Method

Subjects and Experimental Groups

The study was conducted using 38 Long-Evans hooded rats born in the Arts and Sciences animal facility at Appalachian State University. Pups were from four litters and lived with their dams in standard shoebox cages until they were weaned on PND 21. After weaning, the rats were housed three or four to a cage and maintained on a 12 h/12 h, light/dark cycle in a temperature and humidity regulated room. All rats had unrestricted access to food and water throughout the study. All procedures were approved by the Appalachian State University Institutional Animal Care and Use Committee (#13-06, M. Zrull, PI, December 13, 2012, see Appendix).

Before the experiments began, rats were divided into age-matched groups of 20 experimental and 18 control rats. The experimental group \( (n = 20) \) was primed for seizure susceptibility on PND 18 and tested for AGR beginning on PND 32. Control rats were aged-matched to the experimental group.

The experimental, or AGS group, was divided into three smaller groups based upon number of AGR inductions the rats would experience before sacrifice. Experimental rats \( (n = 6) \) in the first group, or AGS 1, experienced a single AGR induction on PND 32. After the induction, AGS 1 rats spent 60 min in a shoebox cage without bedding in a quiet and dark room and then were sacrificed. The quiet and dark room assured that the rat received no further stimulation that would potentially alter expression of the c-fos protein. AGS 1’s age-matched control rats \( (n = 6) \), or Control 32, spent 60 min in the quiet and dark and then were
sacrificed the same day (i.e., all rats were 32 days old). Rats in the second experimental group, or AGS 6, \((n = 6)\) experienced AGR inductions on PNDs 32, 34, 38, 42, 44, and 46 after which they spent 60 min in the quiet and dark after which they were sacrificed. Age-matched controls \((n = 6, \text{Control 46})\) for AGS 6 spent 60 min in the quiet and dark on PND 46 and then were sacrificed. In the third group, or AGS 12, experimental animals \((n = 6)\) experienced AGR inductions on PNDs 32, 34, 38, 42, 44, 46, 49, 53, 57, 59, and 62 after which they spent 60 min in the quiet and dark and then were sacrificed. Corresponding Control 62 rats \((n = 6)\) spent 60 min in the quiet and dark on PND 62 and then were sacrificed.

Data from the experimental group reflects only rats that had experienced the number of AGR and AGS required by their group or if not, as often as possible. Table 1 shows the percentage of possible AGR and AGS for each experimental group. The presence of AGR and AGS as well as seizure severity was determined by timing the latency to wild running, latency to clonic seizure, and duration of clonic seizure in seconds; these data can be found in Table 2. After rats who did not meet the criteria for data collection were excluded, each AGS group (AGS 1, AGS 6, or AGS 12) had a total of 4 rats. For each rat in the experimental, or AGS, group, an age-matched rat was selected randomly to be that specific rat’s control. Each age-matched, PND control group had 4 rats to mirror the AGS groups.

**Priming**

All experimental rats were primed for AGR susceptibility on PND 18. Rat pups were placed with litter mates in a shoebox cage without bedding and were exposed to 120 dB \((re 0.0002 \text{ dyne/cm}^2, \text{A Scale})\), 10kHz tone pips for 8 min. The rate was 8 tone pips per second
(75 ms on, 50 ms off with 5 ms rise and fall times). After priming, the rat pups were returned to their home cages. Tone pips were produced by a TDT, Inc. WG1 waveform generator, amplified by a Realistic MPA-101, and then attenuated back to 120 dB by a HP 350D and presented through Pioneer TS-G1340R speakers.

**AGR Inductions**

AGR inductions began on PND 32 for all the experimental groups (AGS 1, 6, and 12). All inductions occurred in a 29.5 cm x 34 cm cylindrical chamber constructed out of ¾-in. plywood and ¼-in. wire-mesh. Each rat was placed individually in the chamber and then exposed to 120 dB broadband noise (0.1 to 10 kHz) for 120 s or until a clonic seizure began. Noise was generated by a Coulboun S81-02 Noise Generator and amplified with the Realistic MPA-101. The noise was presented through broad-range speakers (Realistic 40-1354A). All trials were videotaped for behavioral coding at a later time. If the rat was to be sacrificed, it was put into a quiet and dark room for 60 min after the appropriate AGR induction (1, 6 or 12). If the rat was due to experience more AGRs, it was returned to its home cage after an induction.

**Histology**

After either the last seizure or the appropriate control PND, the rat would be placed in a cage and spend 60 min in the quiet and dark. After time in the quiet and dark, a rat was given a lethal dose of sodium pentobarbital (100 mg / kg b.w., ip). Upon the absence of a corneal and tail reflexes, the rat was perfused intracardially with 0.9% saline in 7.4 pH 10 mM phosphate buffer (PBS) followed by 4% paraformaldehyde in 10 mM phosphate buffer (PB) in order to fix the brain tissue. The brain was removed and placed in a container of 4%
paraformaldehyde and 10% sucrose at 4 °C for one week and then transferred to 10 mM PB and stored at 4 °C.

Tissue sections were cut in the sagittal plane using a Vibratome. Initially, 100 μm brain sections were removed until the hippocampus became visible and a particular shape, which indicated an approximate depth into the hemisphere. After the predetermined area was reached, 48 50 μm sagittal sections were cut from each brain. Sections were stored in 10 mM PB until they were processed using a floating section immunohistochemistry procedure to strain neurons containing the c-fos protein. First, sections were rinsed twice for 5 min with 10mM PBS and then immersed in 1% hydrogen peroxide followed by PBS rinses (2 x 5 min). Then, tissue was submersed in 15% goat serum (Vector) with 0.2% Triton-X in PBS for 60 min, which blocked non-specific binding sites. Next, the brain tissue was exposed to primary antibody to the c-fos protein (Calbiochem PC-38, made in rabbit, 1:2000) for 40 h at 4 °C.

After incubating in the primary antibody, brain tissue was rinsed in PBS (6 x 10 min) and the sections were placed in biotinylated secondary antibody (Vector, goat anti-rabbit, 1:400 in PBS). Next, tissue was rinsed 3 times, 10 min each, with PBS and placed in peroxidase-labeled avidin-biotin solution (ABC, Vector) for 60 min. The sections were moved to an enzyme substrate (Vector, VIP), which reacts to peroxidase ABC to stain neurons, for 2 to 10 min dependent upon staining. From the VIP solution, the sections were placed in a bath of distilled water for at least 10 min. Sections were mounted on gel-coated slides and air dried. Finally, the sections were dehydrated in graded ethanol, cleared with toluene, and cover-slipped with Permount (Fisher). Alternate sections were Nissl stained with thionin. The
Nissl staining created a set of sections providing a structural reference point corresponding with the sections stained for c-fos.

**Data Analysis**

A member of the Zrull laboratory watched videotapes of the induction sessions and coded the nature and severity of AGRs and AGS. For each induction, a record of the latency to and duration of each wild running phase was recorded as well as the latency to and duration of a clonic AGS if one occurred. Thus, for AGS 1, 6, or 12, there was a record of which of the rats had a clonic seizure and measures of AGR severity (Tables 1 and 2). These results were used to identify rats, and thus tissue, appropriate for inclusion in analyses of neuronal activation following seizures (i.e., rats having at least an AGR on the final induction session). As described in *Subjects and Experimental Groups*, each group consisted of 4 rats with a total of 12 rats across the AGS groups and 12 rats across the control group (Control 32, 46, or 62).

Neuronal activation data was collected from brains of rats from the three seizure groups and age-matched controls. First, c-fos stained slides from each brain were placed on a light box to identify three sections that exhibited BLA and LA most optimally. These sections started at the most lateral part of the brain containing BLA and LA and then moved medially. While all three sections contained the larger BLA, the last and most medial section did not exhibit LA; however, the most medial section exhibited a large portion of the BLA. Following work using the light box, BLA and LA locations were confirmed using the Plan 4 objective on a Nikon® Eclipse microscope. A PixeLink® digital camera was attached to the Nikon microscope and projected a digital image of the microscope visual field on to computer. Once sections were identified and the BLA and LA locations confirmed, the
counting of c-fos positive neurons in the amygdala was performed using a Plan 10 objective and an 800 x 600 pixel image on the computer screen. A transparency sheet with six, 200 x 200 µm sample boxes was placed over the image on the computer. The sample boxes had color-coded lines to identify inclusion areas (black, left and bottom) and exclusion areas (green, right and top) to determine whether cells that fell on lines of the sample box should be counted. The LA was sampled using three sample boxes in a row because of the LA’s shape, which is more narrow than wide. The BLA is more rounded, so it was sampled using a 2 x 2 grid of sample boxes. Each BLA section had four samples and each LA section had three samples.

Two members of the Zrull laboratory, blind to the experimental condition, counted activated neurons that contained c-fos by marking neurons stained for the protein on a transparency sheet placed over the sheet with counting frames with a permanent marker. Because each tissue section has depth, counts were made for 200 x 200 x 50 µm sample volumes from the parts of the amygdala. C-fos positive neurons were categorized into three different levels according to variation in activation, which was indicated by darkness of staining, using the following symbols: slightly activated (●), moderately activated (●/ ●), and strongly activated (+). Counting started in the LA with brains from the experimental group, AGS1, and age-matched control group, PND 32. The same procedure was used to count c-fos positive neurons in LA from experimental group AGS 6 and 12, and corresponding control groups sacrificed on PND 46 and 62. Once the LA was completed, the BLA of experimental and control groups were counted starting with AGS 1 and Control 32 and ending with AGS 12 and Control 62. Marks in the sample areas on the transparency sheets were counted, and the number of fos-positive neurons at each level was recorded in a database. In total, there
were 6 LA samples per brain or 24 LA samples per AGS/Control group and 12 BLA samples per brain or 48 samples per AGS/Control group.

Activated cells in the LA and BLA were averaged together for each of the age-matched groups of control rats. These values served as baseline neuronal activation levels for each experimental group, which accounted for normal maturation from PND 32 to 46 to 62, and allowed for comparisons between AGS and Control groups as well as within the experimental groups. For each rat, an average was computed from all the sampled areas for both the LA and BLA, leaving each of the 12 AGS and 12 Control rats with two averages: one for c-fos positive (i.e., activated) neurons in the LA, and one for activated neurons in the BLA. These data were then used for a two-way ANOVA, which allowed assessment of the hypotheses and examination of differences in neuronal activation in LA and BLA dependent upon the number of seizures.

**Results**

The researcher hypothesized that with increasing AGR and/or AGS events, a significant increased neuronal activation in the LA and BLA would occur between groups AGS 1 and 6; however, the neuronal activation would remain constant between groups AGS 6 and 12. To test the hypotheses, data collected from 24 rats (12 AGS and 12 Control) that reflected c-fos neuronal activation in the LA and BLA was analyzed using a two-way ANOVA followed by Bonferroni post-hoc pairwise comparisons to allow specific investigation of the interaction effect of the AGS/Control group and AGS Number/Control PND on activation of neurons in LA and BLA. Five pairwise comparisons were of interest of each structure, and the Type I error-rate for each Bonferroni comparison was set at .01 to control error at .05 across the tests.
Differences in LA neuronal activation between AGS and control groups

The ANOVA showed an interaction effect between the overall experimental AGS/Control group and AGS Number/Control PND on the number of c-fos positive neurons in LA, $F(2,18) = 3.56, p = 0.05$ (see Figure 2). To explore the significant interaction effect, a post-hoc analysis was conducted to examine the specific differences in LA neural activation between AGS and Control groups for each AGS Number/Control PND and among AGS Numbers within the AGS group. Bonferroni results revealed a significant increase in LA neural activation between groups AGS 1 ($M = 3.7, SD = 2.3$) and AGS 6 ($M = 17.6, SD = 11.2$), $t(18) = 3.10, p < 0.01$). Unlike AGS 1 to AGS 6, there was a non-significant decrease in fos-positive neurons between groups AGS 6 ($M = 17.6, SD = 11.2$) and AGS 12 ($M = 15.2, SD = 9.1$), $t(18) = 0.54, p > 0.05$) (see Figure 2). There were no significant differences in LA neuron activation among Control 32, 46, or 62 groups.

The post-hoc analysis further revealed specific differences in c-fos positive neuron counts between AGS and corresponding age-matched control groups. There was not a significant difference between the means of groups AGS 1 ($M = 3.7, SD = 2.3$) and Control 32 ($M = 3.5, SD = 5.1$), $t(18) = 0.046, p > 0.05$). Importantly, means for groups AGS 6 ($M = 17.6, SD = 11.2$) and Control 46 ($M = 1.6, SD = 1.4$), $t(18) = 3.56, p < 0.01$) were significantly different as well as the means for groups AGS 12 ($M = 15.2, SD = 9.1$) and Control 62 ($M = 1.75, SD = 1.2$), $t(18) = 2.99, p < 0.01$) (see Figure 2).

Statistical analyses supported the observation that rats who experienced an AGR/AGS event exhibited greater neuronal activation in the LA compared to the control rats that did not experience AGR/AGS event or events. Results of the post-hoc analysis supported the hypothesis of this thesis for LA by revealing a significant increase in neuronal activation.
between the first and sixth AGR/AGS event and no statistically significant change between the sixth and twelfth AGR/AGS event.

**Differences in BLA neuronal activation between AGS and control groups**

Interestingly, the ANOVA results revealed that experimental AGS/Control group and AGS Number/Control PND failed to have a significant interaction effect on c-fos positive neuron counts in the BLA, $F(2,18) = 1.57, p = 0.24$ (see Figure 3). While there was no significant interaction effect on BLA neuronal activation, there was an overall difference in activated neurons between AGS and Control groups, $F(1,18) = 6.32, p = 0.02$. The means for activated BLA neurons in AGS/PND by AGS Number/Control PND groups were examined, and some differences in the sample were observed. While not statistically significant, Groups AGS 1 ($M = 1.0, SD = 1.1$) and AGS 6 ($M = 2.0, SD = 1.7$) did differ more than the means between groups AGS 6 and AGS 12 ($M = 2.4, SD = 1.7$). When comparing AGS Number to age-matched Control groups, AGS 1 ($M = 1.0, SD = 1.1$) and Control 32 ($M = 0.8, SD = 0.6$) and groups AGS 6 ($M = 2.0, SD = 1.7$) and Control 46 ($M = 0.8, SD = 0.4$) did not differ with statistical significance. Finally, Groups AGS 12 ($M = 2.4, SD = 1.7$) and Control 62 ($M = 0.3, SD = 0.3$) showed the greatest difference, but the difference did not reach statistical significance (see Figure 3).

Overall, results of ANOVA and post-hoc analyses suggest data does not support the hypothesis for BLA. The trend in increasing c-fos counts across AGS 1, 6, and 12 groups, with the last testing day (AGS 12 on PND 62) resulting in the greatest difference between and AGS and age-matched Control group, suggests that neuronal change in the BLA happens at a reduced rate in comparison to the LA.
Discussion

This study investigated the effects of acquired generalized clonic seizures on neuronal activation in the LA and BLA, which was accomplished using a Long-Evans rat model of AGS. Rats were divided into groups and exposed to 1, 6, or 12 seizure inductions with neuronal data collected only from rats exhibiting abnormal responses to the sound stimulus during most inductions and, at least, an AGR on the 1st, 6th, or 12th induction (AGS 1, AGS 6, AGS 12 groups). Comparison of AGS 1, 6, and 12 groups revealed that between AGS 1 and 6 there was a significant increase in c-fos expression, but further seizure inductions did not significantly increase or decrease c-fos expression within the LA. In the BLA, there was an increasing trend in c-fos expression across AGS groups that were not statistically significant. Thus, for purposes of generalization increasing seizure inductions did not alter neuronal activation in the BLA. Overall, the hypothesis of this study, which suggested that there should be an initial increase of neuronal activation in the LA and BLA between rats having 1 and 6 seizures but that neuronal activation should remain constant between animals having 6 and 12 seizures, was supported partially. Results from the LA supported the hypotheses that there would be an increase in activated neurons between groups AGS 1 and 6, but the number of activated neurons would stabilize between groups AGS 6 and 12.

The increase in c-fos staining between AGS groups 1 and 6 aligns with the results from previous research by Eells, Clough, Browning, and Jobe (2004), who used c-fos as a measurement for AGS-influenced neuronal activity in the LA and found that c-fos staining did not increase after one AGS exposure, and Hirsch et al. (1992) who found that five AGS exposures resulted in the recruitment of the hippocampus. The amygdala is a forebrain area,
like the hippocampus, and also like the hippocampus, is recruited into the AGR/AGS network after some number of audiogenic events (in this case by the sixth exposure) (cf. Eells, Clough, Browning, & Jobe, 2004; Hirsch et al., 1992). The Faingold laboratory has suggested that the neurons of the LA become more efficient over time due to increased activity of glutamatergic systems, thus resulting in a relay nucleus with neurons that are now more sensitive to an audiogenic stimulus (Feng & Faingold, 2002a; Raisinghani & Faingold, 2003; Raisinghani & Faingold, 2005), which can be interpreted as an explanation for the stabilization of LA neuron population in terms of activation from 6 to 12 seizures. Together, previous research suggests that the LA neurons are recruited to propagate the abnormal neural signal of a seizure but that no more neurons are recruited than those required to efficiently propagate the abnormal signal, which results in a stabilizing effect on neuronal activity in the LA.

C-fos has not been used previously to measure the effects of an increasing number of AGR/AGS events on neurons in the LA; however, it has been used to examine function MGN. The MGN is critical in the propagation of AGS (Hirsch et. al., 1996), and it sends efferents to the LA (Feng & Faingold, 2002a; LeDoux, Farb, & Ruggiero, 1990). Hirsch et al. (1996) researched the relationship between increasing AGS exposures and c-fos expression in the MGN and found a positive correlation between AGS and c-fos expression after one AGS, but the extent of c-fos expression did not increase with additional AGS exposures. Previous research supported the MGN as closer to the origin of the abnormal neural signals of an AGS as compared to the amygdala resulting in more rapid and more intense c-fos expression (Pollak, Burger, & Klug, 2003; Simler et al., 1994; Snyder-Keller & Pierson; 1992). Previous research, along with this study’s results, provides support for the
hypothesis that neural activity in LA initially increases and then stabilizes after a number of AGR/AGS.

The researcher hypothesized that the same pattern of neuronal activity seen in the LA would also occur in the BLA as exposure to AGR/AGS inducing stimulation continued. While there was a slight increase in c-fos expression, no significant effects where found between groups AGS 1 and 6 and groups AGS 6 and 12 in number of activated BLA neurons. There were also no significant effects found when comparing AGS groups to the respective age-matched controls. Previous research by Hirsch et al. (1996) measured the c-fos expression in the BLA of rats that experienced 1, 6, or 10 AGS and found that there was an initial increase in c-fos expression between AGS 1 and 6 but remained constant between AGS 6 and 10. While the results of this study are not aligned with Hirsch et al. (1996), it is important to note that they used a model of clonic-tonic seizures whereas this study utilized a model of clonic seizures, suggesting that the clonic AGS network utilizes a slightly different network (see Figure 1). Similar to Hirsch and colleagues (1996), Szyndler et al. (2009) found that there was significant c-fos expression in the BLA after a number of clonic-tonic seizures. It is worth noting that the model in this study, in contrast to Szyndler et al. (2009), includes a behavioral progression from wild running (the initial AGR) to only a clonic seizure of relatively short duration (cf. Ross & Coleman, 1999; Ross & Coleman, 2000). Results from Hirsch et al. (1996) and Szyndler et al. (2009) led to the hypothesis that the BLA c-fos expression would differ significantly between groups AGS 1 and 6 and then remain constant between groups AGS 6 and 12; however, results from this study do not reflect the same findings as Hirsch et al. (1996) and Szyndler et al. (2009). Taking together previous research
and current BLA results, it can be concluded that the developmentally primed model of AGS relies on the LA as a brain stem to forebrain relay center and not the BLA.

Results from this study provide insight into the relationship between the BLA and LA and the AGS neuronal network in the developmentally primed rat. When the AGS-primed rat is exposed to a loud sound, the cochlea of the inner ear is stimulated and converts sound waves into neural signals. The neural signal is carried via the auditory nerve to the cochlea nucleus, which then projects to the medial and lateral superior olive (Coleman & Clerici, 1987; Pollak, Burger, & Klug, 2003). The inferior colliculus (IC) receives input from the medial and lateral superior olive. It is in the IC that the strong neural signal becomes abnormal, thus initiating the cascading effect that results in a seizure (Garcia-Cairasco, 2002; Simler et al., 1994). The IC relays the abnormal neural signal to the MGN (Coleman & Clerici, 1987; Garcia-Cairasco, 1993) from which the MGN propagates the neural signal to various areas in the brain and importantly, the amygdala (Pollak et al., 2003). Analysis of the c-fos expression resulted in the conclusion that the abnormal neural signal that characterizes an AGR/AGS affects neuronal activity in the LA to a much greater extent than the BLA and that this recruitment allows AGR/AGS events to occur more easily up to a level as LA becomes part of the AGR/AGS network.

Researching specific areas of the brain has the potential for results that are influenced by one or more outside variables. Research supports the amygdala as a key brain region in fear-like responses (Davis & Whalen, 2001), which, in regards to this research, raises the issue of whether c-fos expression seen the LA and BLA resulted from a fear-like response to the loud noise used to induce AGR/AGS. This concern has been addressed by previous researchers who use the audiogenic model to study seizures, and they report neuronal activity
results, whether measures of action potentials, $[^{14}\text{C}]$ 2-deoxyglucose (2-DG) uptake, or c-fos expression, from rats who had experienced an AGS that varied significantly from non-primed and not seizure susceptible rats who were exposed to an acoustic stimulus capable of inducing an AGR (Feng & Faingold, 2002b; Hirsch et al., 1996; Simler et al., 1994). Specifically, Hirsch et al. (1996) found that control rats who were exposed to an acoustic stimulus capable of producing an AGR did not exhibit significant c-fos staining in the BLA along with critical AGS brain regions such as the MGN and IC. Simler et al. (1994) found that in non-AGS susceptible rats, exposure to a loud sound stimulus did not produce significant c-fos expression in IC, which is a major area associated with AGS. Pereira de Vasconcelos, Vergnes, Boyet, Marescaux, and Nehlig (1997) researched AGS-induced metabolic activation in the forebrain found that when a loud tone was presented to a group of naïve rat or Wistar rats with acoustically kindled susceptibility to AGS, only the AGS-susceptible rats showed significant increases in optic densities indicative of increased 2-DG uptake, which is a metabolic proxy for increased neural activation, in the BLA. Therefore, previous research demonstrates that although initiation and propagation of the neural correlates of AGS use auditory and fear-response systems and pathways, it is the abnormal neural cascade of an AGS event that results in c-fos expression.

C-fos expression has been well-documented as a measurement for neuronal activity (Dragunow & Robertson, 1987; Ferreira-Netto, Borelli, & Brandão, 2007; Sagar, Sharp, & Curran, 1988; Simler et al., 1994; Snyder-Keller & Pierson, 1992; Szyndler et al., 2009); however, research by Labiner et al. (1993) and Eells, Clough, Browning, & Jobe (2004) questions reliability of using c-fos as a measure of neuronal activity. Eells, Clough, Browning, & Jobe (2004) suggest using both c-fos and 2-DG, a glucose analog, to create a
more conclusive measure of neuronal activity. According to Eells, Clough, Browning, & Jobe. (2004), c-fos has the ability to measure at the neuronal level; however it does not always accurately indicate increased neuronal input. 2-DG measures increased metabolic activity in the brain region in question, but it is not measuring change in individual neurons. Interestingly, neither c-fos nor 2-DG offer any way by themselves to discriminate increased neuronal excitation from increased neuronal inhibition as both methods measure changes related to neuronal metabolism, which does not have a specific connection to either excitation or inhibition. Although there are questions about limitations in the efficacy of c-fos as a measure of neuronal activity in terms of changes in excitation or inhibition, it is still a widely utilized method in seizure research because it provides insight into the effects of abnormal neural signals on metabolic activity within individual neurons (Dragunow & Robertson, 1987; Ferreira-Netto, Borelli, & Brandão, 2007; Sagar, Sharp, & Curran, 1988; Simler et al., 1994; Snyder-Keller & Pierson, 1992; Szyndler et al., 2009).

The amygdala’s role in the AGS network is somewhat perplexing, with experiments suggesting involvement in both tonic and clonic seizure networks (Eells, Clough, Browning, & Jobe, 2004; Raisinghani & Faingold, 2003; Raisinghani & Faingold, 2005; Tupal & Faingold, 2012). Raisinghani and Faingold (2003) explored the AGS clonic seizure network via microinjecting a NMDA antagonist, 2-amino-7-phosphonoheptanoate (AP7), into various areas of the GERP-3 seizure network including the amygdala. Surprising, they found that microinjections into the amygdala blocked AGS in the GERP-3, a genetic rat model for known for acoustically-induced clonic seizures. Raisinghani and Faingold (2005) further researched the specific areas of the amygdala involved in the clonic form of AGS via microinjections of AP7 into the LA of GERP-3s and found that the antagonist AP7 blocked
generalized clonus induced acoustically. Research beyond the Faingold laboratory into the LA is limited, and this study is the only one that uses the developmentally primed Long-Evans AGS model to study the LA and generalized clonus. Findings from this experiment suggest the LA in the Long-Evans model is a component of the AGS network, specifically related to the propagation of generalized clonic seizures. Future experiments following methods put forth by Raisinghani and Faingold (2003, 2005) are needed to test the specific hypothesis that the LA is a necessary relay site in the neural network for generalized clonic seizures in the developmental primed AGS-prone rat, allowing for greater insight into the AGS network in Long-Evans rats. It would be interesting to inject AP7 into the LA of developmentally primed Long-Evans rats and observe whether or not generalized clonic seizures could be induced with acoustic stimulation.

In summary, this thesis included a study of the neuronal activity evoked by 1, 6, or 12 seizures within the LA and BLA of the developmentally primed Long-Evans rat model of acquired generalized clonic seizures. Using the Long-Evans model allowed for precise control over the number of AGR/AGS a rat experienced; rats in this epilepsy model do not experience sporadic, unprovoked seizures. Control over the number of seizures experienced allowed for the rats to be divided into groups specifically defined by the number of AGR/AGS events experienced: 1, 6, or 12. Results from the study revealed a differential effect of AGR/AGS on neuronal activation in the LA and BLA with the LA experiencing greater neuronal activation than the BLA, which was minimally activated, as evident by analyses of c-fos staining. The data also provide evidence for a neuronal activation ceiling within the LA dependent on the number of seizures experienced. Overall, the data collected in this study provide insight into the neural propagation of AGR/AGS from brain stem to
forebrain via the amygdala in the developmentally primed Long-Evans model of acquired reflex epilepsy.
References


Raisinghani, M., & Faingold, C. L. (2005). Neurons in the amygdala play an important role in the neuronal network mediating a clonic form of audiogenic seizures both before and after audiogenic kindling. *Brain Research, 1032*, 131-140. doi: 10.1016/j.brainres.2004.11.007


Table 1

**Percentage of Audiogenic Responses and Seizures for the Three Experimental Groups across all Audiogenic Induction Sessions**

<table>
<thead>
<tr>
<th>Group</th>
<th>Final Induction</th>
<th>N</th>
<th>Possible Responses</th>
<th>Audiogenic Responses</th>
<th>Audiogenic Seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Test</td>
<td>32 days</td>
<td>4</td>
<td>4</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>6 Tests</td>
<td>46 days</td>
<td>4</td>
<td>24</td>
<td>100.0%</td>
<td>95.8%</td>
</tr>
<tr>
<td>12 Tests</td>
<td>62 days</td>
<td>4</td>
<td>48</td>
<td>97.9%</td>
<td>75.0%</td>
</tr>
</tbody>
</table>

Note. All rats exhibited an audiogenic response during the final induction session.
Table 2

*Mean (SD) Audiogenic Response and Seizure Severity Measures in Seconds for Final Induction Session for each Experimental Group*

<table>
<thead>
<tr>
<th>Group</th>
<th>Final Induction</th>
<th>N</th>
<th>Latency to Wild Running (SD)</th>
<th>Latency to Clonic Seizure (SD)</th>
<th>Duration of Clonic Seizure (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 AGS</td>
<td>32 days</td>
<td>4</td>
<td>30.5 (18.6)</td>
<td>46.0 (15.4)</td>
<td>19.0 (4.3)</td>
</tr>
<tr>
<td>6 AGS</td>
<td>46 days</td>
<td>4</td>
<td>17.5 (4.5)</td>
<td>52.7 (3.5)</td>
<td>16.3 (13.3)</td>
</tr>
<tr>
<td>12 AGS</td>
<td>62 days</td>
<td>4</td>
<td>12.5 (2.6)</td>
<td>41.8 (8.3)</td>
<td>10.3 (0.5)</td>
</tr>
</tbody>
</table>

Note. All rats exhibited an audiogenic response during the final induction session.
Figure 1. The drawing is a summary representation of the neural network underlying audiogenic seizures (AGS) based upon descriptions from Garcia-Cairasco (2002), Raisinghani and Faingold (2003, 2005), and Ross and Coleman (1999). Of particular interest in this study were the roles of lateral and basolateral amygdala (LA, BLA) as relay nuclei. While there is agreement that LA relays to forebrain and brain stem in models of inherited epilepsy affecting clonic, and possibly tonic, activity, BLA may be more important as a relay back to brain stem affecting tonic seizure activity (e.g., Raisinghani & Faingold, 2003, 2005).
Figure 2. Results for neuronal activation in lateral amygdala dependent on AGS/Control group and AGS Number/Control PND variables. Mean values reflect c-fos counts between AGS and Control groups across AGS Number/Control PND conditions. Significant differences were found between AGS 1 and AGS 6 groups \((p < .01)\) but not between AGS 6 and AGS 12 groups, and significant differences were found between AGS 6 and Control 46 and AGS 12 and Control 62 groups \((p < .01)\). The error bars are standard deviations.
Figure 3. Results for neuronal activation in basolateral amygdala dependent on AGS/Control group and AGS Number/Control PND variables. Mean values c-fos counts between AGS and Control groups across AGS Number/Control PND conditions. It is worth noting that the mean counts were quite small across all conditions. The error bars are standard deviations.
Figure 4. Digital photographs showing examples c-fos positive neurons in the lateral amygdala of audiogenic response/seizure (AGR/AGS) susceptible and tested rats (A, C, E) and age-matched controls animals (B, D, F). Images were made with a PixeLink camera attached to a Nikon Eclipse microscope using a Plan 10 infinity achromat objective. (A) Rat 1247 following a single AGR/AGS. (B) Control Rat 1239 at postnatal day (PND) 32. (C) Rat 1257 following the sixth AGR/AGS. (D) Control Rat 1251 at PND 46. (E) Rat 1308 after the 12 AGR/AGS events. (F) Control Rat 1301 at PND 62. The bar is 100 μm.
Appendix

TO: Dr. Mark Zrull
    Department of Psychology

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

DATE: December 13, 2012

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

REFERENCE: Effects of environmental enrichment on seizure severity and neural
           activation in a model of acquired reflex epilepsy

           IACUC Reference #13-06

           Initial Approval Date – December 13, 2012
           End of Approval Period – December 12, 2015

The above referenced protocol has been approved by the IACUC for a period of three years.

Best wishes with your research.

JCD/rst
Vita

Haley Kristen Andersen was born in Raleigh, North Carolina to David and Jeanette Andersen. She graduated from East Carolina University in 2010 with her Bachelor of Science degree in Biology and then in 2012, received her Bachelor of Arts degree in Psychology from the same university. In 2012, she attended Appalachian State University in pursuit of her Master of Arts degree in Experimental Psychology. In 2014, she accepted the position of Research Specialist at the University of South Carolina in the lab of Pavel Ortinski, Ph.D. It is here she and her fellow colleagues research the neural mechanisms of cocaine addiction.

Ms. Andersen resides in Columbia, South Carolina with her boyfriend, Steven, and their beloved cats, Dr. Doom, Georgia Mae, Hammurabi, and Klaus.