Developmental Postnatal Neuroplasticity of the Primate Amygdala: a Quantitative Analysis of Parvalbumin Neurons and Perineuronal Nets

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Developmental Postnatal Neuroplasticity of the Primate Amygdala:

A Quantitative Analysis of Parvalbumin Neurons and Perineuronal Nets

A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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Abstract

Neuroplasticity is implicated in the pathology of many psychiatric disorders, abnormal behaviors, and learning disabilities. Additionally, abnormalities in neuronal densities and regional volumes have been reported in psychiatric disorders such as schizophrenia (SZ) and Post Traumatic Stress Disorder (PTSD). Recent research shows parvalbumin (PVB) neurons and perineuronal nets (PNNs) play a crucial role in neuroplasticity. Current understanding of PVB or PNN development in humans, and the onset of their critical point (CP) of plasticity, has been limited to rodent models and human postmortem tissue. This study sought to address this informational gap through observation of the developmental trajectories of PVB neurons and PNNs in the primate amygdala. Tissue for this study was provided from Rhesus Macaques at 3 weeks, 3 months, and adulthood (+3 years) and processed via immunohistochemistry for each hemisphere. Left hemispheres were processed for PVB immunoreactive (IR) neurons, and the right hemispheres were processed using Wisteria floribunda Agglutinin (WFA) immunoreactive (IR) PNNs. After staining, each section of amygdala tissue was divided into subnuclei and we used quantitative microscopy to determine PVB and PNN densities. Our results show an anticipated increase in PVB neuronal density with age. However, we observed an unexpected and notable decrease in PNN density between 3 months and adulthood. We believe the decrease in PNN density may represent synaptic pruning that is occurring deep within brain regions such as the amygdala at this age and thus provides novel insight into the onset of the critical point in neuroplasticity in the
primate amygdala. Overall our results suggest a more applicable model for the development of human neuronal plasticity and more research is required to confirm these findings.
Dedication

I dedicate this thesis to Felicia Hoffman, who has defied all odds and continues to inspire light, love, and wonder in my life each day.

This thesis is also dedicated to my mother, whose love and support knows no bounds. It is to her that I owe my passion for science and wanting to discover the unknown.
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Thank you to my lab mate and friend Joshua Min, for always seeing the best in me.

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Without all of your love and support, none of this would have been possible.
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Chapter I

Introduction

The Research Problem

Although much is known about early (embryonic) brain development, there is a limited understanding of postnatal maturation of the brain. Rodent studies suggest that different parts of the brain mature at different stages, including during late adolescence and early adulthood. Differential maturation between brain regions may have a profound impact on brain function, as well as behavior, and thus be implicated in the pathology of many psychiatric disorders. By observing the development of specific brain regions over a targeted age range, we may better understand developmental psychiatric disorders including schizophrenia (SZ) and autism. This study may also help our understanding of addiction and impulsive behaviors in adolescence as compared to adulthood. Improving our understanding of postnatal brain maturation has broad implications for human behavior and psychiatric diseases.

To date, research on postnatal maturation of the brain has been conducted primarily in rodent models. Despite considerable neural imaging and postmortem studies on human brains, experimental parameters are severely constrained due to lack of full age representation in available brain tissue of humans. Translation of rodent studies to human models may be facilitated via primate research; however, there is a lack of information on differential brain maturation and perineuronal net (PNN) development in primates. Current belief indicates different regions of the human brain mature at varying
points in time and often develop in nonlinear patterns. Moreover, much of this maturation occurs during adolescence, a period when the brain undergoes structural and functional changes.

One specific brain region of interest that is often associated with psychiatric and neurodevelopmental disorders, such as Schizophrenia (SZ) and Autism, is the amygdala (Amaral et al., 1992 and Schumann et al., 2011). The amygdala, located in the anterior portion of the temporal lobe, is responsible for assigning emotional valence to sensory stimuli. Numerous examples of this have been established via rodent fear conditioning studies (Mahan et al., 2012 and Ou et al., 2010). Conditioning and extinction of fear responses in rodent models has helped to establish the role neuronal plasticity plays in the amygdala (Ou et al., 2010). A study by Gogolla et al., has been able to further the role of neuroplasticity in the rodent amygdala through manipulation of PNNs in relation to fear conditioning (Gogolla et al., 2009). Gogolla et al., observed that fear conditioning was more easily reversed during adolescence thus helping them to establish that the critical point of neuroplasticity for the amygdala must develop later into adulthood (Gogolla et al., 2009). Interestingly, this appears to coincide with the later onset of SZ observed in humans. Moreover, Gogolla et al., found that degrading PNNs allowed for full extinction of the fear memory that would have otherwise been protected from fear-erasure by PNNs (Gogolla et al., 2009). Translation of these findings from rodent to primate models will facilitate our understanding of neuroplasticity and possible pathologies for psychiatric disorders in humans. Although not much is known about amygdala development of the primate brain, several Macaque studies have found early postnatal amygdalocortical connections to closely resemble that of mature adults (Schumann et al., 2011; Webster et
Consequently, it is clear something must be done to close this informational gap.

Developmental stages of primates are much longer than that of most other mammals with both in gestation and postnatal development. Primates must provide extensive care for their young post parturition and through early development post a long period of gestation. During this period, the brains of adolescent primates increase dramatically in size and continue to develop throughout early adolescence (Martin, 2013). Similarly, humans go through a prolonged period of altriciality, in which the underdeveloped brains of infants (approximately 30% of adult size) continue to develop postnatally nearly doubling in size within their first year of life (Dunsworth et al., 2012). A comparatively delayed adolescence in humans allows for the human brain to retain a higher level of plasticity, or synaptic flexibility, until synaptic pruning occurs. Recent studies illustrate that a structural component associated with the mature form of plasticity is the formation of extracellular matrix structures known as perineuronal nets (PNNs), which form around neurons at the end of critical developmental periods (CP) of plasticity. PNNs are reticular structures that tightly surround synaptic connections on distinct populations of neurons and appear to significantly reduce synaptic plasticity once their CP has been reached. (Wang and Fawcett, 2012). Scientists believe the maturation of PNNs may be responsible for gating regional activation during key developmental windows (Pizzorusso et al., 2002 and Gogolla et al., 2009). PNNs and onset of their CP require further study and characterization through mapping of their developmental trajectory and maturation.
PNNs typically form around fast firing inhibitory neurons expressing the calcium binding protein parvalbumin (PVB) (Härtig et al., 2017 and Pantazopoulos et. al, 2006). PVB neurons migrate into their proper locations in the brain during early development but achieve their mature electrophysiological properties much later during postnatal stages once PNNs form. Thus, examining the relationship of PNNs around PVB neurons in the primate brain can provide an indication of when certain brain regions mature (Härtig et al., 2017 and Pantazopoulos et. al, 2006). The presence of PVB neurons without PNN maturation would indicate an issue of neuroplasticity and the neuronal firing properties for PVB neurons in that region (Pantazopoulos et. al, 2010). To better understand PNNs, it is crucial to comprehend their development and maturation across varying regions of the brain. Further mapping the trajectory of PVB neuronal development and PNN formation may uncover an underlying pathology of many psychiatric disorders affected by neuroplasticity.

In this study, we have examined PVB neurons and PNNs in a cohort of primate amygdala, across developmental stages, as a proxy for postnatal maturation of selective brain regions in the primate brain. Our study further seeks to address at what point during the development of amygdala do PNNs reach their CDP in primates. Additionally, we have shown that the presence of PVB neurons may indicate the linear, or nonlinear, trajectory of PNN development. Overall, we expect to observe a gradual increase in both PVB neurons and PNNs with age. Specifically, we expect PNNs to observe a similar but delayed developmental trajectory compared to that of PVB neurons. Moreover, we believe formation of PNNs will occur during late adolescence with their appearance marking the end of critical periods of plasticity. Mapping this development
in primates will provide a more accurate indication of PNN maturation in humans than information produced by previous rodent models.

The goal of this experiment has been to track the presence of PVB neurons and subsequent development of PNNs across postnatal developmental stages of the amygdala, a region known to be associated with many psychiatric disorders. To answer these questions, sectioned postmortem tissue of adolescent and adult primate brains, specifically rhesus monkeys, were stained using immunohistochemistry (IHC) for PVB neurons and PNNs. Tissues were then analyzed for the presence of PVB neurons and PNNs via quantitative light microscopy. The tissue provided was from one 3-week-old monkey, one 3 month-old monkey, and two adult (+3 years) monkeys, however, the experimenter (myself) was blind to their ages until all data had been collected and analyzed so as not to create any bias. Ultimately, we hope that this study will provide insight into the developmental trajectory of primate neuroplasticity thus furthering our understanding of the developmental pathology of psychiatric disorders as well as the behavioral differences between adolescents and adults.

Definition of Terms

“Adolescence”: A period of synaptic growth and synaptic pruning in response to a secondary growth spurt, or overproduction and physical growth of cells in early teenage years (10-13) (Casey et al., 2000 and Galvan, 2014).

“Altricial”: Refers to the underdeveloped state at birth of animals in which care and feeding by parents is necessary for survival. For humans, there is a prolonged state of infant altriciality post gestation.
“Amygdala”: A ganglion of the limbic system adjoining the temporal lobe of the brain and involved in emotions of fear and aggression; this is the fight, flight, or freeze section of the brain.

“Basolateral amygdala (BLA)”: The lateral, basal, and accessory-basal nuclei of the amygdala. Receives sensory information from the hippocampus and sends its output to the centromedial nucleus of the amygdala. Together with the nucleus accumbens the BLA stimulates an automatic fear response.

“Bipolar Disorder (BD)”: A manic-depressive illness that is characterized by severe mood shifts or a mix of depression and high-energy phases known as manic episodes. Severity and symptoms of this illness vary; for example, BD Type II involves milder episodes of hypomania coupled with severe depression.

“Brain derived Neurotrophic factor (BDNF)”: A neurotrophin growth factor protein responsible for providing support and ensuring the survival of existing neurons. As a neurotrophin, BDNF also plays a large role in neurogenesis and thusly neuroplasticity.

“Chondroitin Sulfate Proteoglycans (CSPGs)”: A proteoglycan that plays an active role in postnatal brain development as they guide axon growth. CSPGs also play a structural role and interact with the ECM. They have a contributing role in neurogenesis. CSPGs are essential to synaptic plasticity and key contributors in the composition of PNNs.

“Critical period”: The point of time in which PNNs have become fully developed and there is a switch in brain plasticity of the region surrounded by PNNS.
“Classical Conditioning (CC)”: First introduced by Ivan Pavlov and thus also known as Pavlovian conditioning is a process in which a response provoking stimulus (e.g. food) is paired with a neutral stimulus (e.g. a bell). The resulting neutral stimulus will then elicit a conditioned response on its own as the biologically provoking stimulus until/unless the behavior undergoes extinction.

“Conditioned Response (CR)”: A learned behavioral response through the pairing of a biologically provoking stimulus with a neutral stimulus.

“Corpus Callosum (CC)”: The region of the brain that is strongly associated with language and learning. The CC connects the right and left-brain hemispheres through a bundle of nerve fibers allowing for communication between the two hemispheres.

“Endogenous”: Within or internal to the referenced cell, organism, or tissue.

“Extracellular Matrix (ECM)”: A non-cellular, scaffold-like structure that provides physical and biochemical support to tissues. With a complex assembly of macromolecules, including CSPGs and PNNs, the ECM determines the tissue’s physical properties.

“Exogenous”: Outside of the cell, tissue, organism.

“Executive functions”: Cognitive functions necessary for cognitive control. These abilities rely on numerous cognitive processes including working memory and cognitive flexibility. Executive functions are necessary for reasoning and problem solving (Galavan 2013).
“Functional Magnetic Resonance Imaging (fMRI)”: While similar to MRIs, fMRIs use oxygen levels in the blood to monitor brain activity. This allows for real-time imaging of changes in the brain.

“Gamma-Aminobutyric Acid (GABA)”: An amino acid that is the main inhibitory neurotransmitter of the brain. Prevents neuronal excitability.

“GABAergic interneurons”: Main inhibitory neurons of the brain that release GABA in response to excitatory neurons.

“Immunohistochemistry”: The use of antigens (i.e. proteins) to tag a tissue with a primary antibody to the antigen. A secondary antibody is then typically used to promote fluorescence, dye, or otherwise make visible the desired protein expression of the tissue. Antibodies may also be conjugated to an enzyme to initiate a color-producing reaction.

“Limbic System (LS)”: Composed of the amygdala, hypothalamus, thalamus, hippocampus, basal ganglia, and cingulate gyrus. This collection of structures is often referred to as the emotional center of the brain. In addition to controlling the autonomic nervous system and regulating hunger, the LS is involved in fear, aggression, memory, arousal, and sensory information.

“Neural Circuits”: A network of neurons and synapses that produce specific responses.

“Neuroplasticity”: Neuroplasticity is the ability of the brain to change and adapt to new situations and changes within our environment. Such adaptations usually involve a process called neurogenesis: a birthing of new neurons or glial cells. Moreover, neuroplasticity involves forming new connections (synapses) or changes in existing connections (Kay 2012).
“Monocular occlusion”: Restriction of sight out of one eye and limiting it to the other specifically.

“Parvalbumin Neurons (PVB)”: Neurons containing parvalbumin that are thought to serve as precursors or markers to the development of PNNs.

“Perineuronal nets (PNNs)”: PNNs are made up of several components including chondroitin sulfate proteoglycans (CSPGs), hyaluronic acid, link proteins, and glycoprotein tenascin-R (Mauney et al., 2013). PNNs tightly surround synaptic connections on the somata, dendrites, and proximal axon segment of distinct populations of neurons. These extracellular matrix (ECM), mesh-like structures surround and protect certain structures of the brain. In addition to their architectural support, and their possible role as a buffer for cations in the ECM, PNNs are thought to facilitate synaptic plasticity (Mauney et al 2013). Specifically, PNNs are thought to protect PVB and pyramidal neurons from dysregulation and oxidative stress (Cabungcal et al., 2013). A lack of PNNs has been implicated in several psychiatric illnesses.

“Prefrontal Cortex (PFC)”: This area of the brain is highly associated with planning, executive function, personality, and perception of time. As one of the latest regions of the brain to develop (it does not fully mature in humans until approximately the age of 25) it is thought to be highly plastic. The PFC is involved in complex cognitive behaviors including reasoning and inhibition of specific thoughts and actions.

“Schizophrenia (SZ)”: A serious neurodevelopmental disorder affecting an individual’s emotions and often perception of reality. It may be characterized by withdrawal from reality and inappropriate behaviors. Patients with SZ are often
subject to delusions, hallucinations, and anhedonia. Although SZ has long been
associated with changes in neuroplasticity, the notion of neuroplasticity as the disease
pathology is still being explored.

“Transcription factors”: Proteins that involve the central dogma of biology (DNA to RNA
to Protein to Function). These factors are involved in the conversion of DNA into RNA.

“Visual Cortex”: The portion of the cerebral cortex that receives and processes
sensory nerve impulses from the eye. PNNs are plentiful here.

“Westieria Floribunda Agglutinin (WFA)”: a lectin devised from plants that
preferentially binds carbohydrate structures and may be used in the staining and
identification of perineuronal nets.

Evidence for Differential Brain Maturation

As the human brain develops, broad structural changes occur that
profoundly impact functional interactions of specific regions (Lippé et al., 2009). Until
recently, most of our understanding of brain maturation has been the result of numerous
rodent studies. Neural imaging of human adolescent brains has indicated neural
development proceeds from ventral to dorsal regions, with the most critical and basic
physiological functions developing in the most ventral regions first (Casey et al.,
2008). While this has been confirmed in rodent models, translation of any findings
directly from rodent to human models has been questioned as the extent to which
neural imaging may confirm such findings is limited (Casey et al., 2008).
Rodent studies have shown that brain maturation throughout adolescence shifts from deeper to more peripheral layers, especially within the frontal lobe (Andersen et al., 2000). Moreover, several rodent studies have confirmed dendritic pruning throughout puberty with continued growth of the prefrontal cortex into early adulthood (Cunningham et al., 2002, Teicher et al., 1995, and Zehr et al., 2006). While it is difficult to say whether results in rodents will be accurately reflected in humans, neural imaging of human brains has suggested similar developmental patterns (Casey et al., 2008 and Galvan, 2014). In fact, one particular study via fMRIs in teenage and adult humans indicated that higher executive functions are controlled by the more dorsal brain regions that continue to develop throughout adolescence until a person’s mid-twenties (Galvan, 2014 and Hall et al., 2014). Development of grey matter (brain material responsible for thinking and calculating) appears to peak within the 20s, at approximately the same time the PFC finishes its development (Lippé et al., 2009). These findings reflect the results of the aforementioned rodent studies; however, human studies have been limited as to what experimental procedures (if any) may be performed. Consequently, scientists have been left to draw conclusions primarily based on neural images and results from post mortem human tissues in conjunction with their findings in rodent models.

Due to obvious ethical constraints, human studies have been primarily limited to postmortem tissue or neuro-imaging studies. Studies focused on neuro-imaging, such as functional magnetic resonance imaging (fMRIs), are limited in what they may observe. While studies that rely on electroencephalogram recordings (EEGs) and fMRIs have provided much insight into activity of specific brain regions, their
resolution and ability to focus on specific neuron populations is limited. For example, fMRIs may track neuron population activity very quickly and thus may provide insight as to specific brain regions are responsible for specific activities. However, the Voxel Based Morphology (VBM) often utilized by fMRI studies does not have the level of resolution to identify specific cell structures and sometimes even specific brain regions thus impacting the overall statistical analysis of the experiment. Unfortunately, postmortem studies are also not without bias and lack control over environmental factors. Lifestyle information provided by the donors or their family members may not be entirely correct or account for the numerous environmental factors that undoubtedly have an effect on the individuals. By pursuing primate research many of these factors may be eliminated. Not only may we control for environmental factors, but we may also control for additional variables in their lives including the effects of antipsychotics and other potential treatments. Most importantly, the ability to explore these options in primate brains closely represent our own, enables scientists to better understand human neural anatomy and consequently forge more viable treatments. Thus, it is imperative to close the current informational gap between rodent and human studies through primate models.

The Brain in Adolescence

Certain global neuronal changes occur throughout adolescence. For example, a post mortem hippocampal study of individuals ranging from newborn to 76 years of age revealed a significant increase in myelin staining during the first two decades of life (Romer et al., 2010). An increase in myelination, i.e. the fatty tissue surrounding axons, allows for faster communication between neurons and brain areas (Benes 1994,
and Romer et al., 2010). Additionally, postmortem and neural imaging studies of human tissue have revealed an increase in volume and neuronal activity of the PFC, a region involved with high-order functioning and known to be implicated in psychiatric disorders, throughout the first two decades of life (Brody et al., 1987, Kelly et al., 2008 and Casey et al., 2000). A 2006 experiment conducted by Zehr et al., observed synaptic pruning of the medial amygdala occurred in male Syrian hamsters during puberty (Zehr et al., 2006). The authors suggest this is not only evidence of synaptic plasticity, but the coinciding mating behaviors are the result of these neuronal changes (Zehr et al., 2006).

A review of several neuronal imaging studies illustrates that similar to rodent studies, the human brain goes through a slight increase in volume during early adolescence to be followed by a period of synaptic pruning during late adolescence (Casey, 2008). Interestingly, nonlinear patterns of development have been shown in the maturation of adolescent cognitive abilities, specifically grey matter, through both experimental procedures in rodents and neural imaging and electroencephalogram (EEG) studies of humans (Casey et al., 2008). Moreover, white matter and deep grey matter structures do not follow a linear trajectory of maturation and growth throughout adolescence (Casey et al., 2008 and Galvan, 2014). Unfortunately, this may cause many teenagers difficulty with many high order executive functions (Galvan, 2013). For example, a recent comparison study of adults and a group of teens found that teens interpret and process facial expressions in an entirely different brain region than adults (Guyer et al., 2008 and Hall et al., 2014). Brain images provided via fMRIs study revealed adults used the PFC to correctly identify facial micro expressions, whereas
teens processed these expressions in the amygdala, leading them to misinterpret several emotions (Hall et al., 2014). Interestingly, adults correctly identified one of the expressions as fear, while teenagers thought the faces showed anger or shock (Guyer et al., 2008 and Hall et al., 2014). These changes in electrical activity of the brain from adolescence to adulthood suggest that the formation and migration of neurons confirmed in rodent models serve as a good indicator of brain maturation for humans (Inta et al., 2015).

As stated previously, the amygdala plays a crucial role in neurodevelopmental and psychiatric disorders. A postmortem autism study of children found individuals with autism spectrum disorder (ASD) observed early and rapid growth of the amygdala resulting in a larger amygdala volume as compared to controls without ASD (Weir et al., 2007). While the study showed the overall developmental trajectory of the amygdala for both controls and subjects with ASD to be similar, the spine density in individuals with ASD was larger than that of their age-matched controls (Weir et al., 2018). An increase in spine density signifies a higher level of neuronal plasticity and may be an underlying pathology to ASD. This is unsurprising given psychiatric disorders associated with the amygdala, such as SZ and Post Traumatic Stress Disorder (PTSD), have long been implicated with issues of abnormal neuronal density and neuroplasticity (Beretta et al., 2007 and Gilbertson et al., 2002). Similarly, another ASD study found an increase in neurons of the basal and accessory basal subnuclei of the amygdala from adolescence to adulthood in individuals with normal neuronal development (Avino et al., 2018). However, they also concluded individuals with ASD had a rapid increase in amygdala volume to be followed by a decrease in adulthood.
atypical of controls without ASD (Avino et al., 2018). Evidence of these developmental volumetric difference as a possible pathology to ASD is just one example of how the postnatal developmental trajectory of the amygdala plays a large role in neurodevelopmental disorders.

A major developmental benefit unique to humans is the late onset of puberty in comparison to our animal counterparts. During puberty, the brain regions mature and undergo many developmental changes. This delayed human adolescence results in an increase of brain plasticity, or flexibility, for a longer period of time (Martin, 2013). Adolescence is a period of synaptic growth and synaptic pruning in response to a secondary growth spurt, or overproduction and physical growth of neuronal cells in early teenage years (10-13) (Casey et al., 2000 and Galvan, 2014). Synaptic pruning is extremely important as it allows for organization of neural pathways; nevertheless, it has caveat of ‘use it or lose it’ (i.e. foster the growth of certain synapses through experience or potentially lose them forever) (Galvan, 2013). PNNs are structures highly implicated in such neuroplasticity and play a key role in synaptic regulation. Formation of PNNs restricts plasticity and confers the adult or mature form of plasticity. Disruption of normal PNN development may impact excitatory synaptic activation resulting in a perpetual juvenile state of synaptic regulation (Gogolla et al., 2009 and Pizzorusso et al., 2002). In fact, one particular study shows decreases of PNNs in the amygdala and entorhinal cortex of subjects with SZ, indicating potentially higher levels of plasticity and neuronal excitability in these patients (Pantazopoulos et al., 2010). Malformation of PNNs during adolescent development has been recently suggested as a potential pathology of certain psychiatric disorders (Mauney et al., 2013,
Pantazopoulos et al., 2016, and Slaker et al., 2016), thus, characterizing the development of PVB neurons and PNNs in the primate brain is critical for understanding when and how certain disorders develop.

PVB neurons: Maturation in the Rodent Brain and their role in Neuroplasticity

Despite being a relatively new area of study, several discoveries have been made in rodent models regarding the role of PVB neurons. Rodent studies indicate PVB neurons appear late in neonatal development and continue to increase in neuronal density with age (Rio et al., 1994). A developmental parvalbumin-immunoreactive study in mice revealed area specific differences in PVB emergence and maturation (Rio et al., 1994). Furthermore, the authors did not observe expected usual patterns of neocortical neurogenesis but rather first saw the emergence of PVB in layer V of rodent brain which then proceeded to expand to upper and inner cortical levels (Rio et al., 1994). Rodent research shows that with age, PVB neurons become increasingly surrounded by PNNs resulting in a decrease in neuroplasticity thereby indicating the critical period of plasticity (Sugiyama et al., 2008). Following the development of PVB neurons may provide insight as to which brain regions retain their plasticity the longest. Thus, a better understanding of the developmental trajectory of PVB neurons may lead to better understanding of the development of neuroplasticity as a whole.

A 2008 study by Sugiyama et al., really helps emphasize the importance of PVB neurons and their role in plasticity. Authors focused on the embryonic Otx2 homeoprotein and found its expression regulated maturation of PVB neurons (Sugiyama et al., 2008). More specifically, they looked at visual pathways and found the loss or
gain of Otx2 function directly impacted expression of PVB neurons and consequently ocular plasticity. In Otx2 loss-of-function mice, Sugiyama et al., observed hindered PVB expression and consequently ocular dominance plasticity (Sugiyama et al., 2008). Conversely, they found exogenous Otx2 to coordinate PVB neuron maturation and activate visual cortical plasticity (Sugiyama et al., 2008). Moreover, they found Otx2 accumulation can induce PNN formation (Sugiyama et al., 2008). Therefore, Sugiyama et al., concluded the manipulation of Otx2 expression accelerated development of PVB neurons thus leading to onset of CP in plasticity (Sugiyama et al., 2008).

Perineuronal Nets: Differential Maturation in the Rodent Brain and Regulation of Neuroplasticity

PNNs are thought to protect PVB and pyramidal neurons from dysregulation and oxidative stress (Cabungcal et al., 2013). In addition to their architectural support, and their possible role as a buffer for cations in the ECM, PNNs are thought to play a vital role in facilitating synaptic plasticity (Pantazopolous et al., 2010). Moreover, the ECM (and thusly PNNs) affect the diffusion of glutamate receptors as well as receptor clustering within a synapse, both crucial processes of synaptic plasticity and regulation (Sorg et al., 2016). Via restriction of diffusion, PNNs allow for synaptic desensitization during periods of high frequency firing (Pantazopolous et al., 2016). A lack of PNNs has been associated with an increased excitatory state of neurons, and thus thought to contribute to the pathology of several neuroplastic psychiatric illnesses.

While neuroplasticity has become a promising focus for many researchers, there is a surprising dearth of information on what drives its mechanism. Thus far, it is
understood that neuroplasticity in the adult brain is usually confined to specific regions of the brain. However, the mechanism behind remodeling of brain structures and functions needs further exploration. Recent research into the development of PNNs indicates they are largely implicated in the role of neuroplasticity. PNNs are made up of several components including chondroitin sulfate proteoglycans (CSPGs), hyaluronic acid, link proteins, and glycoprotein tenasin-R (Beretta et al., 2012 and Pantazopoulous et al., 2015). These extracellular matrices (ECMs), mesh-like structures, surround and protect certain structures of the brain. Rodent research shows that the organization of CSPGs into PNNs coincides with the developmental switch in fear memory resilience (Gogolla et al., 2009). In other words, PNNs have been shown to protect fear memories from erasure in rodent models.

Previous research has shown the dependence of fear extinction in both juvenile and adult animal models relies on the amygdala (Gogolla et al., 2009). The amygdala appears to undergo maturation and thus changes in plasticity during adolescence in both human and animal models (Galvan, 2013 and Hall et al., 2014). Gogolla et al. proposed that PNNs may underlie the developmental switch to fear extinction (Gogolla et al., 2009). To explore the mechanism behind this plasticity, Gogolla et al. conditioned mice to a fear stimulus (FS). Pairing of a conditional stimulus (CS) with an unconditioned stimulus (US) has proven to cause robust fear memories in rodents (Gogolla et al., 2009). Rodent models show fear conditioning to be more robust in adults if fear extinction does not take place prior to the end the adolescent period (Gogolla et al., 2009). Moreover, the process of fear extinction is not as permanent as fear conditioning; consequently, fear responses may spontaneously recover upon stress.
re-exposure (Gogolla et al., 2009). Injection of the CSPG degrading enzyme chondroitinase ABC (ChABC) into the basolateral amygdala caused the degradation of PNNs; chABC injected mice exhibited a lack of spontaneous fear recovery (Gogolla et al., 2009). Overall, the study found that the absence of perineuronal nets allows for extinction training of the conditioned fear behavior. As a result, Gogolla et al., demonstrate the clear and integral role of PNNs on fear erasure in the amygdala.

Rodent studies have simultaneously suggested the developmental importance of PNNs and their powerful role in neuroplasticity. One particularly successful study in this field has been on the manipulation of the visual cortex in rodents. Synaptic formation in the visual cortex strengthens in direct response to the environment in early postnatal life, during which time ocular-dominance is established. Additionally, the number of PNNs located in the visual cortex appears to grow in direct correlation with its period of synaptic plasticity (Mauney et al., 2013). In a study by Pizzorusso et al., rats were made functionally blind in one eye through monocular deprivation, thus establishing full ocular-dominance in the untouched eye (Pizzorusso et al., 2002 and Sugiyama et al., 2008). After the end of CP of the visual cortex in juvenile rats, Pizzorusso et al., were able to manipulate PNNs via degradation of CSPGs with chondroitinase-ABC (Pizzorusso et al., 2002). In doing so, they were able to return sight to the functionally blind eye (Pizzorusso et al., 2002).

A similar study of neuroplasticity in the visual cortex of mice conducted by Sugiyama et al. in 2008 illustrates the impact of PVB neurons on PNNs and their role in postnatal plasticity (Sugiyama et al., 2008). The authors found that even post maturation of the visual cortex, PVB neurons could still be manipulated via Otx2
homeoprotein expression (Sugiyama et al., 2008). As previously stated, manipulation of Otx2 affects the maturation of PVB neurons, thus impacting the onset of their critical period of plasticity, and subsequently the formation of PNNs (Sugiyama et al., 2008). Through manipulation of Otx2, the researchers were able manipulate ocular dominance and restore vision to functionally blind eyes (Sugiyama et al., 2008). Sugiyama et al., concluded that “experience-dependent transfer of a homeoprotein may establish the physiological milieu for postnatal plasticity of a neural circuit,” (Sugiyama et al., 2008). These studies suggest the maturation of the ECM is responsible for a reduction in neuroplasticity. Moreover, they illustrate an increase of PNNs throughout the visual cortex towards the end of the CP (Pizzorusso et al., 2002 and Sugiyama et al., 2008). Consequently, these studies strongly suggest the key to understanding neuroplasticity is through the understanding of PNN development and maturation.

PNNs have been highly implicated in the role of neural plasticity in rodent models. Little research has been done to explore developmental neuroplasticity in primates and the examination of PNNs in human models has been primarily limited to post mortem tissue. Several neural imaging studies of adolescents have indicated that patterns of neurogenesis and grey matter growth follow a developmental trajectory similar to that of rodents reviewed above. As seen below, Figure 1 depicts volume growth curves taken of different brain regions from children at two-year intervals over the course of 18 years (Giedd et al., 1999). From their data, the researchers concluded grey matter increased before adolescence, peaking at different ages in different regions followed by a decrease in volume across all regions (Giedd et al., 1999). Another longitudinal study followed 13 subjects from ages 4 to 21 and conducted MRIs at two-
year intervals. As illustrated in Figure 2 below, the authors found significant early brain growth in the prefrontal, temporal, and occipital lobes followed by a decrease in total grey matter volume (Gogtay et al., 2004). The graph illustrated in Figure 3 shows a closer look at amygdala neuronal volume for individuals with ASD and controls (TD) without (Avino et al., 2018). As can be seen, an overall increase in neuronal density occurs with age in the controls (Avino et al., 2018). Moreover, Figure 4 shows an overall increase in dendritic spines, thus indicating an increase in neuronal plasticity for TD subjects (Avino et al., 2018). However, characterization for the development of PVB neurons and PNNs in the amygdala has not reported in primates or humans before our study.

Figure #1: Overall Volumetric Changes in Brain Matter (from Giedd et al., 1999)

Figure #2: MRI of Changes in Grey Matter Volume with Age (from Gogtay et al., 2004)

A recent review of normal brain development by Marsh et al., discusses the issues associated with neural imaging studies and their restrictions (Marsh et al., 2008). Unlike rodent studies, certain developmental patterns have been suggested, but the ability to track the development of specific neurons or test these theories is extremely limited. As a result, there is a current need to close the informational gap between neuroplastic development in rodents and humans. Primate research offers the ability to control for environmental factors, track for specific neurons, and test specific theories in brains that more closely resemble our own. Thus, the need for further developmental neuroplastic studies in primates is demonstrated.

Postnatal Neural Development Associated with Psychiatric Disorders

Neuroplasticity is the ability of the brain to change and adapt regarding new situations and changes within our environment. Such adaptations usually involve a process called neurogenesis: a birthing of new neurons or glial cells. Moreover, neuroplasticity involves forming new connections (synapses) or changes in existing connections. An abundance of research illustrates that the highest levels of neuroplasticity occurs during adolescence. However, our perception of the adult brain as being ‘largely fixed’ and overall brain plasticity has changed dramatically over the last few decades (Kays et al., 2012). For example, a study by the University of McGill observed potential adult taxi drivers of London. In procuring a taxi license, individuals must memorize an intricate road map of London. To observe the effects of neural plasticity, researchers used structural MRIs to observe any changes in the posterior hippocampus, the area responsible for spatial representation of one’s environment.
(Maguire et al., 2000). In comparison to their controls, structural MRIs of the taxi drivers showed significantly larger posterior hippocampi, with volume being a direct correlation of time in the field (Maguire et al., 2000). Not only does this study aptly reflect the ability of our brains to remodel itself in response to adaptations in the environment, but it shows that significant rewiring may take place post adolescence.

The role of neuroplasticity both as a cause and possible treatment for many conditions in the field of psychiatry is a very promising field of study. Abnormalities in neuroplasticity have been observed in numerous mental health issues spanning from slight learning disabilities to severe psychosis. Many psychiatric illnesses present with physical differences within the brain. Until recently, many of these physical differences were associated with each condition more as an acquired side-effect rather than a genetic predisposition or developmental disruption of the brain (Mauney et al., 2013). For example, anxiety and depression have long been associated with the ‘side-effect’ of a decrease in overall brain volume. However, new evidence suggests that the volumetric changes in grey-matter in depression results from a decrease in synapses and dendritic regression (Kays et al., 2012). In fact, a recent study on veterans with ongoing PTSD illustrates significantly smaller hippocampal volumes than of those who recovered and veterans who never developed PTSD (Gilbertson et al., 2002). This suggests neuroplasticity has both a role in recovery as well as a possible indicator of those more likely to develop this mental illness. Moreover, a recent study on identical twins indicated that a smaller hippocampal volume increased one’s risk of developing PTSD, not that smaller hippocampal volume was an acquired trait of the illness (Kremen et al., 2012).
Neuroplasticity and Schizophrenia

Synaptic plasticity plays a key role in schizophrenia (SZ), a severe neuro-developmental disorder. In rodents, PNNs in key areas associated with SZ develop during late adolescence, coinciding with the average age of onset for SZ. In SZ, PNN density of the amygdala and the pre-frontal cortex (PFC) were significantly reduced as much as 10-fold and 76% respectively (Mauney et al., 2013). Furthermore, the study showed PNN development in the human PFC increases until early adulthood, a common time in which individuals with SZ become symptomatic (Mauney et al., 2013). Considering SZ as a disorder of neuroplasticity, these findings suggest that the dysfunction of PNNs (and thusly neural circuitry) during development is largely a contributing factor of this psychiatric disorder (Pantazopolous et al., 2016).

Interestingly, the deficit of PNNs seen in SZ subjects appears to be region specific (Pantazopolous et al., 2016). Thus, the implication of PNNs in the pathology of SZ is apparent, but their region-specific pathophysiology is not (Mauney et al., 2013).

In a 2010 study conducted by Pantazopoulos et al., subjects with SZ had decreased numbers of PNNs in the amygdala, lateral nucleus, and basal accessory nucleus compared to that of the normal human amygdala (Pantazopolous et al., 2010). Interestingly, the lack of PNNs in the lateral nucleus suggests that GABAergic projection neurons are also affected by PNN malformation (Pantazopolous et al., 2016).

In fact, the study found that subjects with PNN and ECM abnormalities observed abnormal regulation of glutamatergic and GABAergic inputs on GABAergic neurons thus impacting information processing. Their findings suggest the pathology of SZ may be in part due to PNN disruption and unregulated lateral membrane diffusion.
Further support for this hypothesis stems from a rodent model, in which PNNs were observed protecting PVB neurons from the excitotoxic effects of oxidative stress common in subjects with SZ (Pantazopoulos et al., 2016). In this study, PNNs were enzymatically digested in the hippocampus that resulted in severe functional abnormalities such as an increase in activity of dopamine neurons in the ventral tegmental area (Pantazopoulos et al., 2016). Consequently, the question of PNN development and trajectory throughout the brain comes into question. A better understanding of PNN development through early adolescence to adulthood would provide further insight to the function of neuroplasticity in the pathology of SZ.

Neuroplasticity and Addiction

PNN expression is high in many of the areas implicated in addiction and consequently plays a role in the plasticity of reward-related circuitry. While neuroplasticity may allow the brain to heal damaged areas via neurogenesis and the formation of new synaptic pathways, it may also reinforce bad habits that lead to addiction (Mallin, 2010). Therefore, one may begin to understand addiction as a neural plastic event: as the brain creates a path in support of any habit, the engagement of said habit strengthens that pathway over time. Repeated substance abuse may stimulate dopamine transmission, thus producing long lasting changes in brain circuitry and behavioral responses (Mallin, 2010). As a result, the user typically experiences a feeling of euphoria, strengthening the reward circuitry leading to addiction. Teenagers are even more likely to become addicted to substances than adults as their brains are more attuned to risk-reward circuitry. While the PFC of teenagers is still developing,
their nucleus accumbens (the pleasure and reward zone) matures during early adolescence (Galván, 2014). Neural imaging studies show that when presented with a large reward, adolescent brains light up much more than either children or adults; however, if the reward was small, teen brains barely fired at all (Galván, 2014). In fact, a dramatic increase of brain sensitivity to rewards was seen from children to teenagers, but a sharp decrease was observed from teens to adults. Moreover, fMRIs of teenage against adult brains illustrated that while the striatum was excited by the reward for all subjects, the teenage brain was much more excitable overall (Galván, 2014).

Neural images of substance abusers illustrate the differences in brain activity of the amygdala, prefrontal cortex, and basal-ganglia from their sober counterparts (Kalivas and Volkow, 2011). Unsurprisingly, as a condition impacted by neuroplasticity, these are the same areas affected in SZ, PTSD, depression, and anxiety. One recent study of neural imaging in drug addicts indicates neuroplastic changes towards the ventromedial PFC versus the dorsomedial PFC; these areas have been associated with impulsivity/impaired judgment and internal awareness/ “error detection” respectively (Kalivas et al., 2011). Over time, the drug abuse strengthens the ventromedial pathways resulting in their long-term potentiation (LTP) (Kalivas et al., 2011). Further implication of PNNs and neural plasticity in drug addiction may be seen in a rodent study by Xue et al. in which PNNs were removed prior to extinction training of mice that were addicted to morphine or cocaine (Slaker et al., 2016). Results indicated enhanced extinction training, less spontaneous behavior recovery, and a decrease in condition placed preference (CPP) behavior (Xue et al., 2014). Additionally, removal of PNNs without extinction training yielded similar results, but
yielded greater results when both methods were paired (Xue et al., 2014). Despite the limited research of PNN manipulation as a possible treatment for addiction, the results yielded thus far have been overwhelmingly positive.
Chapter II

Materials and Methods

The purpose of this study has been to better understand both the maturation and developmental trajectory of PNNs and timing of their CP through a closer examination of the amygdala. To study the formation of PNNs we used tissue from primates, specifically rhesus monkeys, procured from varying stages of development. An established cohort of 4 Rhesus Macaques was provided by the lab of Dr. Suzanne Haber at Mclean. Monkeys were raised at Emory University under natural conditions with no behavior training or experimental procedures conducted upon them. Two weeks prior to sacrifice, the monkeys were injected with neural tracers that did not apply to our experiment. One monkey was sacrificed at 3 weeks, another at 3 months, and two at adulthood. The brains were then perfused, sliced, and preserved in cryoprotectant at the facility. Each primate’s age was logged but was kept blind until quantitative microscopy was complete. This was done so as to not create any biases while the tissue was under investigation. The specifics of each monkey case are illustrated in the table below.

Table #1: Cohort Specifics

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Left Hemisphere</th>
<th>Right Hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR 248</td>
<td>M</td>
<td>3 weeks</td>
<td>PVB-IR</td>
<td>WFA-IR</td>
</tr>
<tr>
<td>MR 223</td>
<td>M</td>
<td>3 months</td>
<td>PVB-IR</td>
<td>WFA-IR</td>
</tr>
<tr>
<td>MR 192</td>
<td>M</td>
<td>+3 years</td>
<td>PVB-IR</td>
<td>WFA-IR</td>
</tr>
<tr>
<td>MR 181</td>
<td>M</td>
<td>+3 years</td>
<td>PVB-IR</td>
<td>WFA-IR</td>
</tr>
</tbody>
</table>
Tissue Processing

Primate brains were sliced thinly (40 μm) and sectioned by left and right hemisphere. A small amount of test tissue was run through immunohistochemistry (IHC) to optimize the stain for PVB IR neurons and WFA-IR PNNs for the left and right hemispheres respectively. A small amount of test tissue was utilized to optimize the immunohistochemistry (IHC) protocol for PVB and PNN visualization in primate tissue. While it was suspected the IHC protocol used for human postmortem studies in the Berretta lab would be sufficient, the test segment was run to avoid any possible degradation of the tissue. It was found that use of both antigen unmasking buffers (a lab specific alternative buffer and Citric acid buffer) used by the lab protocol caused too much tissue degradation. As a result, sample tissue was run using each antigen unmasking buffer separately; it was determined the alternative antigen unmasking buffer yielded the best results with optimal staining and minimal tissue damage. The chosen test tissue methods may be viewed in Figure 5 below:

Figure #5: Test Tissue Staining
Immunohistochemistry

Tissue blocks for histochemistry/immunocytochemistry were processed as previously described (Pantazopoulos et al 2008 and Berretta et al., 2007). All sections (40 μm) within a compartment per subject were selected for each marker (i.e. PVB, CSPGs), thus respecting the equal opportunity rule (Coggeshall et al., 1996 and Gundersen et al., 1999). Histochemical labeling for CSPGs was obtained using biotinylated Wisteria floribunda agglutinin (WFA) as described previously (Pantazopoulos et al., 2008b). Wisteria floribunda agglutinin selectively binds to N-acetyl-galactosamine, (Nakagawa et al., 1986, Brückner et al., 1993, Härtig et al., 1992, and Celio et al., 1998) a molecule specifically represented in the glycosaminoglycan chains characteristic of CSPGs (Viapiano et al., 2006). Immunocytochemical detection of PVB was performed as previously described (Pantazopoulos et al., 2008b and 2008a) using monoclonal anti-PVB antibody (48 hours at 4°C, 1:10 000, P3088; Sigma-Aldrich). All sections were cover-slipped and coded for quantitative analysis blinded to diagnosis (Pantazopoulos et al., 2010). Sections from all brains included in the study were processed simultaneously to avoid procedural differences. Omission of streptavidin or bio-tinylated WFA, or replacement of biotinylated WFA with an unconjugated form (Vector Laboratories, Burlingame, California), or omission of the primary (PVB) or secondary antibodies did not result in detectable signal (Pantazopoulos et al., 2010). Following the antigen unmasking step we incubated the tissue in 2% BSA (bovine serum albumin) for 1 hour at room temperature (37 degrees Celsius) for exogenous enzyme block. We then incubated the sections overnight in primary antibody diluted with 2% BSA [1:8k PVB (made in mouse, sigma), 1: k WFA (vector)]. The following day we
incubated the PVB tissue in a secondary raised against mouse for 2 hours at room temperature, followed by an additional 2-hour incubation in streptavidin. The WFA labeled tissue was only incubated in streptavidin HRP-horseradish peroxidase. Diaminobenzadine Nickel Sulfate was used to visualize WFA and PVB specific staining.

Quantitative Microscopy

Tissues were analyzed for the presence of PVB-immunoreactive (IR) neurons and WFA-IR PNNs via quantitative light microscopy. To complete this task, computer assisted microscopes (Stereo Investigator v 10.54) were utilized in recognition and counting of PVB-IR neurons and WFA-IR PNNs in a blind fashion on entire half hemispheres of postmortem primate brain tissue. The number of PVB-IR neurons and WFA-IR PNNs were sampled for each area of tissue and their approximate regions were determined. After data collection, developmental patterns of PBV-IR neurons and WFA-IR PNNs were analyzed from ages ranging from 3 weeks to adulthood.

Research Limitations

Primate tissue is very difficult to procure, and as is typical for primate studies the number of subjects for each age group is low. Low sample sizes impact the overall statistical significance of any potential findings; however, anything significant noted in this experiment would be grounds to continue more research of postnatal maturation of the primate brain. While this is not uncommon in primate studies, and widely accepted, further research is needed to solidify any findings of this study. Furthermore, as this has been a postmortem study, with no abnormalities in the primates’ rearing, and so any findings were strictly due to correlation and not causation. Nevertheless, there have been
very few studies to bridge the gap between rodent and human models. A better understanding of postnatal brain maturation in primates, particularly throughout adolescence, would help to further translate these findings.
Chapter III

Results

Tissue Analysis

Microscopic analysis for this experiment was based on the observation of PVB neurons and PNNs throughout the amygdala. Each section of amygdaloid tissue was broken down into subnuclei. A qualification basis for PVB neurons and PNNs was established prior to counting each section of tissue. Any cell counted was done so without any speculation; those that were uncertain were left uncounted. This process was applied across both tissues. Tissues were counted across Z planes. Counts, as well as their corresponding areas, were recorded for each subnuclei for later data analysis.

Below, Figures 6 and 7 show examples of what was considered countable for both PVB neurons and PNNs respectively.

Figure #6: PVB neurons at 20x

Figure #7: PNNs at 20x
PVB and PNN Densities

PVB and PNN counts for each individual case were totaled and then divided by their total areas to yield overall densities. Data was collected in Excel and a statistical analysis was facilitated through Jmp software. As illustrated in Figure 8, there was an increase in overall PVB density with age, indicating a positive trend. Adult densities for both PVB and PNN densities were calculated using standard error to determine how close the sample mean between the two cases is to the true mean of the population. Density of PNNs observed in Figure 9 shows an increase in density from 3 weeks to 3 months. This is however followed by a large decrease in PNN density between 3 month and adult monkeys (Figure 9).

Figure #8: Total Amygdala PVB Density vs. Age
Oneway ANOVA Analysis

One-way ANOVAs were run between age groups to test for significance of our findings. An analysis of differences in overall PVB density between 3 weeks and adults yielded a strong positive correlation with an R-square value of 0.95 (Table 2). An observed p-value of 0.1385 with an $\alpha = 0.05$ is statistically insignificant and thus we fail to reject the null hypothesis. Results for overall PVB density comparison between 3 months and adults yielded an r-square of 0.46, which suggests a medium positive correlation, and a p-value of 0.5252 (as seen in Table 2). Again, this is insufficient evidence to reject the null hypothesis. A statistical analysis was run for PVB density differences between 3 months and 3 weeks but due to such a small sample size no hard statistics can be provided (Table 4). However, there does appear to be a positive trend and an increase of PNN density with age.
Table #2: ANOVA of total Amygdala PVB Density in 3-weeks vs. Adults

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
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<td>1.463e+11</td>
<td>1.463e+11</td>
<td>20.4723</td>
<td>0.1385</td>
</tr>
<tr>
<td>Error</td>
<td>1</td>
<td>7.1458e+2295</td>
<td>7.1458e+9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>2</td>
<td>1.5344e+11</td>
<td></td>
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</table>

Means for One-way ANOVA

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<tr>
<th>Level</th>
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<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-weeks</td>
<td>1</td>
<td>11.293</td>
<td>8.4523</td>
<td>-10.62800</td>
<td>10.983385</td>
</tr>
<tr>
<td>Adult</td>
<td>2</td>
<td>4797.34</td>
<td>5977.4</td>
<td>-2.97644</td>
<td>12.30317</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance.

Table #3: ANOVA of total Amygdala PVB Density in 3-months vs. Adults

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
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<td>6.0986e+9</td>
<td>0.8535</td>
<td>0.5252</td>
</tr>
<tr>
<td>Error</td>
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<td>7.1458e+2295</td>
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<td></td>
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</tr>
<tr>
<td>C. Total</td>
<td>2</td>
<td>1.5344e+10</td>
<td></td>
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</table>

Means for One-way ANOVA

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tr>
<td>3-months</td>
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<td>1458181</td>
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<tr>
<td>Adult</td>
<td>2</td>
<td>4797.34</td>
<td>5977.4</td>
<td>-2.97644</td>
<td>12.30317</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance.
An overall comparison of differences in PNN density was then run between ages for statistical significance. A One-way Analysis of total PNN density between 3 weeks and adulthood observed a low r-squared value (0.23) indicating a weak correlation (Table 5). Table 5 also indicates a p-value of 0.68; given our 95% confidence interval this evidence is not statistically significant enough to reject our null hypothesis. Upon further analysis of differences in overall PNN density between 3 months and adulthood we discovered a strongly negative correlation with an r-squared value of 0.98 (Table 6). However, the p-value of 0.09 for this graph indicates no statistical significance and suggests we reject our null hypothesis. Again, no standard statistical analysis could be conducted on PNN densities between 3 weeks and 3 months due to the small sample size; however, there does appear to be a positive correlation between PNN density and age (Table 7).
Table #5: ANOVA of total Amygdala PNN Density in 3-weeks vs. Adults

Table #6: ANOVA of total Amygdala PNN Density in 3-months vs. Adults
Table #7: ANOVA of Total Amygdala PVB Density in 3-weeks vs. 3-months

### Analysis of Subnuclei

A closer look at PVB and PNN densities between subnuclei regions yielded the following results. Despite a positive correlation between age and total PVB density, a closer examination of PVB density by nuclei exhibits some different trends. PVB density of adults observed a notable decrease in the Medial (ME), Lateral (LN), Central (CE), and Basal (BN) subnuclei (Figure 10a, b, d, and e). This appears to mimic the trend of overall PNN density (Figure 9). However, it should be noted that Figures 8c and 8e have a large standard error. Figure 10f exhibits a consistent increase in PVB density with age, however, the standard error for adults is large leaving in question the precision of the true mean.
Analysis of PNN density in amygdala subnuclear regions reveals patterns similar to that of Figure 9 above. Figure 11(a-f) examines potential patterns or trends of PNN density between ages within the amygdala subnuclei of our cohort. Medial and Accessory Basal (AB) subnuclei appear to show an overall increase of PNN density with age (Figure 11a and 11f respectively). However, the standard error for adults of the AB region is large and may impact this trend. Closer examination of the other subnuclear regions reveals an increase of PNN density between 3 weeks and 3 months. This trend does not continue but instead we witness a decrease of PNN density from 3 months to adulthood as observed in Figure 11a-d, and e.
Figure #11 (a-f): Amygdala Subnuclei PNN Densities
Chapter IV

Discussion

To date, many of the studies on neuronal plasticity have been limited to rodent models or post-mortem human tissue. As a result, little research has been done to solidify findings of rodent models and make their results more applicable to the human brain. To address this issue, and better understand the development of neuronal plasticity and its far-reaching implications, our study focused on a small cohort of post-mortem primate tissue. Despite our small sample size, our findings indicate some interesting concepts of neuroplastic development. Based off previous rodent research, we had expected to see an increase in both PVB and PNN density with age. Our observed increase in PVB densities with age was consistent with previous rodent models and our aforementioned hypothesis. However, there was an unexpected observed decrease in PNN density between 3 months and adulthood. Interestingly, this decrease in PNN density between the 3-month mark and adulthood was observed in both adult cases leading us to believe this was not a tissue processing or statistical error. Our decrease in adult PNN density may illuminate findings such as the overall decrease of gray matter mentioned in the human neural imaging studies mentioned above (Giedd et al., 1999 and Gogtay et al., 2004). We believe that this unexpected result may potentially be the result of synaptic pruning sometime during late adolescence. Further primate research of a larger cohort of multiple ages is needed to conclude these findings.

Our data indicates previously observed trends of PVB neurons increasing with age in rodent models are consistent with that of primates, as may be seen by the bar graph
of overall PVB densities in Figure 5 (Pantazopoulos et al., 2006). This apparent trend was tested for statistical significance between age groups and no abnormalities were found (Figure 8a-c). Thus, a strongly positive trend was established indicating that PVB neurons do increase with age. Interestingly, a closer look at PVB densities in the subnuclei of the amygdala revealed some regions appeared to decrease from 3 months to adulthood. These individual trends appear similar to that of overall PNN densities (Figure 7). However, this could be perhaps due to an abnormality in our case subjects or the small sample size. Additionally, the standard error for adult groups was often quite large leaving room for both interpretation and further investigation. Nonetheless, our PVB data as a whole supported our hypothesis that PVB neurons would increase with age thus confirming previous findings of rodent models.

Intriguingly, our data for PNNs did not quite fit our hypothesis that PNNs would increase with age. As can be seen in Figure 7, there is a dramatic decrease in PNN density between 3 months and adulthood. While our p-value of the one-way ANOVA between 3 months and adults revealed no statistical significance, an r-square value of 0.98 indicated a strongly negative trend. The large p-value is likely due to our small cohort, which is to be expected in primate studies given the difficulty in obtaining primate tissue.

A closer examination of PNN densities of subnuclei displayed in Figure 9 a-f illustrates consistent decreases in PNN densities from 3 months to 3 years. The most notable decreases may be seen in the LN, BN, and CO of the amygdala (Figures 9b, c, and e respectively). Conversely, Figures 9a and 9f illustrate the PNN density of the ME and AB increase with age as expected. Perhaps this is due to a developmental trajectory that
would be better observed over a larger cohort with an increased age range. While we cannot state any statistical significance behind these trends, mostly due to the small size of our cohort, our evidence strongly suggests further study in the area of primate neuroplasticity development is needed.

Considering the significant decrease in PNN density from 3 months to adulthood is clear in both adult cases, we are inclined to believe this is not an error in tissue processing or a statistical abnormality. Interestingly, the observed decrease of PNNs (and thus neuronal plasticity) occurs during a period between adolescence and adulthood of the rhesus monkeys we have studied. As previously stated, both human and rodent research conclude there is a period of synaptic growth and synaptic pruning during adolescence (Casey et al., 2000 and Galvan, 2014). Again, due to our small cohort we are unable to conclusively state any statistical significance of this observed trend in PNN density (Figure 7). However, PNNs have been recognized as a key factor in synaptic regulation and they ultimately confer the mature form neuroplasticity (Pantazopoulos et al., 2006). Our large decrease in PNN density between 3 months and adulthood suggests not only synaptic pruning but serves as an indicator as to when the critical period of plasticity may occur.

Implications and Further Direction

Our unexpected decrease in PNN density between adolescence and adulthood opens up many new research possibilities for developmental neuroplasticity. Given that our predictions of PVB and PNN densities were based off previously conducted rodent studies, it is clear that something must be done to bridge the gap between rodent and
human models. Primate research is the next logical step in exploring postnatal neuronal development. Unfortunately, due the small cohort of our study it is difficult to conclusively state any findings. Nonetheless, this is normal and was to be expected as early primate studies are usually limited due to ethical practices and the difficulty of procuring primate tissue. Moreover, it is possible some of our tissue may have been lost or damaged during tissue processing as there was not an equal amount of amygdaloid tissue between cases. In an attempt to combat this, densities were calculated for each case as a whole and at a subnuclear level. Furthermore, the staining of one adult tissue did not take and thus was unable to be counted. One slide was broken in tissue during processing and microscopic quantification but was still able to be counted.

Regardless of these issues, future research focusing on the developmental trajectory of PNNs in primates should include a larger cohort and specific range of ages. A larger range of ages would potentially allow future researchers to pinpoint at what point during adolescence synaptic pruning begins but also provide insight as to when the critical period of neuronal plasticity occurs. Another possible approach for future researchers would be to compare the developmental densities of PNNs of primates with neuronal developmental abnormalities to primate models of psychiatric disorders (such as those used in research of SZ) (Mao et al., 2015).

Conclusions

Despite our prediction that PNN density would increase with age, the discovery of their sharp decline during adolescence indicates possible synaptic pruning. If these findings could be replicated, they would provide a better understanding to the timing of
critical periods in neuronal plasticity. Furthermore, a closer look at PVB neuronal and PNN density patterns of development in amygdala subnuclei may indicate a developmental trajectory of PVB neurons and the subsequent development of PNNs. A larger cohort would allow us to further test this theory more conclusively. Our unexpected findings of PNN density may lead to a better understanding of neuroplastic development and in turn lead to a better understanding of the pathology of psychiatric disorders as a whole.
References


