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Elyse L. Morin Date

Early Maternal Care Modulates the Development of Adolescent Emotional Regulation and Neurocircuitry in Nonhuman Primates

By

Elyse L. Morin
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Neuroscience

_____
Mar Sanchez, Ph.D.
Advisor

_____
Jocelyne Bachevalier, Ph.D.
Committee Member

_____
Damien Fair, PA-C, Ph.D.
Committee Member

_____
Tanja Jovanovic, Ph.D.
Committee Member

_____
Brian Dias, Ph.D.
Committee Member

_____
Shella Keilholz, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Early Maternal Care Modulates the Development of Adolescent Emotional Regulation and Neurocircuitry in Nonhuman Primates

By

Elyse L. Morin

B.S. Interdisciplinary Studies & Psychology, University of Florida, 2009

Advisor: Mar M. Sanchez, Ph.D.

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Abstract

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Maternal care is vital for proper primate socioemotional and cognitive development. Childhood maltreatment constitutes a major risk for psychopathology, including anxiety and mood disorders, and social and cognitive deficits, although the underlying neurodevelopmental mechanisms are not clearly understood. This dissertation tests the overall hypothesis that alterations in cortico-limbic circuits are involved, based on their critical role in emotional/stress regulation, and sensitivity to early experience. These studies utilized a well-established, spontaneous, nonhuman primate model of infant maltreatment (MALT) by the mother, which consists of comorbid physical abuse and rejection. In macaques, the highest rates of abuse/rejection happen in the first three months of life, a time of rapid brain development and maturation of prefrontal cortex(PFC)-amygdala circuits. We hypothesize that MALT impacts the developmental trajectory of amygdala circuits, including PFC-amygdala functional connectivity (FC) and relevant emotion/fear regulation behavior into adolescence. Adolescence is a crucial developmental period with physical, neuroendocrine, cognitive, and socioemotional changes linked to neural maturation and remodeling. We first examined long-term effects of MALT on attention bias to threat during adolescence, using cognitive touchscreen testing with the dot-probe task. MALT altered attentional processing of social threat, which could interfere with attention and cognitive processes. Next, acoustic startle testing was performed to measure baseline startle, fear/safety signal discrimination, fear modulation by safety cues, and extinction. Baseline startle in MALT animals was higher than controls, suggesting elevated state anxiety, but showed generalized blunted startle responses when safety and fear cues were presented together. Lastly, we examined the impact of MALT on amygdala FC longitudinally, from infancy through adolescence, using resting state fMRI. We found weaker amygdala FC with PFC and brainstem regions critical for arousal, stress and fear-learning throughout infancy and the juvenile period, some of which was predicted by elevated early exposure to cortisol. Interestingly, these effects on FC normalized by adolescence. Altogether these findings suggest that early adverse experience results in long-term alterations in emotional regulation beyond expected impacts in PFC-amygdala circuits. These studies uncovered lasting effects of MALT and its trajectory throughout development, to identify underlying neurobiological mechanisms that can be the target of and responsive to therapeutic intervention.

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[1] Modified from Wakeford AGP, Morin EL, Bramlett SN, Howell LL, Sanchez MM (2018). A review of nonhuman primate models of early life stress and adolescent drug abuse. *Neurobiology of Stress* 9:199-198.

1.1 Early Life Stress and Childhood Maltreatment

Early life stress (ELS), including adverse caregiving experiences such as childhood maltreatment MALT) , is a major risk factor for the emergence of psychopathology during adolescence, which includes poor emotional and stress regulation seen in anxiety and mood disorders, substance abuse and behavioral disorders (Cicchetti and Toth, 2005; Douglas*, et al.*, 2010; Sinha, 2008). Between 2013 and 2017, the number of children who were under investigation by child protective services increased by 10% (from 3,184,000 to 3,501,000 children), and the number of victims of childhood MALT, which includes abuse (physical & sexual) and/or neglect, increased by 2.7% to 674,000 children(DHHS, 2017). During 2017, approximately 1,720 children died as a result of abuse and neglect, which amounts roughly to a rate of 2.32/100,000 children in the US (DHHS, 2017). About sixty-four percent of the population will have been exposed to at least one trauma or significant stress during their childhood years. But these figures may be highly underestimated due to low rates of disclosure, making it difficult to have an idea of the actual total exposure in the population (Finkelhor, 1994; Pereda*, et al.*, 2009). Even if approximate, these numbers underscore that childhood MALT is a major public health concern and a devastating form of ELS associated with the development of anxiety and depression, including post-traumatic stress disorder (PTSD), drug addiction, criminal behavior, incarceration, social and cognitive deficits, obesity, cardiovascular disease, dementia and Alzheimer, etc. (Asok*, et al.*, 2013; Danese and Tan, 2014; Drury*, et al.*, 2012; Drury*, et al.*, 2016; Gee*, et al.*, 2013; Gunnar and Quevedo, 2007; Howell and Sanchez, 2011; McLaughlin*, et al.*, 2015; Petrullo*, et al.*, 2016a; Rutter*, et al.*, 1999; Sanchez*, et al.*, 2007; Teicher*, et al.*, 2003). The developmental outcomes, though, vary depending on factors such as timing and duration (Kaplow and Widom, 2007; Kisiel, 2014; Spinazzola, 2014; Steinberg, 2014), type and severity – physical abuse, neglect, sexual abuse, which are usually co-morbid-, and co-occurrence with psychological

trauma (Kisiel, 2014; Spinazzola, 2014). Some studies suggest that earlier exposure to adverse experiences, particularly during infancy (below 2 years of age), results in more severe negative outcomes (Kaplow and Widom, 2007) and are also more difficult to reverse with interventions (e.g. (Zeanah*, et al.*, 2017)).

The experience of a solitary stressor during early life may increase the vulnerability to psychopathology such as anxiety or depression by thirty percent during the lifetime (Anda*, et al.*, 2006), with women bearing twice the risk as males for developing such affective disorders (Burt and Stein, 2002; Felitti*, et al.*, 1998; Hankin, 2009; Weissman*, et al.*, 1996). It has been reported that adults that experienced more than six adverse events in childhood, including abuse (physical/sexual/verbal/emotional), social deprivation, neglect, or household dysfunction (substance abuse, poverty, criminality, divorce, witnessing violence) were likely to die 20 years earlier than average (Anda*, et al.*, 2009; Brown*, et al.*, 2009). Chronic ELS, often times from multiple sources of exposure, not only increases one's vulnerability to develop affective disorders and other mental illnesses, but also somatic diseases/disorders into adulthood, such obesity, diabetes, cardiovascular and ischemic heart disease (Dong*, et al.*, 2004). In addition to affective disorders, those that experience chronic and severe ELS early in life are more vulnerable to develop a wide range of behavioral problems that may begin in childhood or adolescence and have lasting impacts into adulthood, such as drug abuse, teen pregnancy, suicidality, risky sexual behavior, and criminal acts/recidivism (Norman*, et al.*, 2012; Pechtel and Pizzagalli, 2011; Shonkoff and Garner, 2012).

Despite the strong link between early life adverse care and the emergence of psychopathology, the underlying neurobiological and developmental mechanisms translating early adversity/stress into emotional and stress dysregulation, are not well understood. Alterations in development of brain networks that control arousal, stress,

threat and emotional responses particularly Amygdala circuits and its connectivity with prefrontal cortex (PFC) have been proposed (Foa and Kozak, 1986; Teicher*, et al.*, 2016; VanTieghem and Tottenham, 2018; Weber, 2008). But understanding the unfolding of these neurobiological changes and underlying mechanisms has been challenging, stemming from difficulties of prospective, longitudinal studies in children at risk. To complicate matters further, many of the children who are exposed to stress and trauma during childhood continue to be exposed to a variety of stressful experiences into their juvenile and adolescent years, and into adulthood (Evans*, et al.*, 2013). Therefore, it becomes challenging to disentangle the effects of early vs. cumulative stress and trauma in studies in humans; this is why we turn to translational animal models that allow more experimental control for our studies. Developmental outcomes from ELS have been described to be highly dependent upon the chronicity, age of onset, and type of exposure, and specifically how these events overlap with sensitive periods in neurodevelopment, when various brain regions may be developing/maturing (Teicher*, et al.*, 2006a; Teicher*, et al.*, 2006b). Currently, the neurobiological mechanisms from which early childhood experiences may lead to various affective, somatic, and behavioral disorders are unknown. Identification of long-term ELS-induced alterations associated with impaired ability to properly attend and regulate emotional responses during adolescence, and the underlying neurobiological substrates and neurodevelopmental mechanisms that translate ELS/early life adversity(ELA) into developmental psychopathology is critical and constitutes the main goal of this Dissertation, This information will increase our potential for developing and optimizing cognitive/behavioral therapeutic interventions for children with early adverse care experiences.

The next sections in this chapter will cover a brief review of: (1) behavioral, endocrine, autonomic and neural aspects of the stress and threat responses, its regulation, development and dysregulation in stress-related disorders, including anxiety,

PTSD and mood disorders; (2) effects of ELS on emotional and stress regulation and measures/paradigms to study attention bias to threat and fear/safety learning using the acoustic startle response and Pavlovian fear conditioning; (3) Given the central question of this thesis on the neurobiological and neurodevelopmental mechanisms translating ELS/ELA/MALT into altered emotional regulation, I will review normative primate brain development, sex differences and how ELS alters brain development; (4) the impact of ELS and Childhood Maltreatment, in particular, on amygdala functional connectivity (FC); and (5) the final section will outline the translational macaque model of infant MALT used in these studies, and the goals, experimental design, specific hypotheses and aims.

1.2 The stress response: neuroendocrine and emotional aspects, regulation and role of ELS on anxiety and mood disorders

The role of the stress response is to meet challenges while maintaining homeostasis, mobilizing energy for survival and inhibiting systems that are not critical at that point. However, chronic exposure to stress can lead to pathology (McEwen, 1998). The stress response involves the coordinated action of different systems to respond to threats: behavioral, autonomic, and endocrine. Behavioral responses to threat include the startle reflex, freezing, increased arousal and vigilance, sharpened attention, and inhibition of eating and reproductive behaviors (Chrousos and Gold, 1992). Autonomic responses involve sympathetic activation and parasympathetic inhibition, via amygdala and hypothalamic projections to the brainstem (Ulrich-Lai and Herman, 2009). Activation of the sympathetic system also causes release of adrenaline/noradrenaline from the adrenal gland by the sympathetic-adrenomedullary (SAM) system, which is part of the neuroendocrine “fight or flight” stress response (Ulrich-Lai and Herman, 2009). The hypothalamic-pituitary-adrenal(HPA) axis is the other major neuroendocrine stress

system that is activated in parallel to SAM activation via specific (e.g. cortico-limbic) pathways to the paraventricular nucleus of the hypothalamus (PVN), resulting in release of corticotropin-releasing factor (CRF) which triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then triggers the synthesis and release of glucocorticoids (GCs: cortisol in primates) from the adrenal cortex (Charmandari*, et al.*, 2005). GCs are catabolic steroid hormones that mobilize energy substrates to respond to the threat. GCs then acts via negative feedback to shut down the HPA axis by binding to glucocorticoid receptors (GR) in the pituitary, PVN and limbic structures such as the hippocampus and prefrontal cortex (PFC) (Charmandari*, et al.*, 2005; Herman and Cullinan, 1997; Ulrich-Lai and Herman, 2009). In primates GRs are strongly expressed in the PFC (Sanchez*, et al.*, 2000b; Sanchez, 2006), explaining how cortisol stress-induced elevations can affect the PFC, particularly during development. Indeed, stress-related disorders such as anxiety, PTSD and depression are associated with chronic activation of stress responses and/or failure of negative feedback or regulatory mechanisms and parallel PFC alterations (Chrousos and Gold, 1992; McEwen, 2007). Stress responses are regulated by several brain regions that include the Amygdala, extended Amygdala and PFC (Herman and Cullinan, 1997)}(Ulrich-Lai and Herman, 2009). These brain regions, as well as their connectivity, have a protracted development, which makes them particularly sensitive to ELS experiences. Thus, although activation of the stress response system to a threatening stimulus, a mechanism which mobilizes biological resources and supports our survival, is an adaptive in response to an acute threat or stressor. However, severe or chronic stress may lead to long term alterations in brain development and support the development of maladaptive behavior and neuropsychiatric disorders (Anda*, et al.*, 2006; De Bellis*, et al.*, 1999a; De Bellis*, et al.*, 1999b; Lupien*, et al.*, 2009) Infants and children that were abused or neglected have been reported to have aberrant responses to negative facial expressions, (Cicchetti and

Curtis, 2005; Fries and Pollak, 2004; Pollak*, et al.*, 2000) and increased activation of attentional resources towards threatening faces compared to positive or fearful face stimuli (Pollak*, et al.*, 2001; Shackman*, et al.*, 2007). It has also been reported that children that have been maltreated may require less information from visual face stimuli in order to detect an expression of anger (Pollak and Kistler, 2002). Given that these children are experiencing more abuse and threatening situations compared to nonmaltreated children, it makes sense that they would devote more attentional focus and increase vigilance towards threatening stimuli, in an effort to quickly identify these threats to avoid or reduce harm that it might inflict on them.

1.3 Impact of ELS on brain development

Regions in the brain, including cortical and subcortical structures, are organized into functional networks (Honey*, et al.*, 2007; Peper*, et al.*, 2011),of which the connectivity can be assessed using neuroimaging, such as functional magnetic resonance imaging (fMRI). Such analyses of functional connectivity (FC), collected during a task or at rest, allow us to study this network, both long range connectivity across the brain and between functional networks, and short-range within network connectivity. During rest, a fMRI scan collects measures of temporal fluctuations in the low-frequency blood-oxygen-level-dependent (BOLD) signal, which reflects local changes in neuronal activity and metabolic demands (Logothetis and Wandell, 2004). FC, an indirect and suggested measure of neuronal communication and connectivity, is a measure of the correlation of neurophysiological activity during the imaging timecourse. Previous studies have reported that high FC seen between regions at rest is recapitulated in awake experimental tasks, suggesting regions that networks that are functionally connected will continue to show temporally correlated activity during rest (Biswal*, et al.*, 1995;

Bullmore and Sporns, 2009; Sporns, 2014). Resting state FC studies can allow us to begin to investigate the impacts of ELS on specific circuitry throughout development.

The neurobiological alterations underlying this increased risk during adolescence, which is reported to be stronger in females than males (Hyman*, et al.*, 2006; Kessler*, et al.*, 2005; Silberg*, et al.*, 1999) are poorly understood. However, some of these neurobiological alterations seem to involve ELS-induced changes in the development of cortico-limbic neural circuits involved in emotional and stress regulation, as well as in reward/motivation processes (Callaghan*, et al.*, 2014; Cicchetti and Toth, 2005; Dannlowski*, et al.*, 2012; Howell BR; Russo and Nestler, 2013). A likely neural circuit affected is the prefrontal cortex (PFC), and its connections with the amygdala, because of its protracted development and intense reorganization/remodeling during adolescence (Anderson*, et al.*, 1995; Brenhouse and Andersen, 2011; Casey*, et al.*, 2010a; Hoftman and Lewis, 2011; Rakic*, et al.*, 1994), and vulnerability to stress early in life (Ansell*, et al.*, 2012; Hanson*, et al.*, 2010; Hanson*, et al.*, 2012; Lupien*, et al.*, 2009; McEwen, 2008; Sanchez, 2006). PFC-limbic circuits are affected in humans with histories of childhood maltreatment and other adverse caregiving experiences (Burghy*, et al.*, 2012; Eluvathingal*, et al.*, 2006; Govindan*, et al.*, 2010; Herringa*, et al.*, 2013; Kumar*, et al.*, 2014), and resting state fMRI studies have reported alterations in FC in these circuits in stress-related disorders (Gimenez*, et al.*, 2012; Harrison*, et al.*, 2009).

Consistent with findings of structural and functional alterations in PFC-Amygdala circuits in humans with adverse caregiving experiences, including reduced connectivity (Eluvathingal, et al., 2006; Gee, et al., 2013; Govindan, et al., 2010; Kumar, et al., 2014), our lab also show a link between higher levels of cortisol exposure during infancy (Howell*, et al.*, 2013; Howell BR, 2012), due to infant maltreatment, and reduced PFC-amygdala white matter tract integrity (dorsolateral PFC (dlPFC) and ventromedial PFC

(vmPFC) with amygdala), using DTI (Howell*, et al.*, 2013; Howell BR, 2016; Howell BR, 2012).

1.4 Impact of ELS on brain connectivity in adolescence

Despite the strong link between human ELS and the emergence of adverse emotional, cognitive and physiological alterations during adolescence, this period is understudied, and the underlying alterations leading to risk, not understood. Adolescence is a crucial developmental period with many physical, neuroendocrine, cognitive, social, and emotional changes (Graber and Brooks-Gunn, 1996; Schulz*, et al.*, 2009; Sisk and Zehr, 2005). It is also a critical period for brain maturation and remodeling, including intriguing synaptic pruning and neural changes, some due to increased levels of gonadal hormones (Bramen*, et al.*, 2011; Paus*, et al.*, 2008), leading to structural and functional changes that increase vulnerability to ELS (Boyce and Ellis, 2005; Goddings*, et al.*, 2014; Steinberg, 2005). In primates, the PFC undergoes substantial reorganization during adolescence, including synaptic pruning (Bourgeois*, et al.*, 1994; Casey*, et al.*, 2010a; Lidow*, et al.*, 1991; Rakic*, et al.*, 1986) and myelination of PFC-limbic circuits (Giedd and Rapoport, 2010; Lebel*, et al.*, 2008; Malkova*, et al.*, 2006; Rakic*, et al.*, 1986). The protracted development of these circuits mirrors delayed emotional and stress regulation in adolescents (Casey*, et al.*, 2010a), increasing their vulnerability to early adverse experiences. The differential timing of subcortical and PFC development, with amygdala and reward centers developing earlier than PFC, so that during adolescence bottom-up (subcortical-to-cortical (PFC) projections are stronger and have accelerated development, while top-down projections that develop later are weaker, has been referred to as the "imbalance" model (Casey*, et al.*, 2011). In addition to their vulnerability to ELS due to the protracted development of these regions, these brain areas play a critical role in modulating stress and emotional reactivity, with the PFC exerting critical top-down control of amygdala activation in response to threats and

stressors (Kim*, et al.*, 2011b). Higher emotional reactivity and risky behavior that is typical of adolescence may be occurring because this time period occurs in the interim between earlier/faster maturation of subcortical regions and the protracted development of cortical regulatory regions such as the PFC (Somerville*, et al.*, 2010). Therefore, the increased sensitivity of these regions to ELS during adolescence may lead to experience-based alterations in PFC-amygdala functional connectivity.

1.5 Impact of ELS on emotional regulation/fear learning in adolescence

According to this model, increased fear during adolescence (Lau*, et al.*, 2011) and attention and vigilance towards socially relevant stimuli (hyperarousal) is due to pubertal maturation of subcortical structures, such as the amygdala, preceding the development of regulatory competence (PFC) regions that control emotion/stress and social behavior (Casey*, et al.*, 2008; Somerville and Casey, 2010; Spielberg*, et al.*, 2014; Steinberg, 2005). Increased activation of the amygdala towards emotional stimuli and insufficient PFC top-down regulation of subcortical structures reflects the on-going fine tuning of excitatory and inhibitory balance in PFC regions during adolescence (Casey*, et al.*, 2010b; Galvan*, et al.*, 2006; Hare*, et al.*, 2008; Knapska and Maren, 2009; Levesque*, et al.*, 2004; Milad and Quirk, 2012; Pattwell*, et al.*, 2012). Fear conditioning studies in rodents show that fear discrimination emerges in adolescence and tracks with this maturation of subcortical structures (amygdala), and PFC (Kim and Richardson, 2010; Rudy, 1993) and adolescents show enhanced fear responses during extinction training (Hefner and Holmes, 2007), and deficits in extinction retention (Baker and Richardson, 2015). Thus, adolescence is a phase when critical changes in fear and reward circuitry occur, emotional reactivity is heightened, and the potential for disorders in fear discrimination are likely to emerge (Kim and Richardson, 2010; Lau*, et al.*, 2011). The

maturational changes taking place during adolescence are also sexually dimorphic due to different maturational and cellular developmental trajectories (Bramen*, et al.*, 2011; Paus*, et al.*, 2008) and effects of gonadal hormones between sexes (Godfrey JR, 2013; Perrin*, et al.*, 2008), which affect fronto-amygdala functional connectivity (FC) (Godfrey JR, 2013), and may account for heightened vulnerability to ELS and psychopathology seen in females (Myers and Davis, 2004; Paus*, et al.*, 2008; Silberg*, et al.*, 1999).

1.6 Translational ELS macaque model of infant maltreatment (MALT): elevated stress and emotional reactivity

The development and utilization of NHP models of ELS has substantially increased our understanding of both healthy and disease states in humans. Rhesus monkeys (*Macaca mulatta*) provide an ethologically valid, translational animal model to study how ELS affects behavioral development and the neurobiological alterations underlying ELS-induced vulnerability to stress and emotional disorders in adolescence. NHP models of ELS are also necessary to address difficult questions in human studies, as 1) infant maltreatment (MALT) occurs spontaneously in NHP species such as rhesus monkeys, resulting in increased anxiety/emotional reactivity similar to reports in children (Sanchez, 2009), 2) ELS effects can be disentangled from heritable traits through cross-fostering and random assignment of infants to caregiving group at birth (used in my experimental design), 3) prospective, longitudinal analysis of neurobehavioral development can be done with high experimental control since birth using neuroimaging and bio-behavioral methods, and 4) infant MALT results in altered brain development in macaques, including structural impact to PFC-amygdala circuits (Howell*, et al.*, 2014; Howell BR, 2016; Morin EL, 2016).

Our laboratory has utilized an infant MALT model of ELS, which displays high translational relevance to humans. Infant MALT is not unique to humans, as it is reported to spontaneously occur in NHP species, such as macaques, baboons and marmosets, both in wild and in captive populations (Brent, Koban, and Ramirez 2002; Carroll and Maestripieri 1998; Johnson, Kamilaris, Calogero et al. 1996; Maestripieri, Wallen, and Carroll 1997; Maestripieri 1998; Troisi, D'Amato, Fuccillo et al. 1982; Troisi and D'Amato 1984). In macaques, infant MALT by the mother is operationalized by two, highly comorbid, behaviors: physical abuse and rejection, which occur at the highest rates during the first two to three months of life (Maestripieri 1998; Maestripieri and Carroll 1998; McCormack, Sanchez, Bardi et al. 2006; McCormack, Newman, Higley et al. 2009). Physical abuse includes aberrant, violent behaviors that the mother exhibits towards the infant (e.g. dragging, throwing, stepping on the infant), leading to pain and distress in the infant (Howell, McMurray, Guzman et al. 2017; Maestripieri 1998; McCormack et al. 2006; McCormack et al. 2009). Although infant rejection is a developmentally typical behavior around the time of weaning (after 3-6 months postpartum), the mother pushing away the infant when it tries to make contact with her is abnormal earlier in life and results in similar distress behaviors as abuse –screams, tantrums- (Maestripieri 1998; McCormack et al. 2006). In addition to intense infant distress, these adverse experiences also lead to elevations in stress hormones (Drury, Howell, Jones et al. 2017; Howell, McCormack, Grand et al. 2013; Howell, Grand, McCormack et al. 2014), long-term HPA axis hyperreactivity (Koch, McCormack, Sanchez et al. 2014; Sanchez et al. 2010), increased emotional reactivity (Sanchez and Pollak 2009), decreased brain serotonin (5HT) function that is associated with increased anxiety (Maestripieri, Higley, Lindell et al. 2006; Maestripieri, McCormack, Lindell et al. 2006; Sanchez et al. 2007), and increased amygdala volume and alterations in structure of PFC-amygdala tracts important for emotional/stress regulation and reward processes

(Howell et al. 2013; Howell et al. 2014; Howell et al. 2017(Howell*, et al.*, 2014; Howell*, et al.*, 2019) Sex differences are also present well before puberty, with females showing heightened vulnerability to emotional and stress alterations in comparison to males (Drury et al. 2017).

To address the challenges of prospective, longitudinal studies in children to understand how MALT derails neurobehavioral development, here we use a translational NHP animal model. Childhood MALT is not unique to humans and is reported in NHPs species with similar rates (Brent*, et al.*, 2002; Johnson*, et al.*, 1996; Maestripieri, 1998; Maestripieri, 1999; Troisi*, et al.*, 1982). More important, some of the developmental outcomes of MALT are also similar in humans and NHPs, including heightened anxiety and emotional reactivity, activation of stress neuroendocrine systems (Howell*, et al.*, 2014; McCormack*, et al.*, 2009; Sanchez, 2009; Sanchez*, et al.*, 1998), and alterations in PFC-limbic connectivity (Howell*, et al.*, 2016a; Howell BR, 2012; Morin EL, 2016), highlighting the translational value of these NHP models.

The selection of rhesus monkeys as an animal model to study the developmental unfolding and neurobiological mechanisms of MALT long-term impact on emotional regulation is justified due to the similarity of macaque and human biological and neurobehavioral processes, mother-infant relationships and maternal care, and neurodevelopment, specifically of the PFC-Amygdala circuits, in comparison to rodents (Drury*, et al.*, 2016; Howell*, et al.*, 2016b; Sanchez*, et al.*, 2001). The study of these animals during adolescence provides unique information of relevance for human development, including specific long-term alterations in emotional regulation (fear and safety learning), attention to (or away from) threat, and their neurobiological correlates. This NHP model allows for quantifiable assessment of maltreatment during a known developmental period (early infancy: first 3-6 months, which is comparable to the first 1-2 years in humans), providing frequency/duration/severity of the adverse caregiving

(e.g. rates of maternal abuse and rejection during early infancy), as well as measures of stress experienced by MALT infants (cortisol accumulation in hair from birth through 6 months) which can be used to determine associations of MALT type/rates and stress signals (cortisol exposure) with specific long-term alterations in emotional regulation and underlying neurocircuitry. The main **goal** of these studies is to test the overall **hypothesis** that ELS in the form of infant MALT alters the developmental trajectory of Amygdala circuits, having long-term impact particularly on functional connectivity (FC) of PFC-Amygdala circuits critical for emotional and stress regulation during adolescence, which results in impaired fear and safety learning, and allocation of attention. I propose an in-depth analysis of the developmental impact on FC of Amygdala-PFC fear/safety circuitry FC, focusing on Amygdala FC with medial PFC (mPFC) -involved in fear expression/modulation, anterior cingulate (ACC) –fear modulation/extinction-, orbitofrontal cortex (OFC) –fear expression/extinction- and dorsolateral PFC (dlPFC) – fear extinction and top-down cognitive control of Amygdala responses) to understand the neurobiological alterations that underlie impact on fear conditioning, extinction and modulation of fear by safety cues, and alterations in attention to threat. This research will significantly impact our understanding of the neurobiological underpinnings of ELS-induced emotional alterations during adolescence.

These goals will be achieved with the following specific aims:

<u>Aim 1:</u> Determine maladaptive emotional functioning in adolescence following ELS (MALT) by analyzing attention bias to threat. *Hypothesis:* MALT animals will show increased attention bias towards threat, reflected in faster reaction times during threat-congruent trials, when the cue is presented in the location of the threat stimulus. Increased attention bias to threat will be associated with weaker PFC-

Amygdala FC. This aim will be accomplished using touchscreen cognitive testing with the dot-probe task, measuring reaction time to a neutral cue following presentation of aversive/threatening stimuli. Human studies have shown increased bias towards threat in anxious individuals, in parallel to atypical disengagement of limbic regions (by fMRI) during trials in which the cue was placed away from threat.

<u>Aim 2:</u> Determine the long-term effects of ELS (MALT) on fear and safety learning in adolescence. *Hypothesis*: Our group has previously reported that MALT infants show increased emotional reactivity (tantrums, anxious behaviors) during early development. Thus, I hypothesize that this ELS experience will have long-term effects during adolescence and MALT animals will exhibit impaired emotional regulation, showing higher baseline and fear-conditioned startle responses, taking longer to learn to discriminate fear and safety cues, and also showing impaired fear extinction and attenuation of fear responses by safety signals, compared to controls. These functions are all regulated by the neurocircuitry examined in aim 3. The AX+/BX- fear-potentiated acoustic startle paradigm will be used because of its translational value (used in human studies to understand alterations in fear and safety learning processes in individuals with childhood trauma-related anxiety disorders). This paradigm is used to examine baseline startle response, fear-potentiated startle to conditioned fear, discriminative conditioning, attenuation of fear-potentiated responses by safety signals, and fear extinction. In addition to alterations in fear and safety learning, specifically fear/safety discrimination, and fear extinction, I also predict that impaired emotional regulation will be related to neurobiological alterations detected in the fear circuitry studied in Aim 3.

<u>Aim 3:</u> Determine the impact of ELS (MALT) on the developmental trajectory of Amygdala circuits, focusing on the long-term effects on functional connectivity (FC) with PFC critical for emotional regulation during adolescence. *Hypothesis*: PFC-Amygdala FC will be weaker in MALT animals compared to Controls. Resting state fMRI scans were acquired longitudinally during infancy and the early juvenile period and again during adolescence and a region of interest analysis will be performed to examine the developmental impact of MALT on FC of Amygdala and subregions of the PFC that are specifically involved in the control of fear acquisition, expression, extinction and safety learning, Thus, I will focus on Amygdala FC with medial PFC (mPFC) -involved in fear expression/modulation, anterior cingulate (ACC) –fear modulation/extinction-, orbitofrontal cortex (OFC) –fear expression/extinction- and dorsolateral PFC (dlPFC) –fear extinction and top-down cognitive control of Amygdala responses) to understand the neurobiological alterations that underlie impact on emotional regulation measures studied in Aim 1 and 2.

Chapter 2 Effects of early maternal care on adolescent attention bias to threat in nonhuman primates[2]

[2] Modified from Morin EL, Howell BR, Meyer JS, Sanchez MM. Effects of early maternal care on adolescent attention bias to threat in nonhuman primates. *Developmental Cognitive Neuroscience*. Accepted.

2.1 Abstract

Attention bias towards threat using dot-probe tasks has mainly been reported in adults with stress-related disorders such as PTSD and other anxiety disorders, in some cases associated with early life stress or traumatic experiences. Studies during adolescence are scarce and inconsistent, which highlights the need to increase our understanding of the developmental processes that predict attentional biases, given that this is a time of emergence of psychopathology. Here, we use a translational nonhuman primate model of early life stress in the form of infant maltreatment to examine its long-term impact on attentional biases during adolescence using the dot-probe task and identify interactions with early life risk factors, such as prenatal exposure to stress hormones and emotional/stress reactivity during infancy. Maltreated animals showed higher reaction times to social threat than animals that experienced competent maternal care, suggesting interference of negative valence stimuli on attentional control and cognitive processes. Higher emotional reactivity during infancy in Maltreated animals predicted attention bias towards threat, whereas higher levels of prenatal cortisol exposure was associated with bias away (avoidance of) threat in maltreated and control groups. Our findings suggest that different postnatal experiences and early biobehavioral mechanisms regulate the development of emotional attention biases during adolescence.

2.2 Introduction

Childhood maltreatment is a major public health concern (Finkelhor*, et al.*, 2013) and a form of early life stress (ELS) associated with increased risk for anxiety and mood disorders -including PTSD-, physiological, neurobiological and cognitive alterations, behavioral disorders, substance abuse, and obesity, not just in humans but in nonhuman primate (NHP) species (Danese and Tan, 2014; Drury*, et al.*, 2016; Gee*, et al.*, 2013;

Gunnar and Quevedo, 2007; Howell and Sanchez, 2011; Kaplow and Widom, 2007; Sanchez*, et al.*, 2001; Sanchez*, et al.*, 2007; Teicher*, et al.*, 2003). Despite the strong link between early adversity and psychopathology, the type and severity of developmental consequences is very complex and depends, in part, on the timing and duration (Kaplow and Widom, 2007; Kisiel, 2014; Spinazzola, 2014; Steinberg, 2014), type and severity of adversity –e.g. physical/sexual abuse, neglect, often co-morbid, and co-occurrence with psychological trauma (Kisiel, 2014; Spinazzola, 2014). The underlying developmental and biobehavioral mechanisms are not well understood, either. Alterations in attentional control and emotional information processing have been proposed to explain some of these alterations (Foa and Kozak, 1986; Weber, 2008), including attention bias toward threat or away from it (avoidance), resulting in interference with processing of other stimuli and disruption of cognitive processes. However, findings from recent human studies are inconsistent and scarce, particularly in children and adolescents, indicating the need to understand the developmental processes that predict attentional biases to emotional valence.

Attention bias toward threat has classically been measured with the dot-probe task (Bradley, 2000; Waters*, et al.*, 2008), measuring reaction time (RT) to respond to a cue (e.g. a red square; see Fig 1) that is presented following, and in the same location (congruent) or opposite location (incongruent) of an emotionally negative image (e.g. threatening face) presented simultaneously with an image of different valence (neutral), from which an attention bias score can be calculated (Price*, et al.*, 2016). Differences in congruent versus incongruent RT to this cue suggests attentional bias (Cisler and Koster, 2010). Biases include vigilance and attention towards threat (shorter RT to the congruent location), avoidance of attention directed away from the threat (avoidance; longer RT to congruent location), and difficulty disengaging from the threatening image. The dot-probe has been used in studies in populations with anxiety or histories of early

adversity/ELS/trauma, showing attentional bias toward threatening images (Aupperle, *et al.*, 2012; Bar-Haim, *et al.*, 2007; Cisler, *et al.*, 2009). In addition, anxious individuals identify the threat image more quickly and, therefore, have a faster RT to the congruent cue, but may also have difficulty disengaging from the threat and respond more slowly during the incongruent trials (Bryant and Harvey, 1997; Fox, *et al.*, 2001; Fox, *et al.*, 2002). Studies in populations exposed to trauma, including those with PTSD (Fani, *et al.*, 2012b; Lindstrom, *et al.*, 2011), also suggest an attentional bias to images related to the trauma (Bryant and Harvey, 1995; Foa, *et al.*, 1991).

Numerous studies have reported altered threat responses in children with a history of maltreatment (Pine, *et al.*, 2005; Pollak, *et al.*, 1997; Pollak, *et al.*, 2001; Pollak and Sinha, 2002; Pollak and Tolley-Schell, 2003; Shackman, *et al.*, 2007), as well as improved memory of angry facial expressions in visual tasks (Rieder, 1989), and increased amygdala activity to threat cues (McCrory, *et al.*, 2011; McCrory, *et al.*, 2013), known to mediate rapid attention to threat (Phelps and LeDoux, 2005). These differences in threat-processing have been detected as early as 15 months of age (Curtis and Cicchetti, 2013). However, inconsistent findings have been reported in dot-probe studies of maltreated children, including attentional bias away from threat (Berto, *et al.*, 2017; Kelly, *et al.*, 2015; Pine, *et al.*, 2005), or towards threat during adolescence (Gibb, *et al.*, 2009). Such attention biases may reflect strategies to improve threat detection, or to avoid exposure to threatening stimuli and attenuate emotional responses in individuals with difficulties with emotional regulation (Wald, *et al.*, 2013), respectively. However, these attentional biases can also promote maladaptive or exaggerated responses to perceived threats, including fear generalization to nonthreatening stimuli (Foa, 1999), interfering with the evaluation of other relevant information in the environment, such as safety cues, and impairing the ability to adapt and cope with situations (Bar-Haim, *et al.*, 2007; Bar-Haim, 2010), or to properly evaluate future risks

(Cisler and Koster, 2010; Messman-Moore and Long, 2003). Altered cognitive processing of threat during development may lead to increased vulnerability for psychopathology later in life, especially in response to adult trauma exposure (Fani*, et al.*, 2010; Gibb*, et al.*, 2009). And, in the case of threat avoidance during stress exposure, this is predictive of later PTSD symptoms (Wald*, et al.*, 2011; Wald*, et al.*, 2013).

NHPs such as rhesus monkeys can provide a translational animal model to help address questions raised in human studies through prospective, longitudinal, studies of (a) emotional attention bias processes, using translational adaptations of the dot-probe task (Lacreuse*, et al.*, 2013; Parr*, et al.*, 2013) and (b) the impact of maternal maltreatment on neurobehavioral development, including vulnerability to emotion and stress regulation. Maltreatment of infants, including abuse and rejection, occurs in NHP populations spontaneously and at similar rates (2-5% prevalence) as in humans (Brent*, et al.*, 2002; Howell*, et al.*, 2016b; Johnson*, et al.*, 1996; Maestripieri and Carroll, 1998; Parr*, et al.*, 2012; Sanchez*, et al.*, 1998; Sanchez, 2006; Troisi and D'Amato, 1984). In macaques, infant maltreatment by the mother includes physical abuse and maternal rejection associated with infant distress (Maestripieri and Carroll, 1998; Maestripieri, 1999; Sanchez, 2006). In addition to transgenerational transmission of maltreatment through the maternal line, these mothers maltreat subsequent offspring in what seems to be a stable maternal trait (Maestripieri and Carroll, 1998; Maestripieri, 2005). Maltreated infant macaques show increased anxiety and emotional reactivity, impaired impulse control, aggression, and social deficits throughout development and into adolescence, as well as elevated levels of the stress hormone cortisol, suggesting chronic stress exposure (Drury*, et al.*, 2017; Howell*, et al.*, 2013; Howell*, et al.*, 2014; McCormack*, et al.*, 2006). These socioemotional alterations and activation of the stress response are consistent with alterations reported in children that experience maltreatment and other forms of adverse early care (Howell*, et al.*, 2013; Koch*, et al.*,

2014; Maestripieri, 1998; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Sanchez, 2006; Sanchez*, et al.*, 2010).

Thus, in this study, we use a translational NHP model of infant maltreatment to (a) examine its long-term impact on attentional biases toward or away from threat during adolescence using the dot-probe task; and (b) potential interactions of postnatal adverse care (maltreatment) with other early risk factors (prenatal stress/cortisol exposure and infant's emotional and stress reactivity) that may increase vulnerability to long-term alterations in threat responses during adolescence, and explain individual variability in the outcomes. Differential attention bias has not been previously studied in maltreated monkeys, particularly during adolescence, which can provide a critical cross-species comparison with findings in human populations of children and adolescents with early adverse experiences (Berto*, et al.*, 2017; Gibb*, et al.*, 2009; Kelly*, et al.*, 2015; Pine*, et al.*, 2005; Pollak*, et al.*, 1997; Pollak*, et al.*, 2001; Pollak and Sinha, 2002; Pollak and Tolley-Schell, 2003; Shackman*, et al.*, 2007). Although this animal model does not span all adverse experiences that human children experience (for instance, sexual abuse), one of its critical strengths lies in its ability to quantify maltreatment during a known postnatal period, providing frequency, duration, and severity of the adverse experience (e.g. abuse and rejection rates) and the concurrent levels of stress it elicits (e.g. cortisol accumulation in hair during the postnatal ELS exposure), difficult to be accurately determined in studies with children with early adverse caregiving experiences. Additionally, NHP models provide strong control over environmental variables that are known confounders of behavioral outcomes of ELS during adolescence in human studies, such as drug use, diet/obesity, prenatal stress/drug exposure, socioeconomic status, and access to medical care or therapy. Our experimental design also allows to disentangle heritability from postnatal experience by utilizing crossfostering and randomized assignment to experimental group (maltreating, competent care; (Drury*, et al.*, 2017;

Howell*, et al.*, 2017)) at birth, which would be unethical in humans. These are just some of the important contributions of this translational animal model with a well-characterized adverse caregiving experience and longitudinal behavioral and biological measures of its developmental impact. Of particular interest here is the role of proper maternal care on emotional attention and regulation. Maternal care is, indeed, critical in regulating the development of emotional/stress neural circuits in both humans and NHP species (Gee*, et al.*, 2014; Gunnar*, et al.*, 2015; Gunnar and Sullivan, 2017; Sanchez*, et al.*, 2015; Tottenham, 2015), and maltreatment experiences take place at a time when critical socioemotional skills are developing, as well as the brain regions that regulate them (Casey*, et al.*, 2010a; LA, 1996; Maestripieri and Carroll, 1998). We are also interested in potential interactions of postnatal adverse care (maltreatment) with other early risk factors (prenatal stress/cortisol exposure and infant's emotional and stress reactivity) that may increase vulnerability to infant maltreatment. Exposure to prenatal stress and elevated cortisol -measured through maternal plasma cortisol, psychosocial stress or amniotic fluid cortisol- predicts increased reactivity and disrupted emotional regulation in human infant (Baibazarova*, et al.*, 2013; Bergman*, et al.*, 2010a; Bergman*, et al.*, 2010b; Davis*, et al.*, 2011); (Bolten*, et al.*, 2013)), and externalizing behavioral problems (Gutteling*, et al.*, 2005). Innate emotional reactivity or temperament have also been reported to affect attentional biases to threat in children, such that biases may be more prominent and fixed in children with fearful temperament (Field and Lester, 2010; LoBue and Perez-Edgar, 2014) and affect-based attention bias and temperament have a synergistic relationship leading to socioemotional maladjustment (Cole*, et al.*, 2016; Morales*, et al.*, 2015; Morales*, et al.*, 2016; Perez-Edgar*, et al.*, 2010; Perez-Edgar*, et al.*, 2011). Thus, and supported by previous evidence in humans (e.g. (Gibb*, et al.*, 2009), we hypothesize that postnatal exposure to adverse caregiving will alter the development of emotional regulation, increasing attention bias towards social threat in adolescence; and

that this will be further worsened by prenatal stress/cortisol exposure and infant reactive temperament. To test these hypotheses we examined differences in RT in the dot probe task in adolescent macaques with and without infant maltreatment, presenting threatening and neutral images (social vs. nonsocial). Next, we examined whether RT in the dot probe task was further predicted by prenatal cortisol exposure, and infant emotional reactivity during infancy.

2.3 Methods

2.3.1 Subjects

Twenty-five adolescent rhesus macaques (*Macaca mulatta;* 13 males, 12 females) between the ages of 4.5-5.5 years old were included in this study. These animals were generated and well-characterized throughout infancy and the juvenile (pre-pubertal period) as part of a bigger longitudinal study by our group on developmental outcomes of infant maltreatment in this species (Drury*, et al.*, 2017; Howell*, et al.*, 2017). They were born and lived with their mothers and families in complex social groups at the Yerkes National Primate Research Center (YNPRC) Field Station breeding colony, consisting of 75-150 adult females, their sub-adult and juvenile offspring, and 2-3 adult males. These groups were housed in outdoor compounds, with access to climate-controlled indoor areas. Standard high fiber, low fat monkey chow (Purina Mills Int., Lab Diets, St. Louis, MO) and seasonal fruits and vegetables were provided twice daily, in addition to enrichment items. Water was available *ad libitum.*

Half of the subjects experienced maternal maltreatment (MALT, n=14; 8 males, 6 females), and the other half received competent maternal care (Control, n=11; 5 males, 6 females); these NHP sample sizes are large in comparison to macaque studies using the dot-probe task, which have published reports with n=6 animals (Lacreuse*, et al.*, 2013;

Parr*, et al.*, 2013). In this model, infant maltreatment is defined by co-morbid experience of maternal physical abuse and rejection of the infant during the first three months of life –never exhibited by Control, competent mothers-, which causes pain, emotional distress and elevations in stress hormones (Drury*, et al.*, 2017; Howell*, et al.*, 2013; Maestripieri and Carroll, 1998; Maestripieri*, et al.*, 2000; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Sanchez, 2006). In this study infants were randomly assigned at birth to be crossfostered to a Control or MALT foster mother in an effort to disentangle and control for effects of heritable factors that may confound the effects of ELS, counterbalancing groups by sex, biological mother, social dominance rank and assigning infants from different matrilines & paternities to provide high genetic/social diversity, as previously reported (Drury*, et al.*, 2017; Howell*, et al.*, 2017). See Table 1 for details of infant crossfostering assignment and counterbalancing of groups. Given that birth weight is a strong predictor of neurobehavioral development in humans and NHPs (Coe and Shirtcliff, 2004; Vohr*, et al.*, 2000) we only studied infants ≥450gr birth weight, which is a safe veterinary clinical cut off to rule out prematurity in rhesus monkeys. While at the YNPRC Field Station, longitudinal measures were collected during the infant and juvenile periods, focusing on the following for this study: (a) behavioral observations of maternal care received and infant emotional reactivity from birth through the first three months postpartum, and (b) HPA axis basal activity, measured as cortisol accumulated in hair from birth through the first 6 months postpartum as well as prenatally (from hair samples collected at birth).

At approximately 4 years of age the 25 adolescents were transferred to the YNPRC Main Station. Upon arrival, animals were pair-housed in home cages and fed Purina monkey chow (Ralston Purina, St. Louis, MO, USA), supplemented with fruit and vegetables daily, and water was available *ad libitum*. Environmental enrichment was provided on a regular basis. The colony is maintained at an ambient temperature of 22 ± 2°C at 25-50%

humidity, and the lights set to a 12-h light/dark cycle (lights on at 7h; lights off at 19h). Following several months of acclimation to the move and new housing environment, the animals underwent several behavioral tasks, neuroendocrine assessments and MRI scans, including the dot-probe task to examine attention bias toward or away from threat as a part of a larger study examining long-term emotional, cognitive and neurobiological consequences of ELS during adolescence.

All procedures and animal care were in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and Use of Laboratory Animals" and approved by the Emory Institutional Animal Care and Use Committee (IACUC).

2.3.2 Behavioral characterization of maternal care & measures of infant emotional reactivity

A detailed description of the infant rhesus maltreatment model and methods for selection of potential mothers and behavioral characterization of competent maternal care (Control) in contrast to infant MALT is provided in previous publications (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Maestripieri, 1998; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009). Briefly, because MALT mothers consistently maltreat their infants, we identified potential multiparous Control and MALT mothers with known maternal care quality towards prior offspring. Following crossfostering, we performed focal observations of maternal care across the first 3 postnatal months to substantiate and measure rates of abuse and rejection towards their fostered infants. These consisted of 30 min long focal observations performed on separate days (5 days/week during month 1, 2 days/week during month 2 and 1 day/week during month 3) for a total of 16 hours/mother-infant pair; this observation

protocol is optimal to document early maternal care in this species, given that physical abuse is the highest during month 1 (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006). Behavioral observations were collected by experienced coders (interobserver reliability >90% agreement, Cohen k>0.8). Competent maternal care is defined as species-typical behaviors such as nursing, cradling, grooming, ventral contact and protection (retrieve from potential danger, restrain) of the infant. In contrast, MALT is aberrant (prevalence rate: 2-5%), defined as the comorbid occurrence of physical abuse (operationalized as violent behaviors directed towards the infant that cause pain and distress, including dragging, crushing, throwing) and early infant rejection (i.e. prevention of ventral contact and pushing the infant away). Both abuse and rejection cause high levels of infant distress –e.g. scream vocalizations- and elevations in stress hormones (Drury*, et al.*, 2017; Howell*, et al.*, 2013; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006; Sanchez, 2006). Control foster mothers in this study exhibited competent/good maternal care (e.g. high maternal sensitivity, infant protection and attachment: (McCormack*, et al.*, 2015) and did not exhibit MALT behaviors -physical abuse or rejection- (Drury*, et al.*, 2017; Howell*, et al.*, 2017). Abuse and rejection rates (frequency/observation time) were calculated across the first 3 postnatal months (see Table 2). In addition to infant abuse and rejection rates, rates of infant scream (distress) vocalizations during the first 6 months of life were also included in the regression models described below as measures of infant emotional reactivity (Table 2).

2.3.3 Dot-Probe Testing Procedure

During adolescence (between 4.5-5.5 year of age), animals were trained and tested in an attention bias task –the dot-probe- in their home cage, using a touchscreen rig attached

to the cage that was fully accessible when the cage door was opened. The dot-probe has been used to assess attention bias to threat in human populations exposed to trauma, including those with PTSD (e.g. (Fani*, et al.*, 2012b; Lindstrom*, et al.*, 2011)) as well as in a few NHP studies to examine attentional bias to social stimuli of varying emotional valence (Lacreuse*, et al.*, 2013; Parr*, et al.*, 2013). Initially, animals were habituated to the touchscreen and behavioral shaping was done using positive reinforcement (food rewards) to guide them to touch the images presented on the screen if they did not immediately do so out of curiosity. Animals were first trained to touch neutral clip art images presented centrally on the screen, and then progressed to touching images that appeared at random locations on the screen, by receiving a nutritionally-balanced food pellet as reward (Bio-Serv®), which was released into an automatic pellet dispenser/hopper below the screen. All animals were on a delayed feeding schedule in order to prevent satiation and decreased motivation for food rewards during testing, which took place 5 days per week. Pair-housed animals were separated by a full panel partition during testing to avoid distractions and interferences due to animals' interactions and to control the number of presentations of experimental (social and nonsocial) images each animal received during testing.

Once the animals were proficient with the touchscreens, they proceeded to the training phase of the dot-probe paradigm, using the Yerkes Cognitive Battery (YCB) software, with neutral clip art images and a modification of previous protocols in macaques (Lacreuse*, et al.*, 2013; Parr*, et al.*, 2013). To initiate each training trial, the monkey touched a central fixation cross, after which two images were presented on either side of the screen for 500 milliseconds. The target cue, a red square, was then presented congruent with one of the images (i.e. in the same location it had been briefly presented), and the animal's RT to touch this cue was recorded. Animals were closely monitored for cheating behavior throughout training, operationalized by the use of two hands to touch

both sides of the screen simultaneously in anticipation of the cue. Shaping was used to enforce responding with one hand and removing the hand from the screen between trials if necessary. Over successive training sessions, the time response interval required to respond and receive the food reward was reduced, encouraging the monkeys to maintain attention, stay engaged with the task, and to respond as soon as the cue was presented. Training criterion was met and animals progressed to the experimental images when two 100-trial sessions (did not need to be consecutive) were completed with ≥80% correct trials at a 1 second response interval.

After reaching training criterion, animals transitioned to the testing phase of the dot-probe with social or nonsocial images of negative or neutral valence. Testing trials followed a similar structure as during training, but one (or both) of the images in the pair was emotionally salient (negative: e.g. threat facial expression, snake) and the other was neutral (see Figure 1). Animals completed one 100-trial session per day over a nine-day testing period. Response intervals were lifted to five seconds in order to allow for potentially delayed responses to the emotional saliency of the images, but still encourage responding and engagement with the task. Two categories of experimental images were presented during testing (social, nonsocial), which were separated into two different tasks. Social images were composed of unfamiliar conspecific faces with two different facial expressions classified into negative (threat) or neutral of 15 different identities (Figure 2). Nonsocial images included familiar and unfamiliar objects with a neutral (i.e. light switch, clock), or negative (i.e. syringe, snake) valence (Figure 2). Trials were randomly balanced per session for image valence (negative vs. neutral), location of cue presentation in relation to image with emotional valence (congruent vs. incongruent), as well as target cue presentation (left vs. right side of the screen). Three days of nonsocial image testing (300 trials) were followed by six days of social image testing (600 trials). Although most studies only use Mean Attention Bias (MAB) -a score calculated by

subtracting RT during congruent trials from RT during incongruent trials- as the dependent variable, we included both MAB score and RT to touch the target cue as dependent variables. Inclusion of RT in the analyses allows to examine potential differences in the way the presentation of the threatening images may interfere with the animals' general performance speed, an issue that has been brought up in recent publications suggesting the use of RT (see (van Rooijen*, et al.*, 2017)) to provide an additional measure more related to difficulties "disengaging" from the emotional stimulus. Bias scores were computed separately for social and nonsocial tasks. Positive scores indicated a bias towards the image with negative emotional valence (i.e. faster RT to threat than neutral images), while negative scores a bias away from threat.

2.3.4 Hair Cortisol

At birth, approximately one square inch of hair was shaved from the back of the infant's head just above the foramen magnum (nuccal area), and the hair that grew in this region was shaved again at 6 months of age. At each time point the hair samples captured chronic cortisol exposure, through its accumulation into the growing hair shaft. Birth hair cortisol samples were collected to examine prenatal cortisol exposure between groups; 6 months hair cortisol concentrations were measured to examine HPA axis activations due to ELS. Both birth and 6 months cortisol measures were also used as predictors in the regression models. Hair samples were processed and assayed using previously described protocols (Meyer*, et al.*, 2014). Each sample was weighed, washed in isopropanol to remove external contamination, ground to powder, and extracted with methanol overnight. After evaporation of the methanol, the residue was redissolved in assay buffer, and cortisol was measured using the Salimetrics (Carlsbad, CA) enzyme

immunoassay kit (cat. # 1-3002) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were <10%.

2.3.5 Statistical Analysis

Data were summarized as mean±standard error of the mean (SEM) and normality tested for each variable using the Shapiro-Wilk test. Student t-test was used to compare means in normally-distributed data; because neither abuse, rejection or scream rates, nor hair cortisol concentrations at birth or at 6 months were normally distributed, the effect of maternal care (Control vs MALT) on these variables was tested using a nonparametric Mann-Whitney Rank Sum Test.

Repeated Measures (RM) ANCOVA with group as fixed factor (Control, MALT), and congruency (congruent, incongruent) as the repeated measures factor were used to examine maternal care effects on RT on each trial type (negative/threat vs neutral). Sex (male, female) was added as a covariate in the statistical models. Biological mother group (Control, MALT) was also added as a covariate to account for the potential effects of heritable/prenatal factors, but was dropped from the final models due to its lack of effects on RT. T-tests were conducted to compare MAB score in Control and MALT groups. Potential confounding/carry over effects of testing day on RT were examined on a separate RM ANOVA for group and congruency. Analyses were performed separately for social and nonsocial stimuli given they were run as separate tasks.

Multiple linear regression was used to assess early predictors of performance in the dot-probe for trial types with significant group effects: (a) abuse and rejection rates received by each infant, (b) infant emotional reactivity (screams), and (c) prenatal and postnatal cortisol exposure (measured as hair cortisol accumulation). Both RT and MAB were

normally distributed. Spearman correlation was used to rule out issues of multicolinearity between regressors.

Statistical significance level was set at $p<0.05$. Bonferroni correction was applied for multiple comparisons (Student t & Mann-Whitney tests; multiple regression models), and we present the results before and after correction, when applicable. Given the sample size of 25, we were powered at 80% power and 5% level of significance to detect moderate to large effect sizes, including for the repeated measures model effects (based on power analysis performed using PASS 15 (NCSS, 2017)) and published criteria (Cohen, 1988). Effects sizes are reported for significant results.

2.4 Results

2.4.1 Early maternal care, emotional reactivity and cortisol

Because abuse, rejection, and scream rates, as well as hair cortisol at birth and 6 months failed the Shapiro-Wilk test for normal distribution, maternal care group differences (Control vs MALT) on these variables was tested using a nonparametric Mann-Whitney Rank Sum Test. As expected based on previous publications with this model of infant maltreatment (Howell et al, 2017; Drury et al, 2017) and as shown in Table 2 there was a significant group difference in maternal abuse and rejection rates received, which were very high in MALT animals and absent or extremely low, respectively, in Control animals (Abuse: $U=0.0$, $n_1=10$, $n_2=14$, $p<0.001$, effect size estimate(r)=0.86; Rejection: $U=2.0$, $n_1=10$, $n_2=14$, $p<0.001$, $r=0.82$), and scream rates were also significantly greater in MALT than Control subjects ($U=18.0$, $n_1=10$, $n_2=13$, $p=0.04$, $r=0.60$). However, there were not significant group differences in hair cortisol levels at birth ($U=50.00$, $n_1=10$,

n_2=14, p=0.254, r=0.24) or in hair cortisol at 6 months of age (U=46.0, n_1=8, n_2=12, p=0.908, r=0.03; Table 2).

2.4.2 Dot-probe test

Trials that the animals initiated but did not respond to within 5 seconds were removed from the analysis. After inspecting histograms of all animal's response times, a lower cutoff of 0.2 seconds was set in order to remove a small peak of responses that occurred immediately after the image was presented, as there is a risk that there were most likely anticipatory responses, consistent with other studies (Joormann and Gotlib, 2007). An upper cutoff of 1.5 seconds was selected based on previous macaque studies (Parr*, et al.*, 2013) in order to capture delayed responding due to image salience and not to distractions (e.g. from other animals). Social and nonsocial trials were assessed in separate tasks, and all trials within these categories were collapsed across days of testing. See Table 3 for details.

Results of the two-way RM ANCOVA conducted to examine the effect of group and congruency on RT (with sex included as a covariate) detected a significant main effect of group on RT ($F_{(1,22)}$=4.5, p=0.0463, η^2=0.17) for the threat vs. neutral social image trials, with MALT animals showing slower RT than Controls (see Figure 3). No main effects of congruency ($F_{(1,23)}$=1.8, p=0.188, η^2=0.07), group x congruency interaction effects ($F_{(1,23)}$=0.0, p=0.998, η^2=0.00) or covariate (sex) main or interaction effects were detected ($F_{(1,22)}$=0.5, p=0.4787, η^2=0.02). The slower RTs in MALT animals seem specific of the threat vs. neutral face trials because no RT group differences are detected during presentations of positive vs. neutral face pairs (unpublished data). No confounding/carry over effects of testing day were detected on RT, either (main day effect: $F_{(1,23)}$=2.4,

p=0.139, η^2=0.093; day x group: $F_{(1,23)}$=2.1, p=0.161, η^2=0.084; day x congruency: $F_{(1,23)}$=0.7, p=0.412, η^2=0.029).

No main or interaction effects of group, congruency or sex were detected for RT in the nonsocial Negative vs. Neutral trials (group when collapsing across testing days ($F_{(1,22)}$=0.5, p=0.506), congruency ($F_{(1,23)}$=0.7, p=0.4237), group x congruency ($F_{(1,23)}$=3.7, p=0.0681), sex ($F_{(1,22)}$=0.02, p=0.878)). However, a significant group x testing day was detected ($F_{(1,23)}$=4.6, p=0.042, η^2=0.168) with control, but not MALT animals, becoming faster with time (no other testing day ($F_{(1,23)}$=0.7, p=0.406, η^2=0.03), or day x congruency ($F_{(1,23)}$=0.06, p=0.809, η^2=0.003) effects were detected).

There was not a significant group difference in MAB score for threat vs. neutral social ($t_{(23)}$=-0.003, p=0.99, g=-0.112; Bonferroni-adjusted p value=0.025) or negative vs. neutral nonsocial images ($t_{(23)}$=-1.9, p=0.08, g=-0.733; adjusted p value=0.025), either.

2.4.3 Early predictors of Reaction Time in the Dot-probe

A multiple linear regression model was used to assess early predictors (infant abuse, rejection and scream rates; hair cortisol at birth and 6 months) of RT during social threat vs. neutral trials in the dot-probe task, where MALT had higher RT than Controls. Because independent variables were not normally distributed there were log-transformed before analysis. No issues of multicolinearity were detected using Spearman correlations. A significant regression equation was found for social threat vs. neutral, congruent ($F_{(5,10)}$=10.0, R=0.95, R^2=0.91, p=0.012 –Bonferroni-adjusted p value: 0.025-), but not incongruent trials ($F_{(5,10)}$=0.7, R=0.64, R^2=0.41, p=0.649). In the significant multiple regression “congruent” model, the RT for threat vs neutral congruent trials was predicted from a linear combination of two of the independent variables: screams during

infancy (β=-0.14, t=-6.1, p=0.002; Figure 4A) and hair cortisol at birth (β=0.27, t=2.8, p=0.037; Figure 2.4B). Higher screams rates during infancy was predictive of faster RT to the threat congruent cue, effect seemingly driven by maltreated animals (Fig. 2.4A), whereas higher prenatal cortisol exposure predicted slower RTs (Fig. 2.4B).

2.5 Discussion

In this study, we used a translational NHP model of infant maltreatment to examine its impact on RT and attentional biases toward or away from threat during adolescence using the dot-probe task. We also examined potential interactions of postnatal adverse experience with other early risk factors, particularly measures of emotional and stress reactivity during infancy and prenatal/postnatal cortisol exposure. RT to a cue following the presentation of two social or nonsocial images of different emotional valence (threatening vs. neutral) was measured. Findings indicated group differences in RT during the social, but not the non-social, threat vs. neutral images presentation, with maltreated animals responding slower than controls, both during congruent and incongruent trials. This suggests potential interference of the social threat image in cognitive processing and attentional control. In the nonsocial trials, control, but not MALT, animals' RT became faster over testing days. Higher emotional reactivity (increased rates of distress vocalizations –screams-) during infancy, predicted faster RTs, whereas prenatal cortisol exposure (measured as hair cortisol at birth), was associated with slower RTs. These findings suggest a complex regulation by postnatal experiences, temperament and prenatal biological factors on emotional attention control during adolescence.

Differences in attention bias have been reported using the dot-probe task in children and adults with stress-related disorders, such as anxiety and PTSD, sometimes associated

with ELS/trauma (Aupperle*, et al.*, 2012; Bar-Haim*, et al.*, 2007; Bryant and Harvey, 1995; Bryant and Harvey, 1997; Cisler*, et al.*, 2009; Dalgleish*, et al.*, 2003; Elsesser*, et al.*, 2004; Fani*, et al.*, 2010; Fani*, et al.*, 2012b; Foa*, et al.*, 1991; Fox*, et al.*, 2001; Fox*, et al.*, 2002; Lindstrom*, et al.*, 2011; Pine*, et al.*, 2005). However, findings from these studies are inconsistent, especially among pediatric maltreated populations, with some individuals showing an attentional bias towards threat (Gibb*, et al.*, 2009), and others away from threat –determined using bias score- (Berto*, et al.*, 2017; Kelly*, et al.*, 2015; Pine*, et al.*, 2005). Studies of the effects of childhood maltreatment on attention bias during adolescence are also scarce, leaving a gap in our understanding of this measure during development. Here we used a macaque model to test the hypothesis that the directionality of the effects may be partially explained by interactions between postnatal adverse caregiving and other early risk factors, namely prenatal stress/cortisol exposure and infant stress/emotional reactivity.

Very few NHP studies have focused on attention bias towards threat using the dot-probe task, mostly to compare human and monkey attention bias and memory for emotional stimuli, and to study the effect of intranasal oxytocin on attention bias to negative facial expressions (Lacreuse*, et al.*, 2013; Parr*, et al.*, 2013). To our knowledge this is the first NHP study of the long-term effects of ELS on adolescent attention bias to threat. Although no group differences were found for attention bias scores, MALT animals showed higher (slower) RT than controls in the social, but not in the non-social, threat vs. neutral trials, independent of congruency. The specificity of the slower RT during social threat vs. neutral trials in MALT than Controls is further supported by the lack of group differences during presentations of positive vs. neutral face pairs (unpublished data). This suggests that threatening social stimuli may specifically interfere with cognitive processing and attentional control in animals with ELS, increasing the time that it takes to process or respond to stimuli that follow the presentation of threatening

faces. Perhaps these aversive emotional faces engage attention control networks, tying up cognitive resources and delaying responses, as previously suggested in individuals with PTSD (Fani*, et al.*, 2012a). Recent publications have suggested to examine RT, in addition to MAB (see (van Rooijen*, et al.*, 2017)) to provide a measure more related to difficulties "disengaging" from the emotional stimulus. Although social threat vs. neutral trial-specific, generalized (i.e., independent of congruency), slower RTs have not been reported in other studies in macaques (Lacreuse*, et al.*, 2013; Parr*, et al.*, 2013), our findings could be attributed to early adversity-related alterations in the development of emotional attention and regulation neural circuits and processes, resulting in interference with cognition.

We also examined the potential interaction of postnatal adverse care (maltreatment) with other early risk factors, specifically prenatal stress/cortisol exposure and infant's stress and emotional reactivity, that could increase vulnerability to long-term alterations in processing and responding to threatening stimuli during adolescence (i.e. differences in RT during the threat vs. neutral face presentations). Despite the high abuse and rejection experienced by MALT animals, and their high emotional reactivity (screams) during infancy, postnatal cortisol accumulation in hair was not significantly higher in MALT than control groups. This finding was unexpected and we believe is a power issue, as we have previously reported significantly higher postnatal hair cortisol accumulation in MALT than control infants in the bigger, full, dataset (n=42), suggesting chronic stress in the adverse caregiving group (Drury*, et al.*, 2017).

Higher emotional reactivity (i.e. distress vocalizations) during infancy was a negative predictor of RT to the cue congruent to threat, particularly in the maltreated group. In contrast, exposure to higher prenatal cortisol predicted slower RT to the congruent cue. For this type of trial, a faster RT to the threat congruent cue suggests an attention bias

towards threat in animals with higher emotional reactivity, (Bryant and Harvey, 1997; Fani*, et al.*, 2012b; Lacreuse*, et al.*, 2013; Parr*, et al.*, 2013), whereas a slower RT could mean either a bias away from threat, or general difficulty disengaging from the threat and therefore slower time responding in animals with higher prenatal cortisol exposure. Neuroimaging studies have shown alterations in activation of the hippocampus during threat disengagement in anxious individuals (Price*, et al.*, 2014). Additionally, in an exogenous cueing task, people with anxiety have been found to have difficulty disengaging from a threat cue, related to non-facilitated attention that is distinct from vigilance (Fox*, et al.*, 2001; Koster*, et al.*, 2004; Yiend and Mathews, 2001). Given the high levels of abuse and rejection that maltreated animals endured over the first few months of life, consistent with previous reports (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006), the long-term effects may be akin to those of complex trauma (Courtois, 2008), and may not present with the classical symptoms of hyperarousal and vigilance (i.e. attention bias toward threat) expected from a traumatized population (D'Andrea*, et al.*, 2013) in all maltreated animals. Thus, MALT animals with more externalizing behavior (i.e. those with higher distress vocalizations – screams-), may be more vigilant/hyperaroused and faster to identify and respond to threat (i.e. exhibit faster RT in the threat trials). A link between emotional reactivity and attention bias to threat has also been shown in maltreated children, suggesting attention bias as a potential marker of later development of psychopathology (Kelly*, et al.*, 2015). Maltreated animals that showed less emotional reactivity during infancy (i.e. screamed less) might exhibit more internalizing problems, blunted autonomic responses and attention bias away from (avoidance of) threat during adolescence. Although there was also some variability in RT among control animals, in general, they responded more quickly than the maltreated animals and had low scream rates.

Higher hair cortisol levels at birth (a measure of prenatal cortisol exposure), predicted longer RT. Previous studies in animal models (Coe*, et al.*, 1996; Coe*, et al.*, 2003; Maccari*, et al.*, 2003; Schneider*, et al.*, 1992; Weinstock, 2001) and humans (Bergman*, et al.*, 2007; Davis*, et al.*, 2007; Huizink*, et al.*, 2003; Laplante*, et al.*, 2004; O'Connor*, et al.*, 2003; Talge*, et al.*, 2007; Van den Bergh and Marcoen, 2004) have shown adverse impacts of high prenatal cortisol on infant cognitive, motor, and socioemotional development. Exposure to prenatal stress and elevated cortisol predicts increased reactivity and disrupted emotional regulation in human infants (Baibazarova*, et al.*, 2013; Bergman*, et al.*, 2010a; Bergman*, et al.*, 2010b; Bolten*, et al.*, 2013; Davis*, et al.*, 2011), and externalizing behavioral problems (Gutteling*, et al.*, 2005), highlighting the programming role that prenatal cortisol plays on the development of emotional attention and regulatory processes. Furthermore, it has been suggested that these effects may be attenuated by secure infant attachment to the mother (Bergman*, et al.*, 2010b).

Despite the strengths of this study, there are also limitations. Notably, although our sample size is large for macaque cognitive studies (Abzug and Sommer, 2018; Acikalin*, et al.*, 2018; Basile and Hampton, 2011; Ferrucci*, et al.*, 2019) – particularly those using the dot-probe task (Lacreuse et al, 2013; Parr et al, 2013)-, it is small in comparison to human studies and limited our statistical power to detect only moderate to large effect sizes (e.g. abuse, rejection, screams, social RT group effects) and to adequately test for other complex relationships (interactions) between factors, such as the effect of (1) genetic/heritable factors (biological mother), (2) sex, and (3) crossfostering and related postnatal environmental mismatches with ancestral environment. Such mismatches have been proposed to result in "recalibration" of emotional regulatory systems – dysfunctional or maladaptive behaviors- from the ancestral programmed pattern ("mismatch theory": (Barker, 1995; Del Giudice*, et al.*, 2011; Gluckman*, et al.*, 2005; Hostinar and Gunnar, 2013)). In this "mismatch theory", individuals are behaviorally

and biologically programmed to benefit from a match of postnatal and ancestral environments, even if they are adverse (Nederhof and Schmidt, 2012). A similar "3-hit hypothesis" has been suggested, such that heritable genetic factors play a role as the first "hit" (de Kloet, *et al.*, 2007).

Smaller sample sizes are used in NHP than in human research based on the high experimental control over environmental factors that are known confounders in human studies such as drug use, diet, prenatal environment, health care, etc. Although our sample size only allowed to test the potential effect of biological inheritance/prenatal factors (via crossfostering) as a covariate in the statistical models, we did not detect significant effects of biological mother on our measures (RT). Although previous crossfostering studies between competent and maltreating macaque mothers have not reported impact of the manipulation on maternal care (Maestripieri, 2005) and our foster mothers displayed similar maternal care to that observed by our group with prior offspring, the potential impact of crossfostering itself on infant's emotional reactivity has not been previously studied. Another limitation is the potential effect of animal transfer to the Main Station; although both groups were exposed to the same relocation stress experience, it is possible that more reactive animals were more impacted by this move.

Lastly, although group differences in RT were found for the social threat vs. neutral trials, the images used in our dot-probe studies were not ranked based on the degree of the stimulus threat, which may have added noise to our measures. Indeed, previous studies have shown differences in attention bias between mild- and high-threat stimuli (i.e. bias towards mild threats, which are more ambiguous and require more attention for evaluation; in contrast to bias away from high intensity-threats, which may be more likely to provoke avoidance) (Bryant and Harvey, 1997; Herzog, *et al.*, 2018). Based on the subgroups of MALT animals identified based on infant emotional reactivity, it would

have been important to examine responses to images with different degrees of threat, in order to determine attention bias to high intensity-threats vs. ambiguous threats, or even whether the effects are generalized/transferred across different types of threatening faces.

In summary, our findings suggest altered attentional processing of threat in maltreated animals, evident in delayed RT in the dot-probe task, which is further modified by emotional reactivity during infancy and prenatal cortisol/stress exposure. We propose that there may be two subgroups of maltreated animals, one that was more emotionally reactive during infancy, outwardly expressing distress, and another that internalized and developed attention bias away from threat during adolescence. Future work is necessary to determine the relationships between these attentional biases and underlying neurobiological functional mechanisms related to emotional regulation and fear learning circuitry, which may be altered during development.

Table 2.1

Groups breakdown based on randomized crossfostering assignment at birth.

The y-axis designates the crossfostering conditions (e.g. C→M identifies infants born to a control biological mother, but fostered to a MALT mother). *All animals were crossfostered except for a male control that was raised by his biological mother.

		FEMALE	MALE	
CONTROL	C→C	3	0	**11**
	M→C	3	5*	
MALTREATED	C→M	1	5	**14**
	M→M	5	3	
		12	**13**	**25**

Figure 2-1

Dot-Probe Paradigm.

In this example of the task, the animal first touches a fixation cross in order to initiate the trial. Two images are presented for 500ms, followed by presentation of the cue (red square). Reaction time (RT) to the final cue is measured. The cue is presented congruent (Left) or incongruent (right) to the threat face.

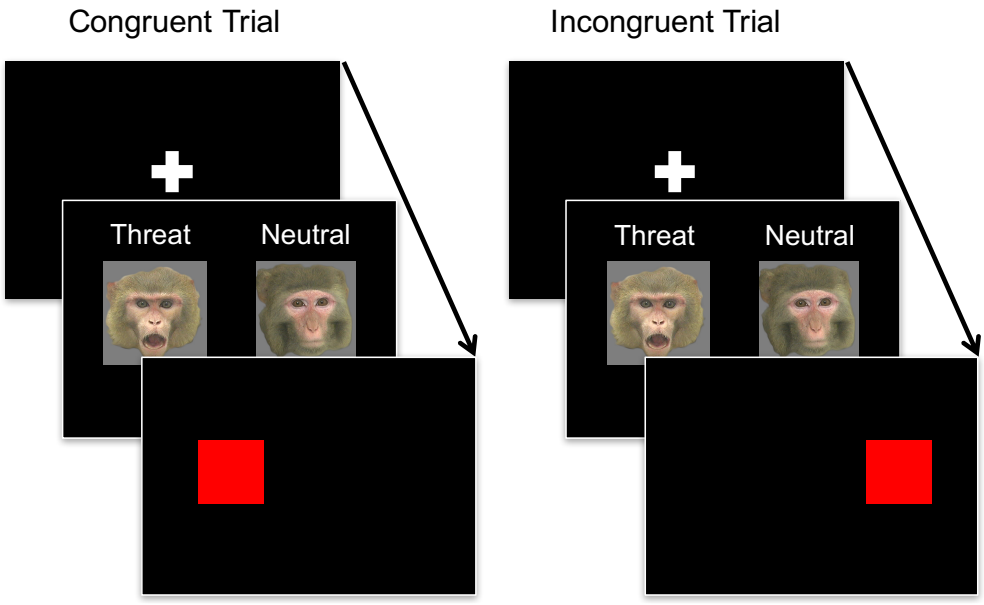


Figure 2-2

Examples of images from the social and nonsocial tasks.

The social task was composed of images of unfamiliar conspecifics with two different facial expressions: threat and neutral. Two images were randomly paired and presented in each trial. The nonsocial task was composed of images of negative and neutral valence and were also randomly paired and presented similarly.

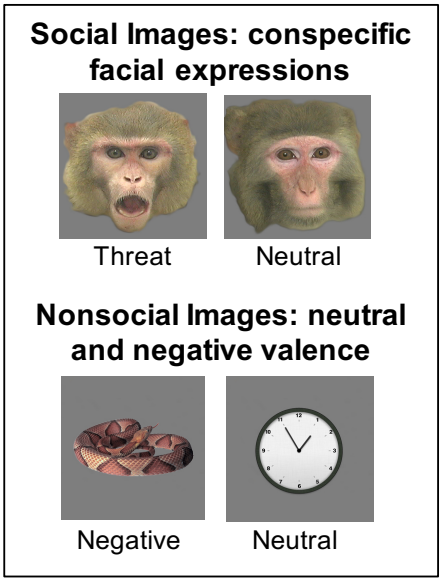


Table 2.2

Descriptive data of adverse caregiving behaviors (abuse and rejection rates/hour), infant emotional reactivity (screams rates/hour) and hair cortisol concentrations (pg/mg) during the first 3-6 postnatal months.

As expected based on previous publications with this model of infant maltreatment (Howell et al, 2017; Drury et al, 2017), Control mothers never exhibit abuse towards offspring and extremely low rejection rates, in contrast to Maltreating females. Data represented as mean±standard error of the mean (SEM). *, **, *** represent significant p values from the Mann-Whitney test.

	Abuse	Rejection	Screams	Hair Cortisol (Birth)	Hair Cortisol (6 months)
Control	0.00±0.00	0.13±0.09	0.67±0.18	515.19±24.93	134.45±9.95
Maltreated	0.98±0.25***	2.14±0.43***	2.85±0.96*	582.24±37.27	154.41±20.71

*<0.05; **<0.01; ***<0.001

Table 2.3

Social & Nonsocial Dot-Probe Test: Cognitive Touchscreen Task Effectiveness.

Summary of usable trials by group and sex, and breakdown of trials rejected on the basis of behavior of behavior (i.e. did not survive the lower -0.2 secs- or upper ->1.5 secs- cutoffs imposed to avoid including anticipatory responding and trials in which too long of a response occurred due to an outside distractor) or of time (i.e. subject did not touch the cue within 5 secs), as described in section 3.2. There was a significant main group effect on trials rejected due to time ($F_{(1,23)}$=4.4, p=0.048, η^2=0.173). Data represented as mean±SEM.

	Total Trials	Usable Trials	Rejected Trials due to Behavior	Rejected Trials due to Time
Control				
Male	900	743.2 ± 5.73	4.2 ± 1.32	152.6 ± 4.96
Female	900	735.33 ± 14.36	7.67 ± 1.56	157.0 ± 13.17
Maltreated				
Male	900	719.38 ± 20.33	10.88 ± 5.57	169.75 ± 15.47
Female	900	681.67 ± 9.54	26.33 ± 11.29	192.0 ± 4.46

Figure 2-3

Social and Nonsocial Dot-Probe: Reaction Time.

Here reaction time (RT) is plotted, subdivided by the location of the cue (congruent or incongruent). A main effect of group was found for the Threat vs. Neutral comparison in social trials, with maltreated animals responding more slowly irrespective of the congruency of the cue. No main or interactions effects of group were found in nonsocial trials when collapsing across trials, but see text for group x testing day interaction effects. Data represented as mean±SEM.

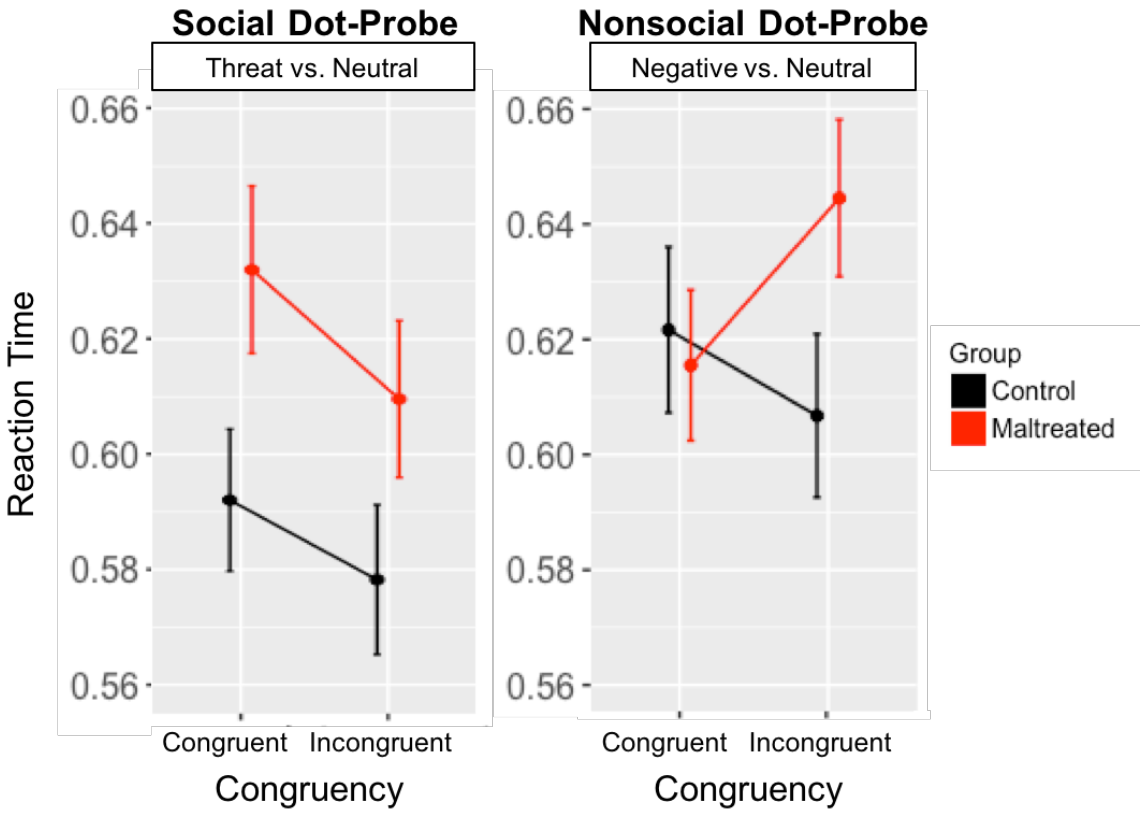
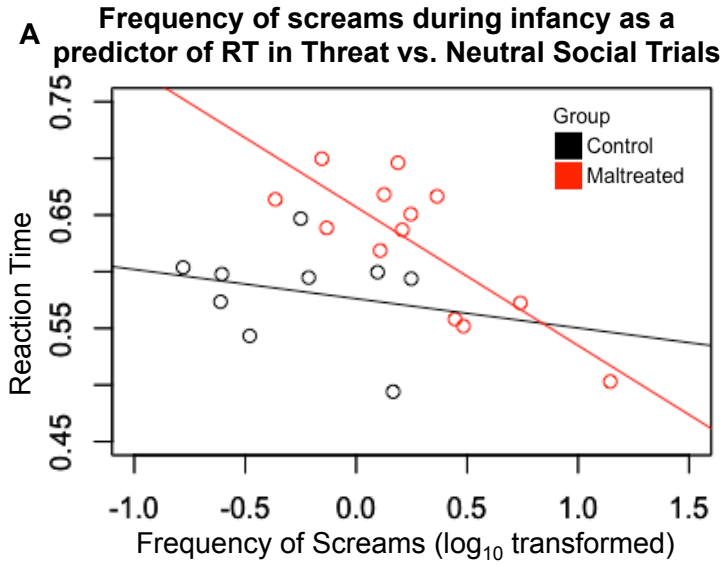
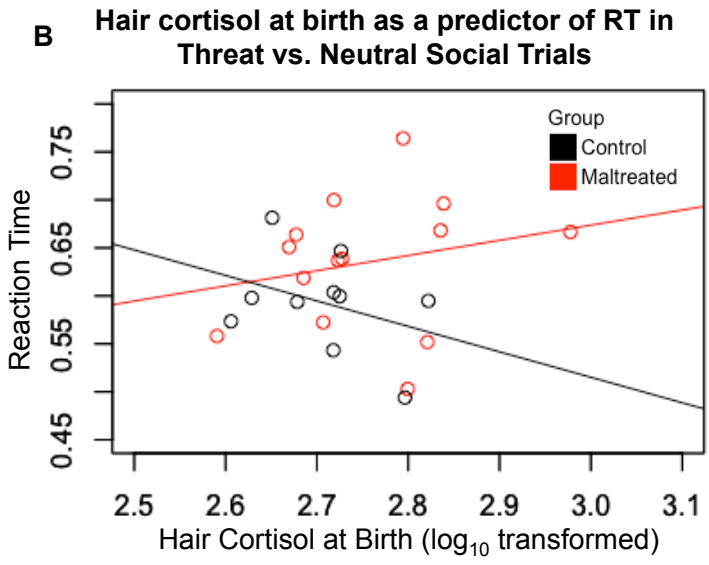


Figure 2-4

Early life predictors of reaction time in Threat vs. Neutral Social Trials: scatterplots per group (from the omnibus multiple regression model).

A. Screams were predictive of RT, such that higher screams rates during infancy was predictive of faster RT to the threat congruent cue (β=-0.14, t=-6.1, p=0.002), and this effect seemed driven by maltreated animals. **B.** Prenatal cortisol exposure (measured as hair cortisol at birth) predicted slower RT (β=0.27, t=2.8, p=0.037), which again seems to be driven by the maltreated group.





Chapter 3 Maternal Care Controls the Development of Fear Learning in Adolescent Nonhuman Primates

3.1 Abstract

Childhood maltreatment is a major risk factor for psychopathology, including anxiety disorders with alterations in fear responses. Consistent with human studies, our lab has reported that infant maltreatment by the mother (MALT) leads to increased emotional reactivity in rhesus macaques. It is unclear if this is due to enhanced fear learning or impaired ability to modulate fear by safety signals in the environment. Using this rhesus model of MALT, we assessed the long-term effects of ELS on state anxiety and fear (and safety) learning in 25 adolescent macaques (4.5-5.5 yrs), 14 MALT and 11 raised by competent mothers, using a translational Pavlovian fear-conditioning paradigm (AX+/BX-) that uses fear-potentiated startle amplitudes as the peripheral measure. The AX+/BX- paradigm measures baseline startle as an indicator of anxiety, fear-potentiated startle, discrimination of fear/safety conditioned cues, attenuation of startle with safety signals, and extinction. Baseline startle in MALT animals was higher than in controls, suggesting elevated state anxiety. During discrimination training, both groups showed differences in fear-potentiated startle to fear/safety cues between early and late acquisition, suggesting discrimination learning. No differences in fear learning or expression (fear-potentiated startle) or extinction were detected in MALT animals, however, the ability to use safety signals to modulate fear-potentiated startle seemed to be affected, where MALT animals showed generalized blunted responses. These findings suggest that adverse postnatal caregiving experiences have a long term impact on the development of emotional regulation during adolescence.

3.2 Introduction

Early life stress (ELS), including adverse caregiving experiences such as childhood maltreatment, is a major risk factor for the emergence of psychopathology during adolescence, which includes poor emotional and stress regulation seen in anxiety and

mood disorders, substance abuse and behavioral disorders (Cicchetti and Toth, 2005; Douglas*, et al.*, 2010; Sinha, 2008). Despite the strong link of ELS and the emergence of anxiety and psychiatric disorders with alterations in fear responses and other cognitive and physiological alterations during adolescence, this period is understudied, and the underlying neurobiological mechanisms of risk not understood.

Adolescence, beginning at the onset of puberty, is a crucial period in development between childhood and adulthood, which involves many physical, neuroendocrine, cognitive, social, and emotional changes (Graber and Brooks-Gunn, 1996; Schulz*, et al.*, 2009; Sisk and Zehr, 2005). In addition to childhood, this is another critical period of brain development and remodeling of brain structures during which cortical gray matter reaches a peak of volume growth before declining to reach adult volumes. This pattern of gray matter maturation is likely due to an increase in synaptogenesis and dendritic elaboration followed by synaptic pruning during adolescence. It may also explain switches in functional connectivity between brain structures within a functional network and alterations in functional neurocircuitry may be caused by ELS (Boyce and Ellis, 2005; Gee*, et al.*, 2013; Goddings*, et al.*, 2014; Sisk, 2017; Steinberg, 2005). Adolescence is also a phase when changes to fear and reward circuitry occurs, and emotional reactivity is heightened, especially among females (Casey*, et al.*, 2008; Myers and Davis, 2004; Silberg*, et al.*, 1999).

Many of the neurodevelopmental changes taking place during adolescence impact fear learning, expression and extinction neurocircuitry. Previous fear conditioning studies in humans have shown that fear-potentiated startle increases in the transition from childhood to adolescence (Glenn*, et al.*, 2012), and that adolescents show attenuated fear extinction learning and retention in comparison to both adults and younger humans and mice (Casey*, et al.*, 2015; Kim*, et al.*, 2011a; McCallum*, et al.*, 2010; Pattwell*, et al.*,

2012). Other differences (at least in rodents) include a transition from simple forms of fear conditioning in pre-adolescence to more complex aspects of fear learning, such that later in adolescence, better discrimination between CSs is expected (Lau*, et al.*, 2011)Failure to develop such complex fear learning capabilities during adolescence may, indeed, suggest increased vulnerability for anxiety disorders throughout one's lifetime (Britton*, et al.*, 2011). The neurodevelopmental mechanisms underlying these striking changes in fear learning and extinction during adolescence are not clearly understood, but there is evidence that changes in PFC-AMY circuits may be involved. Thus, in rodents, suppression or blunting of extinction learning co-occurs with an absence of mPFC extinction learning-induced plasticity (Kim*, et al.*, 2011a; Pattwell*, et al.*, 2012), which may be influenced by an imbalance in inhibitory synaptic transmission during adolescence, due to delayed development of cortical GABAergic transmission (Chattopadhyaya*, et al.*, 2004; Kilb, 2012). Overall, imbalanced developmental rates in adolescent subcortical-prefrontal circuitry likely contribute to developmental shifts in fear regulation, from strictly subcortical-driven fear learning to a more flexible circuit including prefrontal regions in adulthood (Somerville and Casey, 2010).

Physiological changes that occur during puberty, in particular increased levels of gonadal hormones, could be responsible for those neurodevelopmental changes, since they have been linked with underlying neural maturation (Bramen*, et al.*, 2011; Paus*, et al.*, 2008). Puberty is marked by initial significant axon and synapse production, followed by rapid synaptic pruning and myelination throughout the brain, especially in late maturing cortical and limbic regions, such as the PFC and amygdala (De Bellis*, et al.*, 2001; Powell, 2006; Sisk, 2017; Spear, 2000). Thus, this large neural reorganization marks the opening of an adolescence critical window for experience-dependent rewiring of circuits involved in stress/emotional regulation and reward (Casey*, et al.*, 2008; Sisk, 2017).

Consistent with abundant human evidence linking ELS with poor emotional/stress regulation, as seen in anxiety and mood disorders (Cicchetti and Toth, 2005; Douglas*, et al.*, 2010), our group has reported that infant maltreatment also leads to increased emotional reactivity, hyperactivity of stress neuroendocrine systems and impulsive aggression in juvenile (prepubertal) macaques (Howell*, et al.*, 2013; Howell*, et al.*, 2014; Howell BR, 2012; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Sanchez, 2006; Sanchez*, et al.*, 2010); and increased attention to threat during adolescence (Morin*, et al.*, 2019). It is unclear whether this is due to enhanced fear learning/conditioning or impaired ability to modulate fear responses by safety cues, or both. Also, it would be important to understand the potential long-term, pervasive, impact in fear responses during adolescence in this translational NHP model. The fear-potentiated acoustic startle paradigm has been used to examine how fear learning processes are affected by not only stress/trauma experience, psychopathology, but also by neurodevelopmental and neuroendocrine changes (Jovanovic*, et al.*, 2013). Using these paradigms, enhanced acoustic startle and inability to modulate the fear response in the presence of a safety cue, have been proposed to be translational biomarkers for anxiety/fear-related disorders, including PTSD, in humans and animal models (Christianson*, et al.*, 2012; Grillon and Baas, 2003; Jovanovic*, et al.*, 2012; Jovanovic*, et al.*, 2014; Norrholm*, et al.*, 2011). While childhood maltreatment has been associated with increased startle reactivity in adulthood (e.g. (Jovanovic*, et al.*, 2009)), there is disagreement among the few studies on the effects of ELS/trauma on startle during adolescence. For example, baseline acoustic startle in adolescent girls with mixed trauma exposure (some to multiple traumas), showed no differences compared with adolescents that had not experienced trauma (Lipschitz*, et al.*, 2005). Another study, though, including students in late childhood-early adolescence (8-13 year-olds) that experienced sniper fire on the school playground, showed that children with PTSD had decreased baseline startle than

control children (Ornitz and Pynoos, 1989). However, in a study of maltreated children (3-11 years), sex by type of abuse interactions were found on baseline acoustic startle, with physically abused boys showing increased startle compared to non-maltreated boys, whereas girls showed differences related to age, such that older maltreated girls startled more than non-maltreated girls (Klorman*, et al.*, 2003). Increased baseline startle has been proposed as either an adaptation, either short- or long-term, to a threatening environment or a vulnerability towards anxiety (Grillon*, et al.*, 1996). Given the additional evidence in humans of impaired extinction during adolescence, especially among anxious adolescents (Lau*, et al.*, 2008; Pattwell*, et al.*, 2012; Shechner*, et al.*, 2015), maintaining high startle responses regardless of the presence of a threat may prove maladaptive and lead to generalized fear responses (i.e. difficulties discriminating between threatening and safe situations), and also extinguishing fear towards previously threatening stimulus that has become safe.

The Acoustic startle reflex is controlled by a simple circuit, composed of inputs to the cochlear root neurons, which project to the nucleus reticularis pontis caudalis, then to the spinal motor neurons and finally to muscles (Davis*, et al.*, 1982; Lee*, et al.*, 1996). Previous studies have reported that other regions, such as the amygdala, hippocampus, and bed nucleus of the stria terminalis, modulate this startle circuit when an organism is faced with an emotionally valent stimulus (Davis, 2006; Lee and Davis, 1997). Fear-potentiated startle, or the increase in the amplitude of the acoustic startle reflex in the presence of a conditioned stimulus (CS), develops after repeated pairings of the CS and an aversive event (unconditioned stimulus –US) (Davis, 2006), despite stable baseline startle amplitudes (Kazama, et al., 2013). Fear-potentiated startle paradigms are a critical tools for evaluating fear and safety-signal learning and allow for fear potentiation and inhibition to be disentangled and independently assessed, to investigate if the two processes may be uniquely altered following ELS. As an example, previous clinical

studies of humans with PTSD have reported that, despite normal levels of fear potentiation, patients have trouble inhibiting fear responses (Grillon and Morgan, 1999). The AX+/BX- fear conditioned inhibition paradigm has generated consistent findings across species, including studies on rats (Myers and Davis, 2004), nonhuman primates (Kazama*, et al.*, 2012; Kazama*, et al.*, 2013; Winslow*, et al.*, 2008), and humans (Jovanovic*, et al.*, 2005; Jovanovic*, et al.*, 2012). In this paradigm, two cues (visual stimuli: A and X) are presented side by side and paired with an aversive stimulus (a shock in rats, and an air blast in humans and monkeys) in AX+ trials. The X is also presented in separate trials with another visual cue (B), which signals absence of the aversive shock/air blast (BX- trials). With repeated pairings, fear develops towards A, as it is paired consistently with the aversive event. In contrast, decreased fear develops towards B, as it is never paired with the shock/air blast and predicts absence of threat. Some excitement (i.e. aversiveness) may be transferred to the X cue as it is associated with both A (aversive consequences) and B (absence of aversive consequences). The transfer of the inhibition or attenuation of fear by the "safety cue" B can be assessed by the presentation of a pairing with the aversive cue, in an AB trial (Kazama*, et al.*, 2013). Decreased startle amplitude is expected to the AB compared to AX trials, demonstrating the transfer of fear inhibition/modulation. Following this one-time AB test pairing, extinction of fear-conditioning is tested by presenting the AX together, although no longer paired with the aversive stimulus (i.e. no shock/no air-blast), supporting the formation of a new association of cue AX with the absence of threat, and allowing to assess the extinction of fear towards AX over repeated presentations. The AX+/BX- paradigm developed in macaques (Kazama*, et al.*, 2012; Kazama*, et al.*, 2013; Winslow*, et al.*, 2008) takes advantage of the dominance of visual communication in monkeys to discriminate stimuli presented on a computer screen, thus increasing the similarities to paradigms used to study fear learning in humans (Jovanovic*, et al.*, 2012), and therefore

its translational relevance.

Rhesus monkeys provide an ethologically valid, translational animal model to study how ELS affects neurobehavioral development in adolescence, and to address questions unresolved in human studies, as: 1) infant maltreatment occurs spontaneously in macaques, resulting in increased emotional reactivity and attention to threat, similar to reports in children (Morin*, et al.*, 2019; Sanchez, 2009), 2) postnatal effects of ELS can be disentangled from those of heritable and prenatal factors through a cross-fostering design with random assignment to caregiving group at birth (used in the experimental design of this study), 3) prospective, longitudinal analysis of neurobehavioral development can be done with high experimental control since birth, and 4) maltreatment results in neurodevelopmental alterations in cortico-limbic brain circuits involved in emotional and stress regulation in macaques (Howell*, et al.*, 2013; Howell*, et al.*, 2014; Howell*, et al.*, 2019; Morin EL, 2016). Utilizing macaques allows for more experimental control within this paradigm, including testing over an extended timeline so that we can examine the trajectory of learning during discrimination training and extinction of learned associations with fear, over many months of testing, which would be challenging to do in humans. Studies during adolescence with this translational NHP model will provide unique information of relevance for human development, particularly on long-term alterations in emotional regulation, specifically fear and safety learning, in populations with histories of childhood maltreatment.

Using this rhesus model of MALT, the main goal of this study was to test the hypothesis that ELS results in long-term impacts on state anxiety, fear and safety learning, as well as in fear extinction during adolescence using a translational AX+/BX- fear-potentiated startle paradigm developed for macaques. Baseline startle will be used as a measure of state anxiety, followed by measures of fear conditioning, extinction and the ability to use safety signals to attenuate/modulate fear-potentiated startle responses. We hypothesize

that maltreated animals will have higher baseline and fear-conditioned startle, take longer to learn to discriminate fear/safety cues, show impaired attenuation of startle by safety signals, and show impaired extinction –effects that will be more robust in maltreated females than males. This research will significantly impact our understanding of the behavioral underpinnings of ELS-induced emotional alterations during adolescence.

3.3 Methods

3.3.1 Subjects

This study involved 25 post-pubertal, adolescent rhesus macaques (13 males, 12 females) between the ages of 4.5-5.5 years old. These animals were generated and well-characterized throughout infancy and the juvenile period as part of previous studies (Drury*, et al.*, 2017; Howell*, et al.*, 2013; Howell*, et al.*, 2014; Howell*, et al.*, 2019; Howell BR, 2012; Morin*, et al.*, 2019; Morin EL, 2016; Morin EL, 2015; Sanchez MM, 2011). Raised with their mothers/families in large social groups at the Yerkes National Primate Research Center (YNPRC), half of these animals experienced maternal maltreatment (MALT) (MALT, n=14; 8 males, 6 females), and the other half received competent maternal care (Control, n=11; 5 males, 6 females). In this model, infant maltreatment is defined by maternal physical abuse and rejection of the infant during the first three months of life, which causes emotional distress and elevations in stress hormones (Drury*, et al.*, 2017; Howell*, et al.*, 2013; Howell BR, 2012; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Sanchez, 2006; Sanchez MM, 2011). Physical abuse is operationalized as violent maternal behaviors that cause pain and distress in the infants –e.g. crushing/throwing/dragging the infant- (Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006; Sanchez, 2006), never shown by competent mothers.

Each animal was crossfostered at birth and randomly assigned to a control or MALT foster mother, counterbalancing groups by sex, biological mother, and social rank and assigning infants from different matrilines & paternities to provide high genetic/social diversity, as previously reported (Drury*, et al.*, 2017; Howell*, et al.*, 2017). Based on previous studies demonstrating birth weight as a strong predictor of neurobehavioral development in NHPs and humans (Coe and Shirtcliff, 2004; Vohr*, et al.*, 2000), only infants ≥450gr birth weight-a safe veterinary clinical cut off to rule out prematurity in rhesus- were included in this study (see Table 3.1 for details of infant crossfostering assignment and counterbalancing of groups).

At approximately 4 years of age, animals were transferred to the YNPRC Main Station (Atlanta, GA), and housed in pairs in cages to provide social contact with other animals. Animals were fed a monkey chow diet (Purina Mills LCC, St. Louis), supplemented with fresh fruits and vegetables daily, with water *ad libitum*. The animal facility maintains an ambient temperature of 22 ± 2°C with 25-50% humidity, and lighting is based on a 12-h light/dark cycle (lights on - 0700; lights off - 1900). Environmental enrichment, such as toys, were provided in the home cage on a regular basis. Body weights of the animals were monitored on a monthly basis throughout the study. Following an adjustment period of several months to acclimate to the new housing and environment, the animals were tested on several behavioral tasks, including the fear-potentiated startle paradigm and attention bias to threat (Morin*, et al.*, 2019), and underwent stress neuroendocrine assessments (hypothalamic-pituitary-adrenal(HPA) axis function) and MRI scans, as a part of a larger study investigating the long-term socioemotional, cognitive and neurobiological consequences of ELS into adolescence.

All procedures and animal care were in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services “Guide for the Care and Use of Laboratory Animals” and approved by the Institutional Animal Care and Use

Committees (IACUC) of Emory University.

3.3.2 Fear-Potentiated Startle Testing Procedure

All sessions occurred at least 48 hours apart, and session length varied between 23 and 58 minutes depending upon the stage of training. Throughout the testing phase, animals were not deprived of food or water and were provided with fresh fruit and vegetables daily. Additional treats approved by a veterinarian were provided during chair training and following each session. Animals were observed at least every 15 minutes via webcam to monitor for safety. The methods summarized here follow previously published methods in macaques (Antoniadis*, et al.*, 2007; Kazama*, et al.*, 2012; Kazama*, et al.*, 2013; Winslow*, et al.*, 2002; Winslow*, et al.*, 2008). Table 3.2 outlines the stages of training and testing in the AX+ BX- Procedure.

Apparatus: Animals were trained and habituated to sitting in a primate chair mounted on a load cell (Med Associates, St. Albans, VT) in a sound attenuated chamber equipped to deliver unconditioned and conditioned stimuli (visual and tactile), following published procedures (Christianson*, et al.*, 2012; Jovanovic*, et al.*, 2012; Kazama*, et al.*, 2012; Kazama*, et al.*, 2013; Kazama*, et al.*, 2014). Startle displaces the load cell (Sentran YG6-B-50KG-000) creating a signal that is amplified, digitized, and recorded as the startle amplitude (SA). A constant background white noise (65dB) served to further drown out potentially distracting sounds occurring outside the testing room.

Stimuli: Acoustic Probe - A 50-msec white noise burst (5 ms rise-decay time) of intensities ranging from 95-120dB was emitted by a white noise generator and delivered

over top of the background (65dB) white noise, to provoke the acoustic startle reflex. Unconditioned stimuli (US) - The aversive stimulus consisted of a 700-msec jet of air (100 PSI) projected at the face of the animal via four air jet nozzles, which was generated by an air compressor placed outside the chamber. Conditioned Stimuli (CS) – Visual cues consisted of an aversive conditioned stimulus (A), a safety conditioned stimulus (B) and a neutral stimulus (X) that was paired with A or B and concentrated the association to fear or safety onto the A or B cue respectively. The pairing with X to create compound cues AX and BX also served to reduce latent inhibition by habituating the animals to the presence of two stimuli presented on the screen so that during the transfer test (when AB is first presented), seeing a compound stimulus is not novel.

Baseline acoustic startle response: Animals were tested across two days, each session consisting of 60 trials composed of startle activity without an acoustic probe (10 trials), and startle responses to acoustic probes (noise burst) of varying intensities (0, 95, 100, 110, 115, and 120 dB –10 trials each-). All trials were pseudorandomly intermixed within each session, and no visual stimuli or US (aversive stimulus: air jet) were presented during these sessions. Pre-training acoustic startle response was assessed in follicular phase for females to control for variations due to ovarian phase (Wilson*, et al.*, 1982; Wilson*, et al.*, 1988). An additional two days of baseline acoustic startle response testing was performed 48-72 hours following the extinction phase to evaluate any potential effects of the AX+BX- task procedure on state anxiety (post-training baseline startle), which has been reported to increase (Walker and Davis, 1997).

Pre-training without startle (CS only): This phase served to habituate the animals to the presentation of visual stimuli on the computer screen in the same chamber. All animals completed two sessions, which occurred 48-72 hours apart and consisted of 30

trials of which A, B, X, and compound cues AX and BX were presented without an acoustic probe. Each session began with a 5-minute period of acclimation before stimuli appeared for 10 sec at 30-sec inter-trial intervals (ITI). No data was collected during these sessions as no acoustic probes were used to provoke an acoustic startle reflex.

Pre-training with startle (CS & Acoustic Probe): After 72 hours, startle was elicited by a 95-dB startle acoustic probe (noise burst) while single or compound cues were presented on the screen to habituate animals to the presence of visual cues as well as evaluate unconditioned associations with the cues that would influence startle response before conditioning. Sessions consisted of 60 trials, half of which were the acoustic startle probe alone (95 dB) and the other half in which the startle noise occurred in the presence of one of the cues to appear in subsequent conditioning (A, B, X, AX, BX, AB), presented 5 trials each pseudorandomly. During cue-startle trials, the acoustic probe occurred 10 seconds after the onset of the visual cue. Sessions were repeated until presentation of the cues elicited <100% increase in the median startle amplitude to the cue compared to noise alone trials, for a minimum of two sessions.

Discrimination Training (AX+/BX−): In this phase, pairing of the AX and aversive US air jet/puff (+) began, in order to train the animals to learn to associate the compound cue AX with this puff, as the aversive AX+ stimulus. The compound BX was also presented, although not paired with the air puff (-), as the BX- safety cue. Animals were trained to discriminate between cues during 50 minute sessions, beginning with a 10-minute period of habituation to the chamber (no stimuli or noises were presented), followed by pseudorandom presentations of the sequence: acoustic probe trial – 1 minute ITI – CS pairing – 1 minute ITI – CS pairing – 1-minute ITI; where CS pairing will be AX+ (cue/puff pairing), AX (cue/startle noise), or BX- (cue/startle noise). Each

CS pairing was presented in 6 trials each at 95 dB and 6 trials at 105 dB in each session. % Fear-potentiated startle (FPS) was computed as:

$$\frac{(\text{startle amplitude } \textbf{with CS}) - (\text{startle amplitude } \textbf{noise alone})}{(\text{startle amplitude } \textbf{noise alone})} \times 100$$

Animals continued AX+/BX– training (i.e. fear/safety discrimination) until the difference in %FPS between AX and BX reached 100% or more for two sessions, which were not required to be consecutive.

Transfer test (AB)/Conditioned Inhibition: Once animals reached the criteria for fear/safety discrimination, animals underwent a one-day test for conditioned inhibition, or transfer, 48-72 hours following the last day of discrimination training. This test allowed for the evaluation of potential modulatory effects of B on A; that is, whether the presence of the safety cue (B) in combination to the fear cue (A) reduced fear-potentiated startle to the latter, and therefore lowered %FPS to AB in comparison to AX. This 48-trial session consisted of two AX+ air-blast pairings intermixed within (a) 95 & 105 dB acoustic probe alone trials (6 trials each), (b) A, B (5 trials each per probe intensity - 95 & 105 dB), (c) AX, BX (1 trial each per probe intensity - 95 & 105 dB), and (d) AB (5 trials per probe intensity - 95 & 105 dB), all presented in a pseudo-random fashion. Performing the discrimination training in this manner, the transfer of conditioned fear on the AB trial could not be explained by configural learning, or the animals perceiving the AB as a unique stimulus (Myers and Davis, 2004).

Extinction: All animals were presented with 12-trial sessions consisting of the 95 dB startle acoustic probe (noise burst) either presented alone or co-occurring with the presentation of the A and AX stimuli (without the aversive air puff), presented four times

each in an interleaved fashion, in order to evaluate fear extinction. Animals continued extinction sessions until %FPS to the A and AX were both <100%, indicating the animal's startle amplitude had returned to pre-training levels.

3.3.3 Statistical Analysis

Startle data was evaluated following each session for consistent aberrant activity, such as that caused from an animal continuously shaking/spinning in the chair. Since no such behavior was detected, no additional habituation to the chamber was necessary, and data from all animals and sessions was included in the analysis. Because neither startle amplitude (in mV) nor calculated %FPS were normally distributed, statistical analyses were performed on a natural log-transformation of these measures. The first trial of each session was not included in the analysis for each phase to avoid abnormal activity at the beginning of the session following a period of habituation without acoustic probe (noise burst)/visual cues. Abnormal activity was identified during discrimination, with unusually high %FPS on the first trial of each session, likely due to contextual conditioning (Christianson*, et al.*, 2012; Grillon and Morgan, 1999). When analyzing group and sex differences in trajectories of fear learning acquisition and extinction, only the first two days of fear/safety discrimination training and fear extinction were analyzed because these were the only two days with data available for all animals, as some subjects reached criteria for these 2 measures on the second day and then moved onto the next phase of testing. The median was used to quantify the startle amplitudes (mV) central tendency across sessions instead of the mean to avoid bias due to potential movements of the animal during measurement periods unrelated to the startle reflex.

Repeated measures ANOVA were conducted for each phase to examine group, sex, day, trial or dB main or interaction effects on baseline acoustic startle amplitude,

discrimination between fear (AX+) and safety (BX-) cues, attenuation of conditioned fear to A in the presence of B in the transfer test (AB), and reduction in %FPS to the AX when presented without the air puff during extinction.

Two-way ANOVAS were conducted to examine group and sex effects on the number of days to reach criterion to discriminate the fear (AX+) and safety (BX-) cues (i.e.difference in 100 %FPS between AX+ and BX-), and the number of days to reach criterion for extinction (return to pre-training levels of %FPS).

Mauchly's Test of Sphericity was used to test whether the assumption of sphericity had been violated, and if so, a Greenhouse-Geisser correction was used. Sex was included in all models as a factor in addition to group. Days of training to reach criterion for discrimination or extinction were included as covariates in statistical models of phases following training and extinction, respectively. All statistical analyses were performed using R and SPSS 25.0 software (Corp, 2017; Team, 2018), with p value set at <0.05 for significant effects.

3.4 Results

3.4.1 Baseline acoustic Startle Response

Pre-Training Baseline Startle: RM ANOVA conducted to examine the effect of group, sex, acoustic probe intensity (dB) and on acoustic startle amplitude (mV) detected a significant main effect of group on startle amplitude ($F_{(1,21)}=5.0$, $p=0.0367$, $\eta^2=0.19$), with MALT animals having higher startle compared to controls across all dB (Figure 3.1). There was also a significant main effect of dB intensity on startle amplitude ($F_{(1,21)}=11.1$, $p=0.0006$, $\eta^2=0.35$), with startle increasing with increasing dB intensity in both groups. Additionally, a trend was found for the interaction between dB intensity x day on startle amplitude ($F_{(1,21)}=2.4$, $p=0.0558$, $\eta^2=0.10$).

No other main effects of sex ($F_{(1,21)}$=0.5, p=0.47, η^2=0.03), or day ($F_{(1,21)}$=0.01, p=0.92, η^2=0.0005) or interaction effects (group x sex ($F_{(1,21)}$=0.001, p=0.98, η^2=0.000045), dB x sex ($F_{(1,21)}$=0.2, p=0.79, η^2=0.01), dB x group ($F_{(1,21)}$=0.09, p=0.87, η^2=0.004), day x group ($F_{(1,21)}$=2.4, p=0.26, η^2=0.06), or day x sex ($F_{(1,21)}$=2.7, p=0.11, η^2=0.12)) were detected.

An additional two-way RM ANOVA was conducted to rule out group differences in general movement measured during the 0 dB trials (non-startle related). No main effects of group ($F_{(1,21)}$=1.9, p=0.18, η^2=0.08), sex ($F_{(1,21)}$=0.04, p=0.84, η^2=0.002), day ($F_{(1,21)}$=3.4, p=0.080, η^2=0.14), or interaction effects of group x sex ($F_{(1,21)}$=0.8, p=0.38, η^2=0.04), day x group ($F_{(1,21)}$=0.003, p=0.96, η^2=0.0001), day x sex ($F_{(1,21)}$=3.1, p=0.093, η^2=0.13), were detected.

Post-Training Baseline Startle: RM ANOVA was used to examine the effects of group, sex, acoustic probe intensity (dB) and day, on acoustic startle amplitude (mV), with days to reach criterion in discrimination training and in extinction as covariates. A main effect of dB intensity was detected ($F_{(1,21)}$=5.8, p=0.0032, η^2=0.24), with startle increasing with increasing dBs for both groups (Figure 3.2).

No main effects of group ($F_{(1,21)}$=1.0, p=0.34, η^2=0.05), sex ($F_{(1,21)}$=1.3, p=0.28, η^2=0.06) or day ($F_{(1,21)}$=0.08, p=0.79, η^2=0.004) were detected. No interaction effects were detected, either (decibel x day ($F_{(1,21)}$=0.8, p=0.52, η^2=0.04), decibel x group ($F_{(1,21)}$=0.8, p=0.49, η^2=0.04), decibel x sex ($F_{(1,21)}$=1.5, p=0.22, η^2=0.08), day x group ($F_{(1,21)}$=1.2, p=0.29, η^2=0.06), day x sex ($F_{(1,21)}$=0.4, p=0.53, η^2=0.02), or group x sex ($F_{(1,21)}$=1.4, p=0.26, η^2=0.07),).

However, days that to reach criterion during fear/safety discrimination training was a significant covariate ($F_{(1,21)}$=12.4, p=0.0023, η^2=0.4) with higher post-baseline startle

shown by animals that took longer to discriminate fear/safety cues during training. Days to reach criterion for extinction, did not have significant effects as covariate ($F_{(1,21)}$=0.2, p=0.66, η^2=0.01).

An additional RM ANOVA was conducted to rule out group differences in general movement measured during the 0 dB trials (non-startle related). No main effects of group ($F_{(1,21)}$=0.2, p= 0.62, η^2=0.01), sex ($F_{(1,21)}$=0.2, p=0.66, η^2=0.009) or day ($F_{(1,21)}$=1.7, p= 0.20, η^2=0.08), or interaction effects of group x sex ($F_{(1,21)}$=0.1, p= 0.75, η^2=0.005), day x group ($F_{(1,21)}$=0.08, p= 0.78, η^2=0.004), day x sex ($F_{(1,21)}$=0.9, p= 0.34, η^2=0.04), were detected.

Pre vs Post Training Baseline Startle Comparison (95 dB): RM ANOVA used to examine group, sex and Baseline phase (Pre vs Post-Training; averaged across the two days per Baseline phase), detected a trend for the factor group ($F_{(1,21)}$=4.3, p= 0.0512, η^2=0.17) (Figure 3.3A) and for group x sex interaction effects ($F_{(1,21)}$=3.7, p= 0.0677, η^2=0.15), suggesting higher startle in MALT females than control females (Figure 3.3B). No other main sex ($F_{(1,21)}$=1.2, p= 0.29, η^2=0.05), baseline phase ($F_{(1,21)}$=2.6, p= 0.12, η^2=0.11), or interaction effects (baseline x group ($F_{(1,21)}$=0.5, p= 0.48, η^2=0.03), baseline x sex ($F_{(1,21)}$=1.4, p= 0.25, η^2=0.06)) were detected.

3.4.2 Fear/safety signal discrimination learning: AX+/BX– training

A two–way ANOVA conducted to examine the effects of group and sex on AX+/BX- discrimination training days to criterion (≥100% difference in %FPS to the fear (AX+) in comparison to the safety (BX-) cue) detected no significant main effects of group ($F_{(1,21)}$=0.1, p=0.75, η^2=0.005), sex ($F_{(1,21)}$=0.006, p=0.94, η^2=0.0003), or interaction effect of group x sex ($F_{(1,21)}$=0.06, p=0.82, η^2=0.003) (Figure 3.4).

The RM ANOVA conducted to examine the effect of group, sex, CS (AX vs. BX), day and trials (after removing the first trial of each day) on changes in %FPS throughout the AX+/BX- discrimination training, detected a significant main effect of trial ($F_{(1,21)}$=9.0, p=0.000004, η^2=0.3), day x trial ($F_{(1,21)}$=5.7, p=0.0004, η^2=0.21) and a trend in group x day interaction ($F_{(1,21)}$=3.7, p=0.0666, η^2=0.15) with MALT animals showing lower %FPS for both AX and BX on day 1 were detected (Figure 3.5). A significant CS x day x trial interaction ($F_{(1,21)}$=0.2, p=0.0160, η^2=0.21) suggests that fear/safety discrimination learning takes place from day 1 to day 2. No other main effects (group ($F_{(1,21)}$=0.05, p=0.83, η^2=0.002), sex ($F_{(1,21)}$= 0.00002, p=1.0, η^2=0.000001), CS ($F_{(1,21)}$=0.0003, p=0.99, η^2=0.00001), or day ($F_{(1,21)}$=1.4, p=0.25, η^2=0.06)) or interaction effects (group x sex ($F_{(1,21)}$=0.1, p=0.75, η^2=0.005), group x CS ($F_{(1,21)}$=2.2, p=0.15, η^2=0.09), CS x sex ($F_{(1,21)}$=0.2, p=0.64, η^2=0.01), day x sex ($F_{(1,21)}$=0.4, p=0.54, η^2=0.02), trial x group ($F_{(1,21)}$=1.4, p=0.26, η^2=0.06), trial x sex ($F_{(1,21)}$=0.5, p=0.71, η^2=0.03), CS x day ($F_{(1,21)}$=0.7, p=0.40, η^2=0.03), CS x trial ($F_{(1,21)}$=1.0, p=0.44, η^2=0.04), were detected. Based on the significant interaction effect of CS x day x trial, day 2 of training was stratified by early versus late acquisition for additional analysis, based on previous studies (Norrholm*, et al.*, 2011), averaging trials 7 and 8 as 'early acquisition' and trials 9 and 10 as 'late acquisition' (see Figure 3.5). Results of the RM ANOVA used to examine the effects of group, sex, CS (AX vs. BX) and Acquisition (early vs. late) on %FPS, detected a main effect of acquisition ($F_{(1,21)}$=35.8, p=0.000006, η^2=0.63, Bonferroni-adjusted p value=0.025) with higher %FPS during early than late acquisition, and an acquisition x CS interaction effect ($F_{(1,21)}$=5.7, p=0.0270, η^2=0.21, adjusted p value=0.025) with %FPS to BX decreasing from early to late acquisition, indicating discrimination learning (Figure 3.6). Upon visualization, figure 3.6 indicated a potential difference between control and MALT groups during late acquisition, so a post hoc

comparison of the means (AX vs BX) was performed separately for each group. However, there were no significant differences in %FPS between AX and BX in the control ($t_{(1,19.9)}$= 1.01, p=0.3229, d=0.43, Bonferroni-adjusted p value=0.025) or MALT groups ($t_{(1,25.8)}$= 0.98, p=0.3352, d=0.37, Bonferroni-adjusted p value=0.025).
No other main effects (CS ($F_{(1,21)}$=0.6, p=0.43, η^2=0.03), group ($F_{(1,21)}$=0.4, p=0.53, η^2=0.02), sex ($F_{(1,21)}$=0.2, p=0.68, η^2=0.008)) or interaction effects (acquisition x group ($F_{(1,21)}$=0.02, p=0.89, η^2=0.001), acquisition x sex ($F_{(1,21)}$=0.2, p=0.63, η^2=0.01), CS x group ($F_{(1,21)}$=0.6, p=0.43, η^2=0.03), CS x sex ($F_{(1,21)}$=0.6, p=0.45, η^2=0.03), group x sex ($F_{(1,21)}$=0.02, p=0.89, η^2=0.001), group x acquisition x CS ($F_{(1,21)}$=0.03, p=0.8718, η^2=0.001)) were detected.

3.4.3 Transfer Test (Conditioned Inhibition)

The two-way RM ANOVA conducted to examine effects of group, sex and CS (AX vs. AB) on startle response amplitude (mV), with days of discrimination training as a covariate, detected significant main effects of CS ($F_{(1,21)}$=4.9, p=0.0387, η^2=0.20), with higher %FPS to AX than AB, group ($F_{(1,21)}$=8.1, p=0.0099, η^2=0.29) with higher %FPS in Control than MALT animals, and sex ($F_{(1,21)}$=4.8, p=0.0405, η^2=0.19) with males displaying higher %FPS than females (Figure 3.7). Upon visualization, figure 3.7 showed a potential group difference in conditioned inhibition (AX vs. AB) that was tested via post hoc comparisons of the means. However, there were not significant differences in startle to the AX vs. AB among control ($t_{(1,19.6)}$=1.5, p=0.1493, d=0.64) or MALT ($t_{(1,22.3)}$=0.1, p=0.9178, d=0.000003) animals. No interaction effects were detected (group x sex ($F_{(1,21)}$=1.7, p=0.20, η^2=0.08), CS x group ($F_{(1,21)}$=1.5, p=0.23, η^2=0.07), CS x sex ($F_{(1,21)}$=0.3, p=0.60, η^2=0.01), CS x training ($F_{(1,21)}$=3.1, p=0.096, η^2=0.13)). Days to reach criterion during discrimination training did not have a significant covariate effect, either ($F_{(1,21)}$=1.0, p=0.33, η^2=0.05).

3.4.4 Extinction

A two–way ANOVA was conducted to examine the effects of group and sex on extinction days to criterion, with days to reach criterion during AX+/BX- discrimination training as a covariate. No significant main effects of group ($F_{(1,20)}$=1.1, p=0.32, η^2=0.05), sex ($F_{(1,20)}$=2.6, p=0.13, η^2=0.11), or group x sex interaction effect ($F_{(1,20)}$=2.6, p=0.12, η^2=0.11) were detected (Figure 3.8). Days to criterion during AX+/BX- discrimination training was not a significant covariate, either ($F_{(1,20)}$=0.06, p=0.80, η^2=0.003).

A RM ANOVA was conducted to examine the effects of group, sex and Extinction phase ("early" vs. "late") on %FPS to AX (without air puff pairing), with days to criterion for AX+/BX- discrimination as a covariate. Trials 2 and 3 of day 1 were averaged and examined as 'early extinction' and trials 3 and 4 of day 2 (Figure 3.9) were averaged and examined as 'late extinction' (Figure 3.10). No main effects of extinction phase (early vs. late; $F_{(1,18)}$=2.1, p=0.16, η^2=0.10), group ($F_{(1,18)}$=0.01, p=0.91, η^2=0.001) or sex ($F_{(1,18)}$=0.6, p=0.44, η^2=0.03), were detected. No interaction effects were detected, either (group x sex ($F_{(1,18)}$=0.2, p=0.66, η^2=0.01), extinction phase x group ($F_{(1,18)}$=0.7, p=0.41, η^2=0.04), extinction phase x sex ($F_{(1,18)}$=1.6, p=0.23, η^2=0.08)). Days to criterion for AX+/BX- discrimination did not have a significant covariate effect, either ($F_{(1,18)}$=0.06, p=0.81, η^2=0.003).

3.5 Discussion

The goal of this study was to examine the long-term impact of ELS on state anxiety, fear and safety learning and fear extinction in adolescent macaques using a translational AX+/BX- fear-potentiated acoustic startle paradigm. The main findings from these

behavioral tests were that MALT animals showed higher baseline startle compared to controls (a measure of state anxiety (Brown*, et al.*, 1951)) prior to fear conditioning and fear/safety discrimination training. During fear (AX+) versus safety (BX-) discrimination training, higher fear-potentiated startle to the fear than to the safety cue emerged between early and late acquisition phases, suggesting discrimination learning in both groups of animals. Although no differences in fear learning or expression (fear-potentiated startle) or extinction were detected in MALT animals, the ability to use safety signals to modulate fear-potentiated startle responses seem to be affected in the transfer (AB) test, where MALT animals showed generalized blunted responses to both CS (AX) and the AB cue in comparison to control animals. These findings suggest that adverse caregiving experiences have a long-term impact on emotional regulation of macaques during adolescence, leading to elevated state anxiety, as well as impairments in early phases of fear/safety discrimination and generalized blunted startle responses when both safety and fear cues are presented together.

An important long-term impact of ELS, including infant maltreatment, is adolescence psychopathology, which includes poor emotional and stress regulation typical of anxiety and mood disorders (Cicchetti and Toth, 2005; Douglas*, et al.*, 2010). This could result from impaired development of underlying regulatory neurocircuits up to adolescence, combined with drastic remodeling taking place during adolescence in fear learning, expression and extinction pathways. It is unclear, though, whether the poor emotional regulation reported in maltreated adolescents is due to enhanced fear learning, expression, extinction and/or impaired ability to modulate fear responses by safety signals in the environment. Addressing these questions was the goal of our study in this NHP translational model of infant maltreatment. Childhood maltreatment has been linked to increased startle reactivity in adult humans (Jovanovic*, et al.*, 2009; Klauke*, et al.*, 2012; Metzger*, et al.*, 1999). Our findings of increased baseline startle (pre-training)

in maltreated animals is consistent with those reports in human adults that experienced child abuse (Jovanovic*, et al.*, 2009); Klauke*, et al.*, 2012; Metzger*, et al.*, 1999). However, while childhood maltreatment has been associated with increased startle reactivity in adults, the startle effects are inconsistent in the studies in children and adolescents with childhood maltreatment or early adversity, ranging from no differences in baseline startle (Lipschitz*, et al.*, 2005) to increased baseline startle, at least in maltreated boys (Klorman*, et al.*, 2003). Our findings are consistent with the Klorman et al (2003) report of increased startle in maltreated children, although we also observed it in females, not just males. Increased baseline startle has been proposed to reflect state anxiety, sometimes as result of long-term adaptations to threatening environments, and it is high in individuals with anxiety disorders, such as PTSD or panic disorder, despite normal fear-potentiated startle (Grillon*, et al.*, 1996; Grillon, 2008) which is what we found in this study. The interpretation of high state anxiety in the maltreated adolescent macaques seems consistent with the developmental pattern of heightened emotional and stress reactivity that was reported during infancy and the juvenile period by our group in a larger cohort of animals, including those in this study (Drury*, et al.*, 2017; Howell*, et al.*, 2013; Howell*, et al.*, 2014; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009) and enhanced inference of threatening stimuli on attentional processes (Morin*, et al.*, 2019). Following pre-training baseline startle, AX+/BX- discrimination training, the transfer test and extinction, we repeated the baseline startle (post-training) to test for stability of the startle response throughout testing. Although there were no significant pre- vs. post-training or group differences in baseline startle, days to reach discrimination criterion was a significant covariate, suggesting animals that took longer and received more training had higher post-training baseline startle. The otherwise stable baseline startle amplitudes throughout testing is consistent with previous baseline acoustic startle in monkey (Winslow*, et al.*, 2007) and human (Cadenhead*, et al.*, 1999; Schwarzkopf*, et al.*,

1993) studies without a history of maltreatment. Studies in mice have described a failure of contextual fear retrieval that occurs during adolescence, which may explain why contextual fear is uniquely suppressed during this period, but returns once these animals reach young adulthood (Pattwell*, et al.*, 2011). Perhaps we do not see significant differences across pre- vs. post-training baseline startle due to this suppression of contextual fear, which may be beneficial during adolescence, a period of increased exploration and novelty-seeking (Lee*, et al.*, 2016). Fear generalization to context later increases with age, following maturation of the hippocampal formation and prefrontal cortex (Kim and Richardson, 2010; Rudy, 1993). Thus, it is possible that animals that took longer to discriminate fear/safety cues during training were more vulnerable to develop a generalized anxiety to the testing environment, which may be why, despite normal extinction, they showed higher post-training baseline startle compared to pre-training baseline.

Our findings, however, rejected the rest of our initial hypotheses that MALT animals would have higher fear-conditioned startle, take longer to learn to discriminate fear/safety cues, show impaired attenuation of startle by safety signals or show impaired extinction-. During fear (AX+) versus safety (BX-) discrimination training, higher %FPS to the fear (CSs) than to the safety cue emerges between early and late acquisition phases, suggesting discrimination learning in all animals, although the MALT group showed a trend for impaired discrimination during the early phases. This may be due to the introduced pairing of the aversive air puff with the AX cue on day 1, and increased general fear associated with both cues because the animals are just beginning to learn how to discriminate between the fearful AX and safe BX cues. Because the X cue is included in both the compound AX and BX in this paradigm, X may become slightly aversive because it is paired with the AX half of the time, therefore potentially enhancing the inhibitory effect of B (Kazama*, et al.*, 2013). As expected %FPS to BX decreases from

early to late acquisition on day 2 in both groups, suggesting that there is already some learned inhibition of fear to the BX among both groups as early as the second day of testing, inconsistent with studies in humans with PTSD, in which patients had higher %FPS to the BX compared to the AX (Jovanovic*, et al.*, 2010; Jovanovic*, et al.*, 2012).

Although no differences in fear learning or expression (fear-potentiated startle) or extinction were detected in MALT animals, the ability to use safety signals to modulate fear-potentiated startle responses seem to be affected in the transfer (AB) test (conditioned inhibition), during which MALT animals showed generalized blunted responses to both the CS (AX) and the AB cue in comparison to control animals. In this test, when AB was presented to the animals for the first and only time, %FPS to the AB cue was significantly lower than to the AX, suggesting that there was an inhibitory effect of B on fear responses towards AB, collapsing across groups. The higher %FPS in control than MALT animals, could not be explained by a ceiling effect due to higher MALT baseline startle to the acoustic probe. The generalized blunted FPS response of MALT animals during the transfer test trials could be due to a generalized suppression or "external inhibition" in the presence of ambiguous stimuli (i.e. AB). External inhibition can occur when a novel configuration of cues results in a reduction of the conditioned response to the cue (i.e. AX) (Pavlov, 1927). However, the additional trend towards blunted FPS to both the AX+ and BX- cues in MALT animals during day 1 of discrimination training (Fig. 3.5) strengthens the interpretation of potential blunted FPS responses in the MALT group. Similar blunted physiological responses (skin conductance) to threat cues during fear conditioning and impaired threat vs. safety discrimination have been recently reported in maltreated children -6-18 years old- (McLaughlin*, et al.*, 2016). The authors interpreted those findings as either related to issues of fear generalization –in this case we would suggest generalized suppressed fear responses- or deficits in associative learning (McLaughlin*, et al.*, 2016). Similar blunted

physiological responses to threat have been previously reported following childhood maltreatment, whereby increased corticotropin-releasing hormone (CRH) following trauma results in downregulation of CRH receptors in brainstem and pituitary, resulting in reduced cortisol production and blunting of sympathetic responses to challenges nervous system reactivity (McMillan et al, 2009; McLaughlin et al, 2014a). Thus, blunted emotional and physiological reactivity to threats may be a sequelae of maltreatment, similar to reports in in infants raised in impoverished socioemotional environments (Carlson and Earls, 1997). However, these measures are extracted from one single trial of each CS, which may not be optimal to provide an accurate examination of subtle modulation of %FPS to these cues, as discussed below.

Finally, there were no group effects on extinction (days to criterion or in %FPS differences between early and late extinction). Additionally, no effects of the covariate (days to reach criterion to discriminate fear and safety cues during training) were observed on fear extinction to the AX. This suggests that, even if animals that received more days of training formed a more solid memory of the association of fear to the AX over more sessions, compared to animals that took less days, this did not affect the formation of an inhibitory memory that suppresses fear associations. These findings may conflict with previous human studies reporting difficulties in extinguishing fear in anxious adolescents (Lau*, et al.*, 2008; Shechner*, et al.*, 2015), based on which we would expect maltreated animals that showed higher pre-training baseline startle, suggestive of increased state anxiety, to also show impaired extinction. These maybe species differences or differences in the paradigms and need to be explored further in future studies.

The use of nonhuman primates in this study had the distinct advantage of experimental control, allowing for a long, consistent testing timeline, in which monkeys could be tested every other day throughout the paradigm – which would be incredibly challenging

to do with human subjects. Additionally, we were able to set a criterion level of discrimination performance to be relatively certain that the animal learned to discriminate between fear/safety cues, which would be difficult to establish in the human paradigm, as all training sessions usually occur within the same testing day, and the ability to train to discrimination over a number of days would be constrained, therefore, criterions are not commonly utilized.

Despite the strengths of this study, there are some limitations. Although this study includes a smaller sample size than is seen in human fear-potentiated startle studies, it is a large sample size for similar studies in macaques. The smaller sample used here allows for increased experimental control over environmental variables that confound studies in humans, such as drug use, prenatal exposures, health care, drug use, diet, etc. Another limitation to this study is the conceivable impact of the animals' relocation from the field station to the main station. Although all animals underwent this change, it may have impacted individuals differently, such that more emotionally reactive animals may have experienced more stress to the move, compared to other animals. Amplitude of baseline startle in these control animals are similar to those previously reported (Kazama*, et al.*, 2012; Kazama*, et al.*, 2013; Winslow*, et al.*, 2008). However, these studies also include animals that have been relocated from the field station to the main station (Kazama*, et al.*, 2012; Kazama*, et al.*, 2013; Winslow*, et al.*, 2008), and were also surrogate-nursery-reared (Kazama*, et al.*, 2012). Therefore, to the best of our knowledge there are no published reports that provide levels of startle in "true control" animals that have not experienced this relocation stress for comparison to the control animals in this study.

therefore, there are no true control animals that have not experienced this stress to compare to the control animals in this study. Within, the AX+/BX- paradigm itself, although pre-training baseline startle was collected during the luteal phase for all

females, such control over the timing of testing in respect to ovarian cycle was not feasible for subsequent testing due to variation in testing days per animal (notably discrimination training). Previous studies have shown that the phase of the menstrual cycle can impact acoustic startle response in humans (Armbruster*, et al.*, 2014) and safety-signal learning rodents (Toufexis*, et al.*, 2007), therefore the timing of testing in relation to cycle phase is a likely confounder. Next, there was a wide range of performance in discrimination training, varying from 2-20 days) to reach criterion. Animals that received more days of training, and therefore more exposure to the pairings, could have potentially formed more solid associations of fear and safety to the CSs, and the number of training days was in fact a significant covariate in the post-training baseline startle measures. Additionally, within the discrimination training phase, the 100% difference in FPS between CSs were not required to be consecutive and in some animals, multiple days passed between sessions they met discrimination criterion. Perhaps, restricting this criterion to necessitate consecutive days would more confidently demonstrate learned fear/safety associations. Alternatively, criteria for testing could be removed and all animals could be trained/extinguished the same number of days to examine the trajectory of learning and extinction over the same period of testing, such as is currently used in human paradigms (Jovanovic*, et al.*, 2005; Jovanovic*, et al.*, 2009; Jovanovic*, et al.*, 2012). Finally, within the transfer test, only one presentation of each AX and BX compound cues occurred, constraining us to rely on the accuracy of one measurement of each the AX and BX. Perhaps calculating the median over multiple measurements of startle to the AX and BX would minimize potential aberrant behaviors, as is done in other phases.

In summary, these findings suggest MALT animals have increased state anxiety compared to control animals, and may show blunted emotional reactivity, however, MALT animals show no differences to control animals in their ability to begin to

discriminate between fear and safety, and to form a new memory of safety to the previously fearful cue, extinguishing this association. Further studies are necessary to assess the potential of cycle phase (follicular, luteal, estrous) and gonadal hormone levels on the rest of phases during fear-potentiated startle testing. Likewise, there may also be individual differences in developmental stage at the onset of testing, as the first menarche, ovulation or spermatogenesis cycle is unknown in these animals, it is difficult to determine at what stage in post-pubertal development animals were tested. We did, indeed, collect blood samples from both males and females at critical phases of testing (pre- & post-training baselines, first day of AX+/BX- discrimination training, transfer test), and are currently being analyzed for estradiol and testosterone levels. Adding the levels of these hormones as covariates in the above statistical models will allow to examine the effects of these hormones and more biologically-informed sense of developmental stage on fear learning given a history of early life stress. Additionally, future work is necessary to examine the relationships between these attentional biases and underlying neurobiological functional mechanisms related to anxiety, fear learning, and emotional regulation learning circuitry, which may be altered during development.

Table 3.1

Randomized crossfostering assignment at birth and counterbalancing of groups. The y-axis describes the crossfostering conditions (e.g. C→M identifies infants born to a control biological mother, and fostered by a MALT mother). *Out of n=25 animals, only one control male was not crossfostered. From Morin et al, 2019., with permission.

		FEMALE	MALE	
CONTROL	M→C	3	0	**11**
	C→C	3	5*	
MALTREATED	C→M	1	5	**14**
	M→M	5	3	
		12	**13**	**25**

Table 3.2

AX+BX- Task Summary

Sequential behavioral training stages are listed here with the types of cues (A, B, X) presented and the Acoustic Probe ('Noise' - decibel of the 0.05-sec startle trigger). Adapted from (Kazama*, et al.*, 2012).

Training stages	**Stimuli**	**Startle noise (dB)**
Baseline acoustic startle (pre-training)	Noise alone (NA)	95, 100, 110, 115, 120
Pretraining without startle	All cues (A, B, X, AX, BX)	None
Pretraining with startle	All cues, NA	95
AX+/BX– training	AX/Airpuff, AX/Noise, BX/Noise, NA	95, 105
Transfer test	A/Noise, B/Noise, AX/Airpuff, AX/Noise, BX/Noise, AB/Noise, NA	95, 105
Extinction	A/Noise, AX/Noise, NA	95
Baseline acoustic startle (post-training)	Noise alone (NA)	95, 100, 110, 115, 120

Figure 3.1

Pre-training baseline acoustic startle. Plotted here is the median startle amplitude to the acoustic probe at various dB intensities, across two days of testing. A main effect of group was detected, with MALT animals having higher startle compared to controls. A main effect of dB was also found, with startle increasing with increasing dB intensity. A trend was found for the dB intensity x day interaction. No other main or interaction effects were found for group, sex, day, or dB. Data represented as mean±SEM.

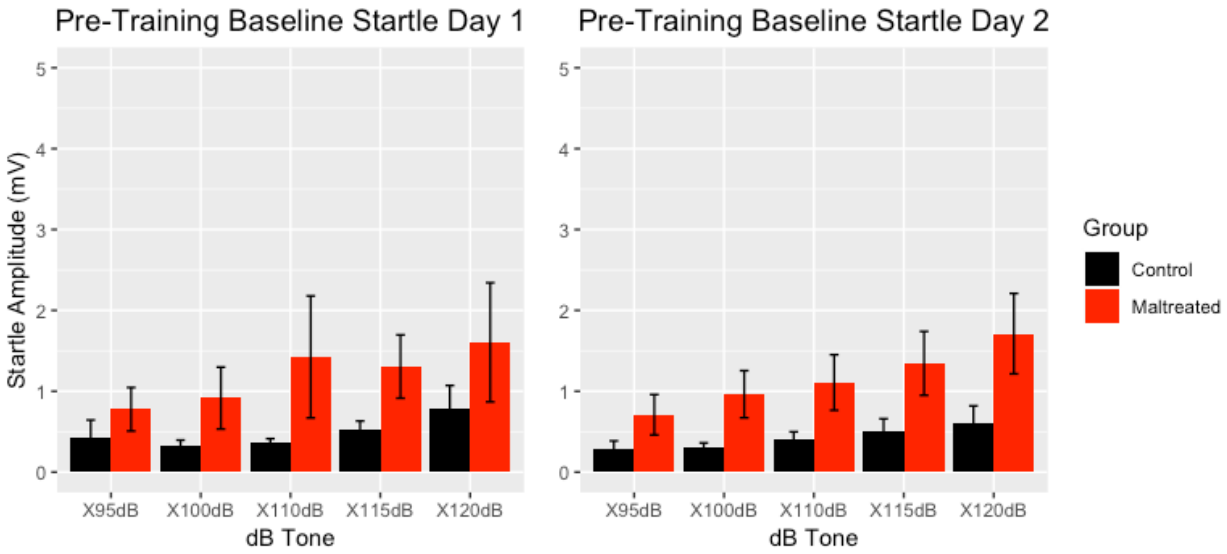


Figure 3.2

Post-training baseline acoustic startle. Startle amplitude to the acoustic probe at various dB intensities, across two days of testing following extinction sessions. A significant main effect of dB, with startle increasing with increasing dB intensity was found. No other significant effects were found, except for a significant covariate effect of days to reach fear/safety cue discrimination criterion during training, with higher baseline startle in animal that took longer to reach criterion. Data represented as mean±SEM.

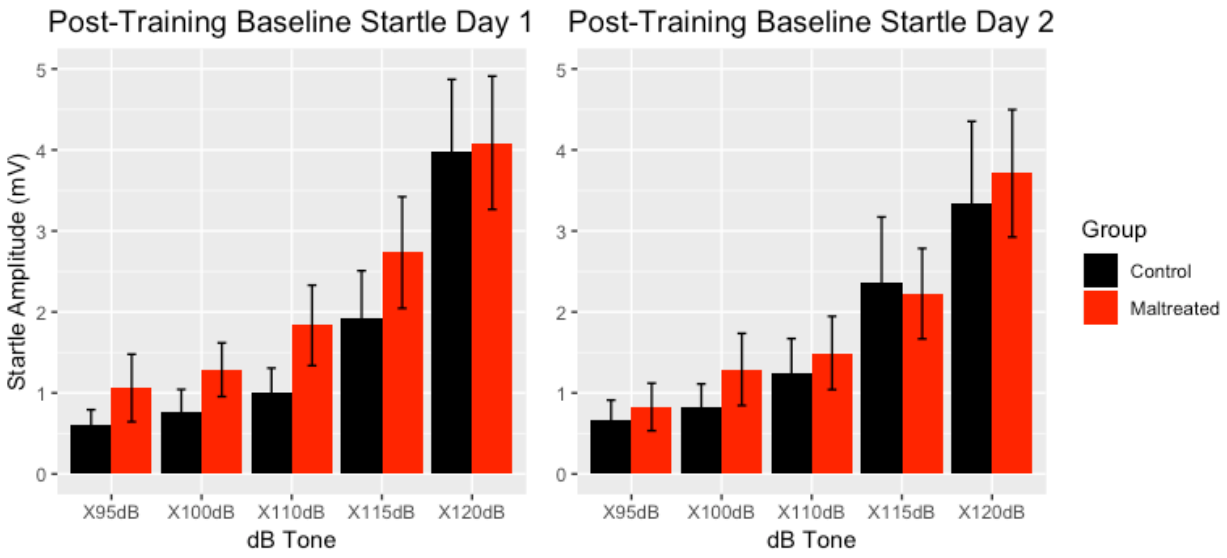
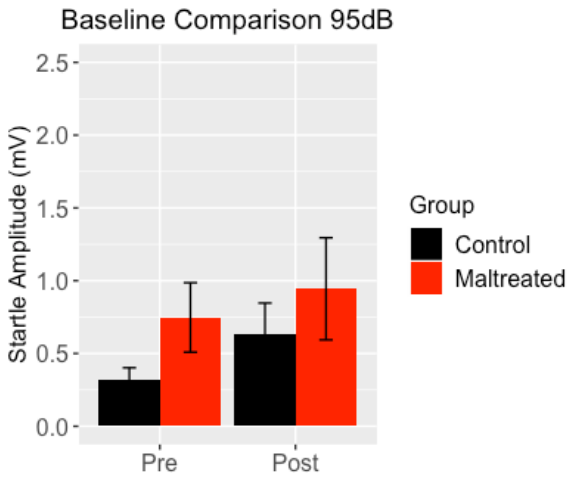


Figure 3.3

Pre vs. post-training baseline acoustic startle. **(A)** Startle amplitude to the 95dB probe, averaged across the two days of baseline startle testing, stratified by pre- versus post-training baseline startle, and collapsed across sexes. Trends for main group and group x sex interaction effects were found. No other effects were found. **(B)** Startle amplitude plotted separately by sex to visualize the group x sex trend, with MALT females displaying higher startle amplitude. Data represented as mean±SEM.

A.



B.

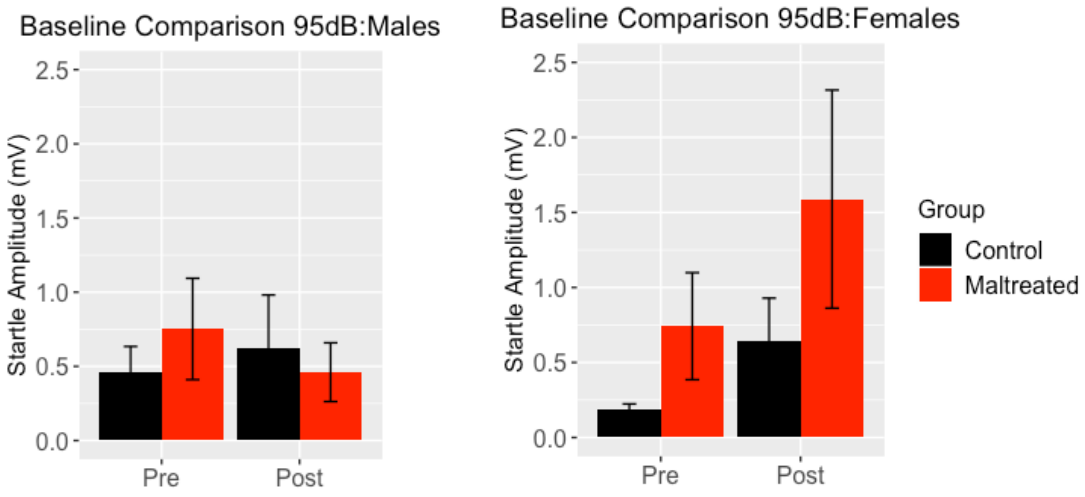


Figure 3.4

AX+/BX- discrimination training, days to criterion. Number of days to reach ≥100% difference in %FPS to the fear (AX+) in comparison to the safety (BX-) cues, subdivided by sex. No main effects of group or sex, or interaction effects were found. Data represented as mean±SEM.

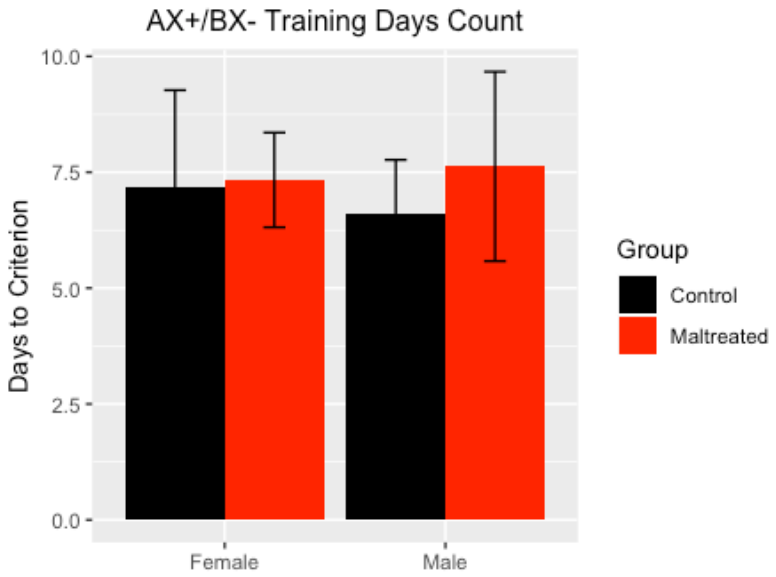


Figure 3.5

AX+/BX- discrimination training trajectory. %FPS to the AX+ and BX- cues is plotted here by trial over the first two days of testing (the blue vertical dotted line divides trials in day 1 from day 2), after removing the first trial of each day. A significant main effect of trial, day x trial interaction and trend in group x day, with MALT animals showing lower %FPS for both AX+ and BX- on day 1, was found. A significant CS x day x trial interaction was detected, too. No other main or interaction effects were found. Data represented as mean±SEM.

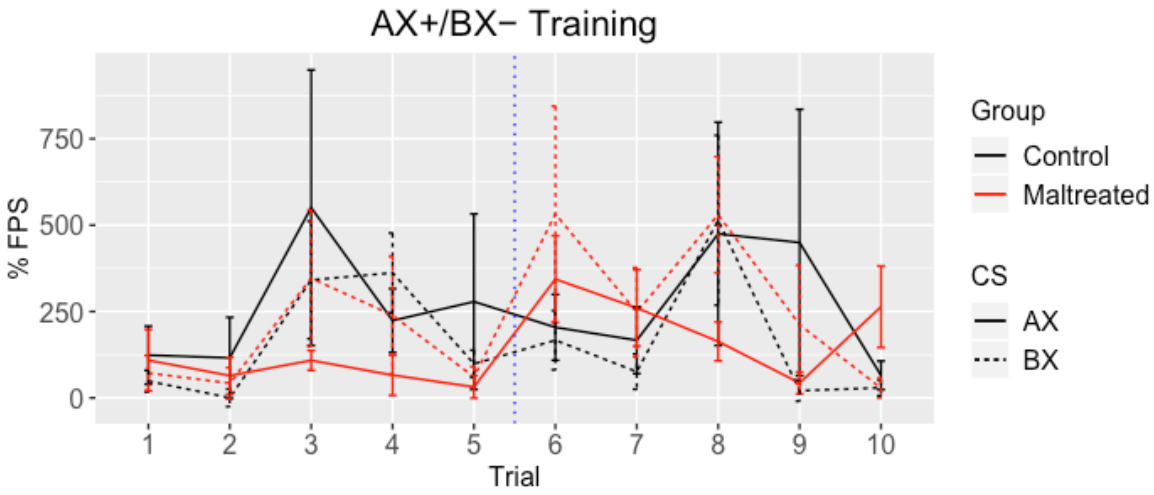


Figure 3.6

AX+/BX- discrimination training trajectory. Day 2 of training was stratified by early versus late acquisition for additional analysis, averaging trials 7 & 8 as 'early acquisition', and trials 9 & 10 as 'late acquisition' (see Figure 3.5). %FPS to the fear (AX+) and safety (BX-) cues are plotted here by 'early' vs. 'late' acquisition levels. A main effect of acquisition, with higher %FPS during early than late acquisition was found. Also, an acquisition x CS interaction effect was found, with %FPS to BX- decreasing from early to late acquisition, indicating discrimination learning. Data represented as mean±SEM.

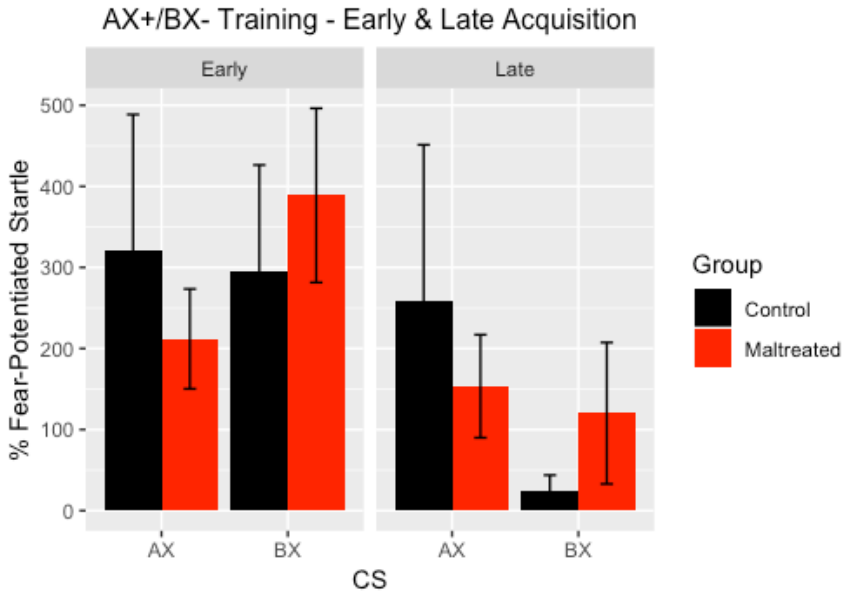
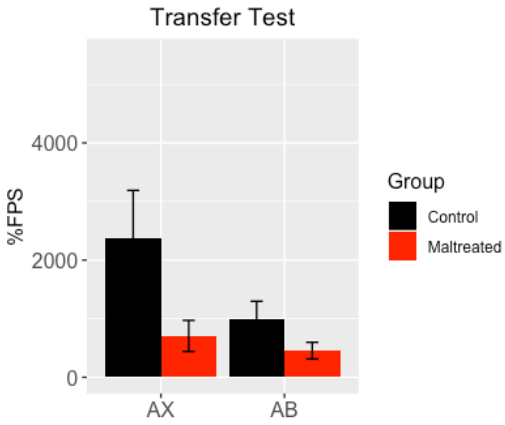


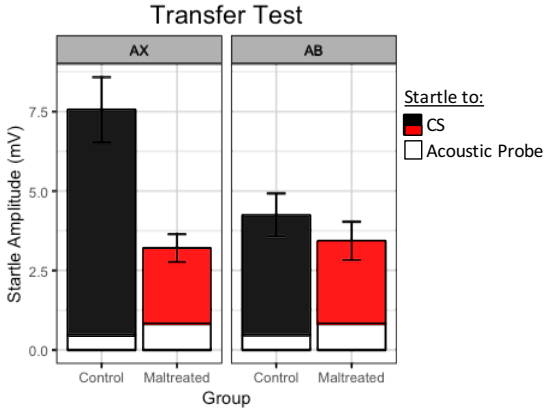
Figure 3.7

Transfer Test. **(A)** %FPS to the AX versus AB cues during the transfer test, collapsed across sexes. Significant main effects of group (higher %FPS in control than MALT animals), sex, and CS (higher %FPS to AX than AB) were found. No interaction effects were detected, and days to reach criterion during discrimination training did not have a significant covariate effect. **(B)** Same data plotted in mV instead of %FPS, with the lower white portion of each bar representing the startle amplitude to the acoustic probe/noise alone and the colored bar portion representing startle amplitude to AX or AB beyond the noise alone amplitude Although startle to the acoustic probe alone seems slightly higher in MALT than control animals, it does not seem to generate a ceiling effect that would explain the blunted responses of this group to both the AX and AB pairings, such that an animal could not physically startle much more. **(C)** %FPS to the AX and AB cues plotted separately by sex to visualize the main sex effect, with males displaying higher %FPS than females.

A.



B.



C.

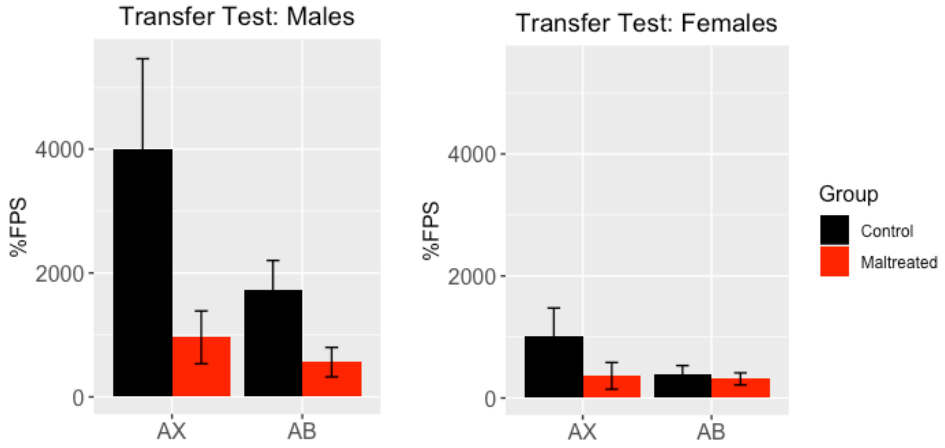


Figure 3.8

Extinction, days to criterion. Number of days until %FPS of the A and AX were both <100%, similar to pre-training levels, stratified by sex. No main effects of group or sex, or interaction effects were found, and days to criterion for AX+/BX- discrimination was not a significant covariate, either. Data represented as mean±SEM.

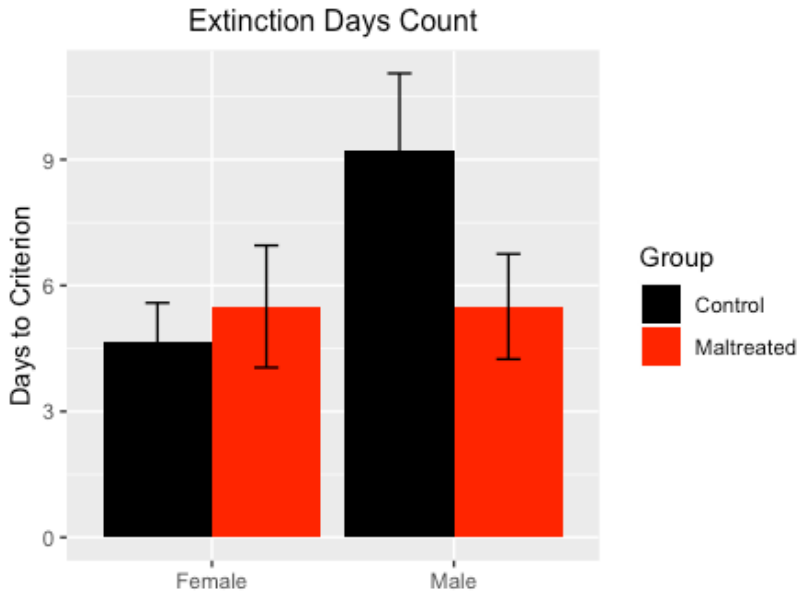


Figure 3.9

Extinction trajectory. %FPS to the AX cue plotted by trial over the first two days of testing (separated by the blue vertical dotted line) after removing the first trial per day, to avoid noise. Trials 2&3 of day 1 were averaged and examined as 'early extinction' and trials 3&4 of day 2 were averaged and considered 'late extinction'. No main or interaction effects were detected. Data represented as mean±SEM.

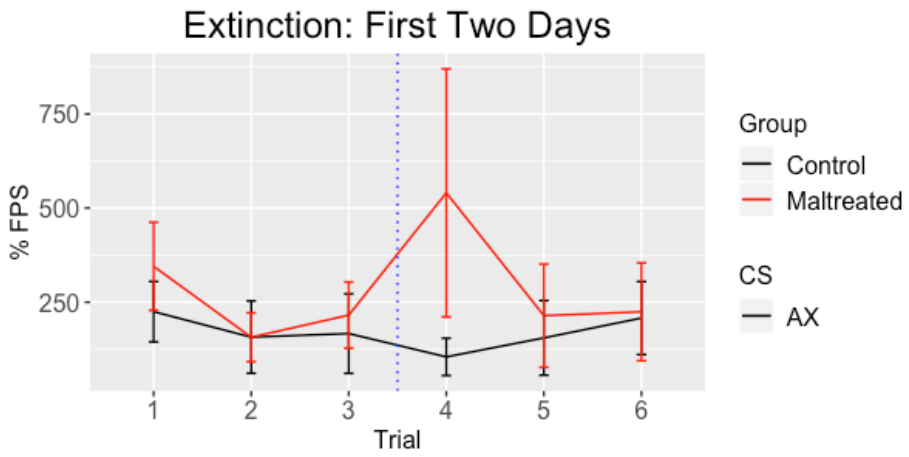
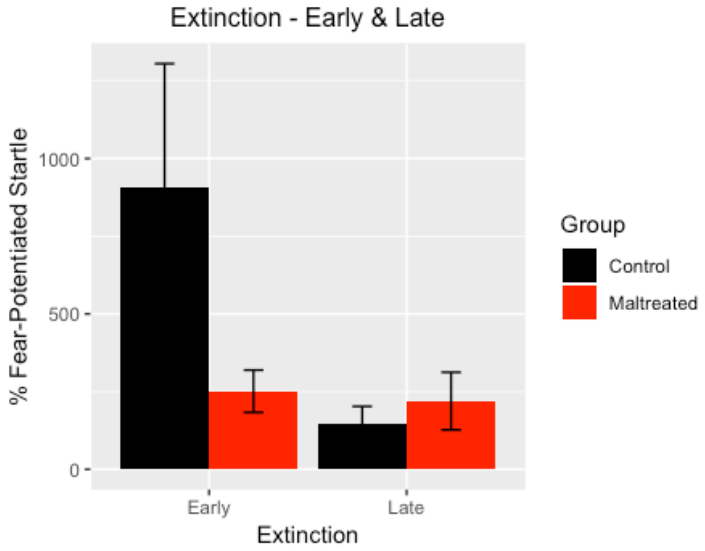


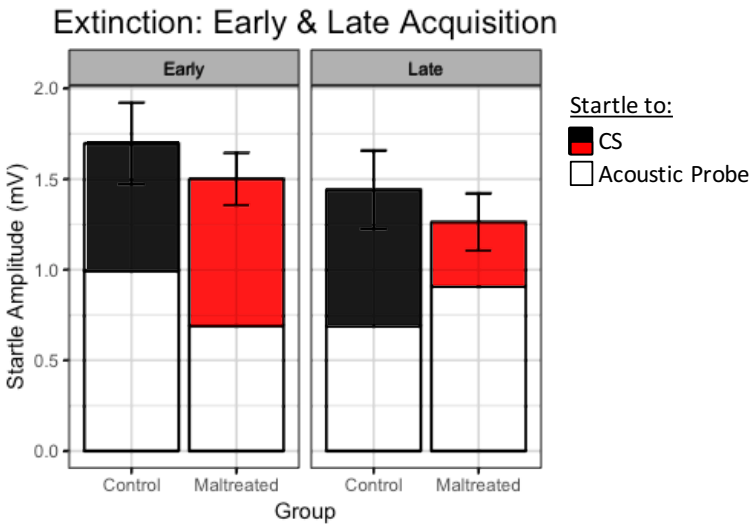
Figure 3.10

Extinction trajectory phases (early vs. late). (A) %FPS to the AX cue plotted as 'early' vs. 'late' extinction levels. No main, interaction or covariate effectswere found. . **(B)** Same data plotted in mV instead of %FPS with the lower white portion of each bar representing the startle amplitude to the acoustic probe/noise alone and the colored bar portion representing startle amplitude to AX beyond the noise alone amplitude. Data represented as mean±SEM.

A.



B.



Chapter 4 Developmental Outcomes of Early Adverse Care on Amygdala Functional Connectivity in Nonhuman Primates

4.1 Abstract

Early life stress, including adverse caregiving experiences such as childhood maltreatment, is a major risk factor for psychopathology and social and cognitive deficits. However, the neurodevelopmental changes involved are poorly understood and challenging to disentangle from genetic/heritable factors. This study utilized a well-established nonhuman primate model of infant maltreatment (MALT) by the mother leading to infant distress, and a crossfostering experimental design with random assignment of infants to either Control or MALT caregiving group at birth. In this model, the highest rates of abuse and rejection take place at a time of drastic cortico-limbic maturation, leading to long-term structural alterations in amygdala (AMY) and related white matter tracts that control emotional and stress responses affected in MALT macaques throughout development. This study examines the developmental impact of infant MALT on Amygdala functional connectivity longitudinally, from infancy through adolescence, to further understand its potential role in the emotional and stress outcomes reported previously by our group. For this, we collected resting state functional MRI (rsfMRI) scans longitudinal to examine AMY functional connectivity (FC) and performed an exploratory voxel-wise AMY seed-based FC analysis at the group level. Most developmental changes involved increased FC of mPFC, ACC, and OFC regions with amygdalae with age, which may reflect increased processing of socioemotional stimuli from infancy to the juvenile period, especially related to fear-learning. MALT effects on AMY FC emerged with age. Thus, while controls' AMY's functional coupling with regions implicated in arousal and stress (i.e. Locus Coeruleus, laterodorsal tegmental area) increased during the juvenile period, it weakened in MALT animals, and it was associated with increased exposure to the stress hormone cortisol during infancy. This uncoupling may be promoting detachment or emotional supression/blunting as a survival mechanism in response to threatening environments. We also performed a

region of interest (ROI) analysis to examine specific alterations in FC in prefrontal(PFC)-AMY circuits that could underlie the higher emotional reactivity, attention bias to threat (chapter 2), and elevated state anxiety and fear/safety discrimination issues (chapter 3) reported in MALT animals. From infancy to the juvenile, prepubertal, period MALT animals showed weaker AMY FC with subgenual cingulate (in females), anterior cingulate cortex, insular cortex, dorsolateral PFC, but stronger FC between left and right AMY than control animals, which suggests developmental alterations to these circuits begin to emerge early in life. During adolescence we collected rsfMRI scans in a subset of animals and the group effects in PFC-AMY FC seem to have normalized by that time. In contrast, we uncovered several unexpected effects of biological mothers (ancestral experience, heritable factors). Together, these findings suggest that MALT results in developmental alterations of AMY FC withPFC and brainstem arousal centers, although some of them seem to be transient and normalize by adolescence. Since MALT animals show elevated state anxiety and problems with fear-safety discrimination during adolescence, the question remains: what neural circuits are responsible for these problems with emotional regulation? Understanding the dynamic developmental changes and alterations of these circuits could generate useful neural biomarkers for future studies testing interventions in individual with childhood MALT and other adverse experiences.

4.2 Introduction

Childhood maltreatment (MALT), including neglect and physical, emotional, or sexual abuse, is a major public health concern and experienced by approximately 1 in 4 US children throughout childhood (Finkelhor*, et al.*, 2013). It is a devastating form of early life stress (ELS) associated with an increased risk anxiety and depression, including

post-traumatic stress disorder (PTSD), cognitive dysfunction, behavioral disorders, substance abuse, obesity and inflammation in humans and nonhuman primates –NHPs- (Asok*, et al.*, 2013; Danese and Tan, 2014; Drury*, et al.*, 2012; Drury*, et al.*, 2016; Gee*, et al.*, 2013; Gunnar and Quevedo, 2007; Howell and Sanchez, 2011; McLaughlin*, et al.*, 2015; Petrullo*, et al.*, 2016a; Rutter*, et al.*, 1999; Sanchez*, et al.*, 2007; Teicher*, et al.*, 2003). However, the developmental consequences vary in complex ways depending on factors, such as the timing and duration of exposure (Kaplow and Widom, 2007; Kisiel, 2014; Spinazzola, 2014; Steinberg, 2014), type and severity of MALT – physical abuse, neglect, sexual abuse, which are often co-morbid-, and co-occurrence with emotional/psychological trauma (Kisiel, 2014; Spinazzola, 2014). Some studies suggest that earlier exposure to early adverse experiences (not just MALT), particularly during infancy (below 2 years of age), results in more severe negative outcomes (Kaplow and Widom, 2007) that are also more difficult to reverse with interventions (e.g. (Zeanah*, et al.*, 2017)). Despite the strong link between early life adverse care and the emergence of psychopathology, the underlying neurobiological and developmental mechanisms translating early adversity/stress into emotional stress, are not well understood, either. Alterations in development of brain networks that control arousal, stress, threat and emotional responses, particularly Amygdala and its connectivity with prefrontal cortex (PFC), have been proposed as potential neural structures associated with behavioral alterations (Foa and Kozak, 1986; Teicher*, et al.*, 2016; VanTieghem and Tottenham, 2018; Weber, 2008). But, understanding the unfolding of these neurobiological changes and underlying mechanisms in humans has been challenging, stemming in part from difficulties of prospective, longitudinal studies in children at risk, lack of experimental control, and complex comorbid conditions and environmental confounds.

As an alternative, rhesus monkeys (*Macaca mulatta*) provide an ethologically valid, translational animal model to address the questions raised in human studies through

longitudinal, prospective, experimental designs that include random assignment of infants to rearing group at birth and collection of highly dense neurobehavioral sampling from infancy through adolescence. Additional strengths of the animal model are the studies of emotional regulation covered in Chapters 2 and 3 using translational paradigms and approaches used in children and adolescents, such as the dot-probe and AX+/BX- fear conditioning, and our ability to examine the unfolding impact of maternal maltreatment on neurodevelopment using longitudinal MRI techniques on the same animals. NHPs share complex social behaviors with humans and are suitable for longitudinal developmental studies of the impact of early life stress (Howell and Sanchez, 2011; Sanchez*, et al.*, 2001; Sanchez, 2006). The relationship and bond between a macaque mother and its infant is highly influential in early neurodevelopment (Hinde and Spencer-Booth, 1967), which develops about four times faster than in human children. Maltreatment of offspring, including abuse and rejection, occurs in NHP species spontaneously and with similar prevalence rates (2-5%) and developmental consequences, as in humans, includes activation of the stress response system and alterations in socioemotional functioning (Brent*, et al.*, 2002; Drury*, et al.*, 2017; Howell*, et al.*, 2016b; Johnson*, et al.*, 1996; Maestripieri and Carroll, 1998; Sanchez*, et al.*, 1998; Sanchez, 2006; Troisi and D'Amato, 1984). In rhesus infant maltreatment by the mother is operationalized by early life physical abuse and maternal rejection, both associated with infant distress (Maestripieri and Carroll, 1998; Maestripieri, 1999; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Sanchez, 2006). As in humans, there is transgenerational transmission of maltreatment through the maternal line in macaques (Maestripieri and Carroll, 1998; Maestripieri, 2005) and these mothers reliably maltreat subsequent offspring, which seems to be a stable maternal trait (Maestripieri and Carroll, 1998; Maestripieri, 2005). This stability allowed for the identification of mothers for assignment to experimental group in our study,

followed by mother-infant dyad observations to confirm that maltreatment occurred, for how long and at what rates. Such generational maltreatment perpetuation parallels findings in humans with a history of childhood adverse care, which further validates using this NHP model to help understand the mechanisms underlying developmental outcomes in human health (Franklin*, et al.*, 2010a; Huizinga*, et al.*, 2006; Maestripieri, 2005; Tarullo and Gunnar, 2006). Macaque infant studies have recapitulated alterations reported in maltreated children (and other adverse caregiving experiences), including increased anxiety, emotional reactivity and aggression, impaired impulse control, social deficits, elevated levels of stress hormones –indicative of chronic stress exposure-, activation of pro-inflammatory pathways, structural alterations in cortico-limbic tracts, and larger amygdala volumes (Howell*, et al.*, 2013; Howell*, et al.*, 2014; Howell*, et al.*, 2019; Koch*, et al.*, 2014; Maestripieri, 1998; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Morin*, et al.*, 2019; Petrullo*, et al.*, 2016b; Sanchez, 2006; Sanchez*, et al.*, 2010). Of particular interest for this study, maltreatment affects the structural integrity of cortico-limbic white matter tracts involved in socioemotional processing and responses during rhesus development (Howell*, et al.*, 2013; Howell*, et al.*, 2017; Howell*, et al.*, 2019), including those connecting PFC and amygdala, such as the uncinate fasciculus (UF). Furthermore, weaker measures of UF structural integrity (fractional anisotropy: FA), were associated with higher hair cortisol accumulation from birth through 6 months of age (Howell BR, 2012) detected in maltreated infants compared to controls (Drury et al, 2017), which is the time when they experienced the highest rates of abuse and rejection (Howell*, et al.*, 2016b; McCormack*, et al.*, 2006). Myelination of axons in tracts is a critical cellular change that serves to increase conductance of action potentials, supporting functional connectivity between brain regions (Fields, 2008; Thomason and Thompson, 2011; Zatorre*, et al.*, 2012), and also undergoes massive growth during early development. This maturation increases its vulnerability to

environmental influences during development, including adverse care and early life stress (Deoni*, et al.*, 2012; Dubois*, et al.*, 2014; Eluvathingal*, et al.*, 2006; Geng*, et al.*, 2012; Govindan*, et al.*, 2010; Kumar*, et al.*, 2014), and the associated increased glucocorticoid (cortisol) levels resulting from these stressful experiences (Kumar*, et al.*, 1989). In addition to being stressful experiences for the infant, suboptimal maternal care also impacts the ability of the mother to buffer the infant's stress and fear responses (Sanchez*, et al.*, 2015), which is a function that follows its own developmental trajectory, with maternal signals losing their potency to act as a stress buffer as animals become more independent during weaning and acquire self-regulation. The infant's stress/fear/arousal responses are buffered through maternal signals that regulate emotion/stress circuitry, especially amygdala connections with the hypothalamus and brain stem regions involved in stress/arousal/fear responses and with PFC, for top-down emotional regulation (Moriceau and Sullivan, 2006). Thus, maternal care is critical in regulating the development of these amygdala circuits, which are involved in self-regulation in primates as they reach independence (Drury*, et al.*, 2016; Gee*, et al.*, 2014; Gunnar*, et al.*, 2015; Gunnar and Sullivan, 2017; Sanchez*, et al.*, 2015; Tottenham, 2015), explaining why infant maltreatment impacts neural and socioemotional development (Casey*, et al.*, 2010a; LA, 1996; Maestripieri and Carroll, 1998). Given this maternal impact on emotion/stress regulatory systems development, the quality of the care may also play a role in how these circuits continue to develop through childhood and adolescence.

In this study, we use a translational NHP model of infant maltreatment to examine (a) its long-term impact on development of AMY functional connectivity, which may underlie the increased emotional reactivity of MALT animals; and (b) potential biological mechanisms that underlie those neurobiological effects, specifically whether the higher cortisol exposure during infancy reported in MALT animals (measured as hair cortisol

accumulation from birth through 6 months) was a predictor. Although this MALT animal model does not span all adverse experiences that MALT children experience (for instance, sexual abuse), one of its critical strengths lies in its ability to quantify maltreatment during a known postnatal period, providing frequency, duration, and severity of the adverse experience (e.g. abuse and rejection rates) and the concurrent levels of stress it elicits (e.g. cortisol accumulation in hair during the postnatal ELS exposure), difficult to be accurately determined in studies with children with early adverse caregiving experiences. Additionally, NHP models provide strong control over environmental variables that are known confounders of behavioral outcomes of ELS during adolescence in human studies, such as drug use, diet/obesity, prenatal stress/drug exposure, socioeconomic status, and access to medical care or therapy. Our experimental design also allows to disentangle heritability from postnatal experience by utilizing crossfostering and randomized assignment to experimental group (maltreating, competent care; (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Howell*, et al.*, 2019); Howell et al, 2019) at birth, which would not be possible in humans. These are just some of the important contributions of this translational animal model with a well-characterized adverse caregiving experience and longitudinal behavioral and biological measures of its developmental impact.

Thus, we use this translational NHP model of infant MALT to examine developmental alterations in amygdala FC longitudinally, from infancy through the juvenile, prepubertal, period (at 3, 6, 12, 18 months of age) and again in adolescence (at 4.5-5.5 years), which may underlie behavioral and stress outcomes. For this, we collected resting state functional MRI data to examine the developmental impact of MALT on AMY FC (1) across the brain, using an exploratory voxel-wise AMY seed-based FC analysis at the group level; in parallel to (2) region of interest (ROI) analysis to examine specific alterations in PFC-AMY FC that could underlie the higher emotional reactivity, attention

bias to threat (chapter 2), elevated state anxiety and fear/safety discrimination issues (chapter 3) reported in MALT animals in adolescence.

In addition, we examined whether the higher hair cortisol accumulation from birth through 6 months of age reported in MALT infants -a marker of chronic exposure to stress- (Drury et al, 2017), predicted developmental changes in amygdala functional connectivity.

4.3 Methods

4.3.1 LONGITUDINAL (INFANCY THROUGH JUVENILE –PREPUBERTAL-PERIOD) RESTING STATE fMRI FUNCTIONAL CONNECTIVITY STUDIES

4.3.1.1 Subjects

These studies included 20 rhesus macaques (*Macaca mulatta*; 10 males, 10 females), raised by their mothers in large social groups at the Yerkes National Primate Research Center (YNPRC, Lawrenceville, GA) and studied from birth through 18 months of age (juvenile, prepubertal age) as part of a larger longitudinal study of developmental consequences of infant maltreatment (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Howell*, et al.*, 2019; McCormack*, et al.*, 2015). This is a spontaneous macaque model of maternal maltreatment that leads to infant distress (Maestripieri and Carroll, 1998; Maestripieri, 1999; Sanchez, 2006), and with translational value to humans. Half of the animals in this study experienced maternal maltreatment, including physical abuse and rejection (MALT, n=10; 6 males, 4 females), and the other half received high quality maternal care (Control, n=10; 4 males, 6 females). Subjects lived with their mothers and families in

large social groups with a matrilineal social hierarchy consisting of 75-150 adult females, their sub-adult, juvenile and infant offspring, and 2-3 adult breeder males. Based on this social complexity we were able to use a balanced distribution of social dominance ranks (high, medium and low social status), in addition to sex, across experimental caregiving groups and assigned infants from different matrilines and paternities to provide high genetic/social diversity, as previously reported (Drury*, et al.*, 2017; Howell*, et al.*, 2017). Given that birth weight is a strong predictor of neurobehavioral development in humans and NHPs (Coe and Shirtcliff, 2004; Vohr*, et al.*, 2000), we only studied infants ≥450gr birth weight, which is a safe veterinary clinical cut off to rule out prematurity in rhesus monkeys. Furthermore, half of the animals were raised by their biological mothers (10 MALT: 5 males, 5 females; 10 Control: 5 males, 5 females) and the other half were randomly assigned at birth to be fostered to either mothers with a history of nurturing maternal care (Control) or to maltreating foster mothers (10 MALT: 6 males, 4 females; 10 Control: 4 males, 6 females), following published protocols by our group (Drury*, et al.*, 2017; Howell*, et al.*, 2017). This design allows us to disentangle the effect of caregiving experience from heritable/prenatal factors (Drury*, et al.*, 2017; Franklin*, et al.*, 2010b; Maestripieri, 2005). Social groups were housed in outdoor compounds, with access to climate-controlled indoor areas. A standard, high fiber and low fat monkey chow diet (Purina Mills Int., Lab Diets, St. Louis, MO) as well as seasonal fruits and vegetables were provided twice daily, in addition to enrichment items. Water was available *ad libitum*. All the procedures were in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and Use of Laboratory Animals" and approved by the Emory Institutional Animal Care and Use Committee (IACUC).

4.3.1.2 Behavioral characterization of maternal care

A detailed description of the infant rhesus MALT model and methods for selection of potential mothers and behavioral characterization of competent maternal care (Control) in contrast to infant MALT is provided in previous publications (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Howell*, et al.*, 2019; Maestripieri, 1998; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Morin*, et al.*, 2019). Briefly, because MALT mothers consistently maltreat their infants, we identified potential multiparous Control and MALT mothers with consistent maternal care quality towards prior offspring. Following birth and crossfostering, we performed focal observations of maternal care across the first 3 postnatal months to substantiate and measure rates of abuse and rejection towards biological or fostered infants. These were 30 min long focal observations of mother-infant interactions performed on separate days (5 days/week during month 1, 2 days/week during month 2 and 1 day/week during month 3) for a total of 16 hours/mother-infant pair. This observation protocol is optimal to document early maternal care in this species, given that physical abuse is the highest during month 1 and declines by the third month, after which it is rarely observed (Drury*, et al.*, 2017; Howell*, et al.*, 2017; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006). Observations were performed between 7:00-11:00 AM, when monkeys are most active. Behavioral observations were collected by experienced coders (interobserver reliability >90% agreement, Cohen k>0.8). Competent maternal care is defined as species-typical behaviors, such as nursing, cradling, grooming, ventral contact and protection (retrieve from potential danger, restrain) of the infant. In contrast, MALT is aberrant (prevalence rate: 2-5%), defined as the co-morbid occurrence of physical abuse (operationalized as violent behaviors directed towards the infant that cause pain and distress, including dragging, crushing, throwing) and early infant rejection (i.e. prevention of ventral contact and pushing the infant away). Both abuse and rejection cause high levels of infant distress –e.g. scream vocalizations- and elevations in stress hormones (Drury*, et*

al., 2017; Howell*, et al.*, 2013; Maestripieri and Carroll, 1998; McCormack*, et al.*, 2006; Sanchez, 2006). Control foster mothers in this study exhibited competent/good maternal care (e.g. high maternal sensitivity, infant protection and attachment: (McCormack*, et al.*, 2015) and did not exhibit MALT behaviors -physical abuse or rejection- (Drury*, et al.*, 2017; Howell*, et al.*, 2017). Abuse and rejection rates (frequency/observation time) were calculated across the first 3 postnatal months.

While at the YNPRC Field Station, longitudinal measures were collected during the infant and juvenile periods, focusing on the following for this study: (a) behavioral observations of maternal care received through the first three months postpartum, (b) resting state functional MRI (rsfMRI) scans during infancy (3, 6 months) and the juvenile, prepubertal, period (12, 18 months) to examine the developmental impact of MALT on AMY FC, and (c) whether the higher levels of cortisol accumulated in hair of MALT animals than controls from birth through the first 6 months postpartum -a marker of chronic exposure to stress- (Drury*, et al.*, 2017), predicted developmental changes in AMY functional connectivity.

4.3.1.3 Resting state functional MRI (rsfMRI) Image Acquisition

MRI images were acquired longitudinally, at 3 and 6 months of age (infancy) and at 12 and 18 months (juvenile period) using a 3T Siemens Magnetom Tim Trio MRI scanner (Siemens Med. Sol., Malvern, PA, USA), and an eight-channel phase array knee coil (YNPRC Imaging Center). Subjects were transported from the YNPRC Field Station on the day of the scan or the day before (infants were transported with their mothers) to the YNPRC Main Station on Emory campus. Data were acquired during a single scanning session, which included T1- and T2-weighted MRI structural scans for anatomical registration of functional images to standard, age-specific, rhesus monkey atlas space.

Two 15-minute rs-fMRI T2*-weighted scans with a gradient-echo echoplanar imaging (EPI) sequence (400 volumes, TR/TE = 2060/25msec, voxel size: 1.5mm^3 isotropic) were acquired to measure temporal changes in regional blood oxygen level dependent (BOLD) signal. An additional short, field map, scan was also acquired for unwarping distortions in the EPI scans. The first 3 volumes were removed from each EPI scan to ensure scan environment stabilization, resulting in a total of 794 concatenated volumes. These protocols and scan sequences have been optimized at the YNPRC for infant macaque longitudinal imaging (Kovacs-Balint*, et al.*, 2018; Mavigner*, et al.*, 2018; Shi*, et al.*, 2016; Zhang*, et al.*, 2017). Briefly, animals were scanned supine in the same orientation, with their head placed and immobilized in a custom-made head holder with ear bars and a mouthpiece to minimize motion artifacts. A vitamin E capsule was placed on the right temple to mark the right side of the brain. Following initial induction of light anesthesia with telazol (3.9±0.83 mg/kg BW, i.m. mean±standard deviation) and intubation, scans were collected under the lowest possible level of isoflurane anesthesia (1.0±0.1%, inhalation; mean±standard deviation) to minimize its reported dampening effect on BOLD signal. MRI images were acquired and processed following approaches optimized by our group for studies of macaque neurodevelopment (Kovacs-Balint*, et al.*, 2018; Mavigner*, et al.*, 2018) and protocols developed and widely-used for macaques (Grayson*, et al.*, 2016; Hutchison*, et al.*, 2012; Li*, et al.*, 2013; Margulies*, et al.*, 2009; Sallet*, et al.*, 2011; Vincent*, et al.*, 2007). This level of isoflurane is lower than that used in previous macaque studies of sensory, motor, visual and cognitive-task related systems, reporting patterns of coherent BOLD fluctuations and similar to those observed in awake and behaving monkeys (Vincent et al. 2007; Hutchison et al. 2013; Li et al. 2013; Miranda-Dominguez 2014b; Tang and Ramani 2016). Ideally, these animals would have been scanned awake, but training socially housed infants for such scanning is not currently feasible. Each animal was fitted with an oximeter, rectal thermometer, blood

pressure/heart rate monitor for physiological monitoring during the scans, an i.v. catheter was placed in the saphenous vein to administer dextrose/NaCl (0.45%) to maintain hydration. Then, each animal was placed on an MRI-compatible heating pad to maintain normal body temperature and monitored throughout the scan procedures by veterinary staff. Infants were immediately returned to their mothers after full recovery from anesthesia and mother-infant pairs (or juveniles) returned to their social groups the day after the scan.

4.3.1.4 Structural MRI acquisition

Structural images were acquired for registration of functional data to the age-specific atlases space. High-resolution structural MRI images (T1-weighted) were collected with a 3D magnetization prepared rapid gradient echo (3D-MPRAGE) parallel image sequence (TR/TE = 3000/3.31 ms, 116 mm FOV, voxel size: 0.6 mm^3, 6 averages, GRAPPA, R=2). T2-weighted scans were also collected in the same direction as the T1 during the same scanning session (TR/TE = 2500/84 ms, 128 mm FOV, voxel size: 0.7x0.7x2.0 mm, 1 average, GRAPPA, R=2) to aid with registration and delineation of anatomical borders.

4.3.1.5 Rs-fMRI data preprocessing

Data pre-processing was done using the FMRIB Software Library (FSL, Oxford, UK (Smith*, et al.*, 2004; Woolrich*, et al.*, 2009), 4dfp, and an in-house pipeline built using Nipype (Gorgolewski*, et al.*, 2011), modified based on published fMRI analysis methods (Fair*, et al.*, 2007; Fair*, et al.*, 2009; Fair*, et al.*, 2012; Iyer*, et al.*, 2013; Miranda-Dominguez*, et al.*, 2014a), and optimized for macaques (Miranda-Dominguez*, et al.*,

2014b), including rsfMRI studies in infant rhesus by our group (Kovacs-Balint*, et al.*, 2018; Mavigner*, et al.*, 2018). This procedure consisted of 1) quantification and correction of dynamic field map changes, 2) slice-time correction of intensity differences as a result of interleaved slice image acquisition 3) combined resampling of: within-run rigid body motion correction, registration of the EPI to T1 (linear) and registration of the T1 to 6 month infant atlas (nonlinear) 4) BOLD signal normalization to mode of 1000, to scale BOLD values across participants at an acceptable range 5) BOLD signal detrending 6) regression of rigid body head motion (6 directions), global brain signal, BOLD signal of the ventricles and white matter (derived from manually drawn masks), and first-order derivatives of these signals 7) low-pass ($f < 0.1$ Hz) temporal filter (second order Butterworth filter (Fair*, et al.*, 2007; Fair*, et al.*, 2009; Fair*, et al.*, 2012; Miranda-Dominguez*, et al.*, 2014b).

Regression of the global signal (GSR) was performed based on previous literature showing the importance of removing systematic artifacts, including global artifacts that arise as a consequence of movement, respiration, and other physiological noise (Burgess, et al., 2016; Ciric, et al., 2017; Nalci, et al., 2017; Power, et al., 2017; Yan, et al., 2013), including macaque rs-fMRI FC studies (Grayson, et al., 2016; Miranda-Dominguez, et al., 2014b). Without doing so, spurious artifacts would become problematic, especially in longitudinal studies of FC throughout development. Notwithstanding the above defense of GSR, we acknowledge that controversy regarding this method persists (Murphy and Fox, 2017). Because of this, we previously compared infant macaque FC with and without GSR and obtained similar results throughout development (Kovacs-Balint*, et al.*, 2018), including the current dataset, in which similar effects of MALT were obtained with and without GSR and similar relationships were found (Morin EL, 2015).

Motion artifacts were further minimized by removing frames displaced more than 0.2 mm (Power, et al., 2012; Power, et al., 2014). Scans that contained significant artifacts throughout the scan were removed from the analysis. Of the 20 animals included in the study, 17 had viable scans at 3 months, all had viable scans at 6 months, 15 at 12 months, and 8 at 18 months (at the latter age missing scans was due to problems with animal assignment to the protocol). Concatenated EPI functional time series for each subject were rigid-body co-registered to the subject's own averaged T1-weighted structural image and transformed (linear (FLIRT) and nonlinear (FNIRT) FSL methods) to conform to age-specific T1-weighted rhesus infant and juvenile brain structural MRI atlases developed by our group (publicly available at: https://www.nitrc.org/projects/macaque_atlas/, (Shi*, et al.*, 2016)) using non-linear registration methods in FSL (FNIRT). These infant atlases were previously registered to the 112RM-SL atlas (publicly available at: http://brainmap.wisc.edu/monkey.html) in F99 space (McLaren*, et al.*, 2009; McLaren*, et al.*, 2010) as shown in (Miranda-Dominguez*, et al.*, 2014b) and were templates of scans acquired longitudinally at 3, 6 and 12 months of age on 40 infant rhesus monkeys from the YNPRC social colony, balanced by sex and social rank. Based on best match of neuroanatomical characteristics, we registered the earliest scan age (3 months) to the 3 months atlas, the 6 months scans to the 6 months atlas and the later ages (12, 18 months) to the 12 months rhesus atlas. All the atlases were transformed to conform to the T1-weighted 112RM-SL atlas image in F99 space, following previously described protocols (Miranda-Dominguez*, et al.*, 2014b) and allowing the EPI images to be transformed into F99 space in one interpolation step for the region of interest (ROI) analysis (see below). Global brain signal was regressed (GSR) based on literature supporting this method for removal of systemic artifacts due to movement, respiration, and other physiological noise, especially in human developmental longitudinal data (Ciric*, et al.*, 2017; Nalci*, et al.*, 2017; Power*, et al.*,

2017; Yan*, et al.*, 2013) and monkey BOLD data (Grayson*, et al.*, 2016; Miranda-Dominguez*, et al.*, 2014b). Finally, all data were inspected following preprocessing to identify scans with poor registration, significant distortion, or BOLD signal loss, which would alter the connectivity analysis.

4.3.1.6 Definition of Amygdala and Prefrontal Cortex (PFC) Regions of Interest (ROI)

ROIs were selected from published anatomical parcellations (Lewis and Van Essen, 2000; Markov*, et al.*, 2011; Paxinos G, 2000), anatomically defined using macaque atlases (Saleem KS, 2012; Schmahmann JD, 2006), and mapped onto the cortical surface of the 3, 6 and 12 months UNC-Emory rhesus infant atlases (Shi*, et al.*, 2016) registered to the F99 space. The amygdala (AMY) ROI was drawn by neuroanatomists onto cytoarchitectonic maps in the UNC-Wisconsin adolescent atlas (Styner*, et al.*, 2007), and propagated to the UNC-Emory rhesus 3, 6 and 12 months infant atlases in F99 space (Shi*, et al.*, 2016). After that, all ROIs were manually edited in each age-appropriate infant atlas according to established anatomical landmarks (Paxinos G, 2000; Saleem KS, 2012) and under guidance of an expert on AMY and PFC developmental neuroanatomist (Jocelyne Bachevalier, Emory University), before removing voxels that covered regions where at least one animal showed signal dropout, determined as the mean intensity of each subjects' BOLD signal intensity across the whole-brain mask minus two standard deviations (Figure 4.2A). AMY ROI included all amygdaloid nuclei, excluding perirhinal cortex, and ROIs for PFC subregions included the dorsolateral PFC (dlPFC) (Brodmann areas 9 & 46), medial PFC (mPFC) (Brodmann areas 25 & 32), orbitofrontal cortex (OFC) (Brodmann areas 11 & 13), anterior cingulate cortex (ACC) (Brodmann area 24).

4.3.1.7 Whole Brain Voxel-wise Amygdala (AMY) functional connectivity (FC) Analysis

Seed-based maps of AMY FC with other voxels in the brain were created for each subject across all ages. A secondary seed-based analysis was limited to voxels which were significantly correlated with AMY activity (FDR corrected q=0.05, cluster size ≥ 10 voxels). These FC maps were included in a mixed linear model (MLM), AFNI's 3dLME statistical model described in more detail in the Statistical Analysis section (below). Statistically significant voxel clusters after multiple comparisons correction using False Discovery Rate (FDR) were displayed on the infant atlases (Shi*, et al.*, 2016) and their anatomical localization identified with established stereotaxic/MRI combined rhesus macaque brain atlases (Paxinos G, 2000; Saleem KS, 2012); see Figure 2B).

4.3.1.8 Amygdala-Prefrontal Functional Connectivity (FC): ROI-ROI analyses

ROIs were selected and defined as described above, based on published anatomical parcellations (Lewis and Van Essen, 2000; Markov*, et al.*, 2011; Paxinos G, 2000), anatomically defined using macaque atlases (Saleem KS, 2012; Schmahmann JD, 2006), and mapped onto the cortical surface of the UNC-Emory rhesus infant atlases (Shi*, et al.*, 2016) registered to the F99 space. BOLD time series were then parcellated and averaged across all voxels within each ROI, and averaged across the time course. Pearson correlations were calculated between AMY and subregions of the PFC (dlPFC: Brodmann's Area (BA) 9, BA 46; mPFC: BA 25 -subgenual cingulate-, BA 32; ACC: BA 24; OFC: BA 11, BA 13 for each age and Fisher Z-transformed to stabilize variance.

4.3.2 ADOLESCENCE RESTING STATE fMRI FUNCTIONAL CONNECTIVITY STUDIES

4.3.2.1 Subjects

A subset of 25 adolescent rhesus macaques studied above, during infancy and the juvenile period, were studied again during Adolescence, between the ages of 4.5-5.5 years. These animals were characterized as part of the bigger longitudinal study described above to examine the developmental outcomes of infant MALT in this species (Drury*, et al.*, 2017; Howell*, et al.*, 2017); Howell et al, 2019; Morin et al, 2019). See above for rearing and housing conditions from infancy through the juvenile, prepubertal, period *("Subjects").* Half of the subjects experienced maternal MALT (n=14; 8 males, 6 females), and the other half received competent maternal care (Control, n=11; 5 males, 6 females). See Table 1 for details of infant crossfostering assignment and counterbalancing of groups in the Adolescent animals.

At approximately 4 years of age, the animals were transferred from the YNPRC Field Station to the YNPRC Main Station, where they were pair-housed in home cages and fed Purina monkey chow (Ralston Purina, St. Louis, MO, USA), supplemented with fruit and vegetables daily, and water was available *ad libitum*. Environmental enrichment was provided on a regular basis. The colony is maintained at an ambient temperature of 22 ± 2°C at 25-50% humidity, and the lights set to a 12-h light/dark cycle (lights on at 7h; lights off at 19h). Following several months of acclimation to the move and new housing environment, the animals underwent several behavioral tasks, neuroendocrine assessments and the MRI scans described in the section below, as part of a larger study examining long-term emotional, cognitive and neurobiological consequences of MALT during adolescence.

All procedures and animal care were in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and Use of Laboratory Animals" and approved by the Emory Institutional Animal Care and Use Committee (IACUC).

4.3.2.2 Rs-fMRI Image Acquisition during Adolescence

All animals were scanned during adolescence (4.63±0.51 years of age) using a 3 T Siemens Magnetom Trio Tim system scanner (Siemens Med. Sol., Malvern, PA, USA), and an 8-channel phase array knee coil. Images were acquired during a single session, during which T1- and T2-weighted MRI structural scans were collected for registration purposes, and two 15-minute rs-fMRI (T2*-weighted) scans measured regional temporal changes in the BOLD signal. Functional BOLD-weighted images were acquired with a T2*-w gradient-echo echoplanar imaging (EPI) sequence (200 volumes, TR/TE = 2290/25ms, voxel size: 1.5mm3 isotropic). These two scans occurred following collection of the T1-w sequence (~30 min), so that the collection of the functional EPI scans was standardized to ~45 min from initial anesthesia in all subjects. Additionally, a short reverse-phase encoded scan was acquired for unwarping distortions in EPI images, as previously described (Andersson*, et al.*, 2003). To allow time for the scanner to reach equilibrium, the first three volumes were removed from each EPI sequence, resulting in a total of 794 volumes.

Animals were scanned in a supine position, with their head immobilized in a custom-made head holder fitted with ear bars and a mouthpiece. The right side of the brain was marked with a vitamin E capsule placed near the right temple. Following initial induction of light anesthesia with telazol (3.86 ± 0.02 mg/kg BW, i.m.) and intubation, scans were collected under the lowest possible level of isoflurane anesthesia (isoflurane

0.8–1%, inhalation; mean±standard deviation) to minimize its reported dampening effect on BOLD signal, as discussed above. Physiological signals were monitored with an oximeter, ECG, rectal thermometer, and blood pressure monitor. An i.v. catheter was placed in the saphenous vein to administer dextrose/NaCl (0.45%) in order to maintain hydration. Animals were placed under an MRI-compatible heating pad to maintain normal body temperature during scanning.

Structural MRI acquisition: High-resolution T1-w structural scans were acquired using a 3D magnetization prepared rapid gradient echo (3D-MPRAGE) parallel image sequence (TR/TE = 2600/ 3.38 ms, FOV: 128 mm, voxel size: 0.5mm^3 isotropic, 8 images, GRAPPA, R = 2). T2-w scans were acquired in the same direction as the T1 (TR/TE = 3200/338ms, FOV: 128mm, voxel size: 0.5mm^3 isotropic, 3 averages, GRAPPA, R = 2).

4.3.2.3 Rs-fMRI data preprocessing: adaptation of the Human Connectome Project (HCP) Pipeline for macaques

The HCP pipeline (Glasser*, et al.*, 2013) is a preprocessing pipeline that conforms to the best standards and practices in the field for fMRI data. The use of surface based procedures in preprocessing pipelines, galvanized by the HCP pipeline, has shown improvements through spatial artifact/distortion removal to minimize signal loss and maximize noise removal, cross-modal/multi-modal (MRI, fMRI) registration, alignment to standard space utilizing both surface and volume based registration for precision in between-subject comparisons. Because these pipelines do not work with NHP primate data, the Fair Neuroimaging Laboratory at OHSU has developed HCP pipeline adaptations for use with macaque data. A summary of the stages of the HCP

preprocessing pipeline and modifications/additions to the HCP pipeline made to accommodate macaque brains is provided in Table 2. Control points were also utilized in all animals during the Freesurfer stage in order to improve delineation of the pial surface in areas with poor image contrast. Quality control was performed with two raters by assessing the output of the executive summary, visualizing gray/white surfaces on T1w, surfaces on T2w, functional data registered to T1 (to check field of view, functional to anatomical registration, signal dropout), and greyplots (for assessing resting state data quality). Frames with no greater than 0.2 mm displacement were included in the final analysis (Power, et al., 2012; Power, et al., 2014).

4.3.2.4 Amygdala-Prefrontal Functional Connectivity (FC): ROI-ROI Analyses

As described above, ROIs were selected from published anatomical parcellations (Lewis and Van Essen, 2000; Markov*, et al.*, 2011; Paxinos G, 2000), that were converted into surface labels for compatibility with the CIFTI format of the time series functional data. The Amygdala ROI was extracted from the segmentation of each subject's subcortical structures, which was created for registration purposes. Therefore, each amygdala is unique to each subject, whereas PFC subregions are consistent across all animals and originated from the Markov atlas (Markov*, et al.*, 2014). In order to generate FC, Pearson correlations were calculated between BOLD temporal courses of AMY and PFC subregions, as described above (dlPFC: BA 9, BA 46; mPFC: BA 25, BA 32; ACC: BA 24; OFC: BA 11, BA 13) for each age and Fisher Z-transformed to stabilize variance.

4.3.2.5 Postnatal Hair Cortisol Measures

At birth, one square inch of hair was shaved from the infant's head just above the foramen magnum (nuccal area), which was shaved again at 6 months of age. Hair samples at 6 months were assayed to measure cortisol accumulated in the growing hair shafts from birth through 6 months of age as a measure of hypothalamic-pituitary-adrenal (HPA) axis activation, and entered as predictor of FC in the regression models. Hair samples were processed and assayed using previously described protocols (Meyer, *et al.*, 2014). Hair was first weighed and washed in isopropanol to remove external contamination, then ground to powder, and extracted with methanol overnight. After the methanol evaporated, the resulting residue was re-dissolved in assay buffer, and cortisol was measured using the Salimetrics (Carlsbad, CA) enzyme immunoassay kit (cat. # 1-3002). Intra- and inter-assay coefficients of variation were <10%.

4.3.3 STATISTICAL ANALYSES

4.3.3.1 Infancy-Juvenile Period Longitudinal PFC-Amygdala FC: ROI-ROI Analyses

Linear mixed models (LMM) were used to assess the fixed effects of caregiving group (Control, MALT), sex, age (3, 6, 12, 18 months) and hemisphere (right, left), on PFC-Amygdala FC from infancy to the juvenile (prepubertal) period. Biological mother group (control, MALT) was included as a covariate in the statistical models. Post-hoc comparisons of the means were conducted using Student t-tests when significant caregiving group interaction effects were detected in the LMM, and were Bonferroni corrected for multiple comparisons where applicable. Linear regression was used to test whether postnatal cortisol exposure (6 month hair cortisol concentrations) were predictive of PFC-Amygdala FC when significant caregiving group effects were detected in the LMM. Statistical significance level was set at $p<0.05$.

4.3.3.2 Infancy-Juvenile Period Longitudinal Whole Brain Voxel-wise Amygdala FC

The seed-based maps of Amygdala FC with other voxels in the brain were included in a mixed linear model (MLM), AFNI's 3dLME group analysis program with age (3, 6, 12, 18 months), and hemisphere (Right, Left) as the within subject variables and caregiving group (Control, MALT) as the between subject variable. Statistically significant voxel clusters following multiple comparisons corrections using False Discovery Rate (FDR-corrected q=0.05, cluster size ≥ 2 voxels) were displayed on the infant atlases (Shi*, et al.*, 2016) and their anatomical localization identified with established stereotaxic/MRI combined rhesus macaque brain atlases (Paxinos G, 2000; Saleem KS, 2012); see Figure 2B).

Post-hoc comparisons of means were conducted using Student t-tests on clusters with significant main group or group x age interaction effects resulting from the MLM AFNI 3dLME analysis, after FDR multiple comparisons correction, and were Bonferroni corrected for multiple comparisons where applicable. We then performed regression analyses to test whether early postnatal cortisol exposure (6 month hair cortisol concentrations) were predictive of Amygdala FC in clusters with significant group or group by age post-hoc effects. FC in these clusters (Fischer's Z-transformed) was normally distributed. Statistical significance level was set at $p<0.05$.

4.3.3.3 Adolescent PFC-Amygdala FC: ROI-ROI Analyses

Repeated measures ANCOVA were conducted for each PFC-Amygdala ROI-ROI BOLD temporal correlation to examine differences in adolescence FC based on group (Control, MALT) and sex (male, female) as fixed factors and hemisphere (left & right) as the repeated measure. Biological mother group (bio-Control or bio-MALT) was included as a covariate. Mauchly's Test of Sphericity was used to test whether the assumption of sphericity was violated, and if so, a Greenhouse-Geisser correction was used. Post-hoc comparisons of means were conducted using Student t-tests on tests with significant group interactions. Bonferroni correction was applied for multiple comparisons, and we present the results before and after correction, when applicable. Effect sizes are reported for significant results. Statistical significance level was set at $p<0.05$.

4.4 Results

4.4.1 LONGITUDINAL INFANCY-JUVENILE AMYGDALA FUNCTIONAL CONNECTIVITY STUDIES

4.4.1.1 Amygdala-PFC ROI-ROI Functional Connectivity (Figure 4.1)

Left Amygdala-Right Amygdala FC

A significant main effect of group ($F_{(1,20.4)}=4.4$, $p=0.0496$, $\eta^2=0.951$) was detected with MALT animals showing higher FC compared to Controls across age. The main effect of group remained after adding biological mother as a covariate ($F_{(1,23.2)}=4.3$, $p=0.0487$, $\eta^2=0.949$) and a significant group x sex x biological mother emerged ($F_{(1,19.8)}=7.6$, $p=0.0124$, $\eta^2=0.983$) when biological mother was included as a covariate in the model, with stronger (positive) FC in MALT females born to biological Controls than MALT females born to biological MALT mothers, although post-hoc comparisons of the means

were not significant due to small sample sizes. Additionally, a trend toward main effect of age ($F_{(3,20.0)}$=2.7, p=0.0764, η^2=0.879) was found. No other significant main or interaction effects of sex ($F_{(1,20.7)}$=0.4, p=0.5176, η^2=0.138), group x age ($F_{(3,17.8)}$=0.6, p=0.6017, η^2=0.265), group x sex ($F_{(1,34.8)}$=0.2, p=0.6645, η^2=0.036), or age x sex ($F_{(3,17.7)}$=0.3, p=0.8157, η^2=0.083) were detected.

Amygdala-mPFC FC

Amygdala-BA 25

A significant interaction effect of group x sex ($F_{(1,48.3)}$=5.3, p=0.0258, η^2=0.966) with higher FC Control than MALT females was detected; however, post hoc t-tests were not significant ($t_{(1,24.8)}$=2.0, p=0.0540, η^2=), likely due to lack of power. A sex x hemisphere interaction effect that was just short of significance ($F_{(1,53.8)}$=3.6, p=0.0641, η^2=0.928), and a group x sex x hemisphere interaction ($F_{(1,48.3)}$=6.1, p=0.0169, η^2=0.974) were found, with higher positive FC in Control than MALT females in the right hemisphere (t(1, 12.0)=2.3, p=0.0431, η^2=). No other significant main or interaction effects of group ($F_{(1,53.7)}$=0.6, p=0.4548, η^2=0.265), age ($F_{(3,26.7)}$=0.8, p=0.4886, η^2=0.390), sex ($F_{(1,53.8)}$=0.02, p=0.8957, η^2=0.0004), hemisphere ($F_{(1,53.5)}$=0.6, p=0.4481, η^2=0.265), group x age ($F_{(3,30.8)}$=0.4, p=0.7726, η^2=0.138), group x hemisphere ($F_{(1,53.7)}$=0.4, p=0.5508, η^2=0.138), age x sex ($F_{(3,30.9)}$=0.6, p=0.6284, η^2=0.265), or age x hemisphere ($F_{(3,26.7)}$=1.6, p=0.2114, η^2=0.719), were detected. Including biological mother as a covariate to the model did not affect the statistical findings.

Amygdala-BA 32

A significant main effect of age ($F_{(3,36.5)}$=3, p=0.0426, η^2=0.900) was detected, with the highest (positive) FC seen at 3 months, and decreasing through 18 months, although

remaining positive. A significant interaction effect of sex x hemisphere was found ($F_{(1,56.3)}$=4.4, p=0.0401, η^2=0.951) with females showing higher FC than males in the right vs. left hemisphere. No other significant main or interaction effects of group ($F_{(1,55.5)}$=0.09, p=0.7714, η^2=0.008), sex ($F_{(1,56.3)}$=0.02, p=0.9010, η^2=0.0004), hemisphere ($F_{(1,55.4)}$=1.0, p=0.3230, η^2=0.5), group x age ($F_{(3,41.4)}$=0.9, p=0.4339, η^2=0.448), group x sex ($F_{(1,58.9)}$=0.3, p=0.5700, η^2=0.083), group x hemisphere ($F_{(1,55.5)}$=0.4, p=0.5208, η^2=0.138), age x sex ($F_{(3,41.3)}$=0.9, p=0.4329, η^2=0.448), or age x hemisphere ($F_{(3,36.5)}$=1.2, p=0.3357, η^2=0.590), were detected. Including biological mother as a covariate to the model did not affect the statistical findings.

Amygdala- ACC(BA 24)- FC

A significant age x group x sex effect ($F_{(2,42.3)}$=3.7, p=0.0337, η^2=0.932) followed by post hoc t-tests, showed significantly different functional coupling between female groups at 12 months of age (t(1,3.9)=3.2, p= 0.0334, η^2=), with positive FC in Control but negative FC in MALT females. This interaction effect remained after adding biological mother as a covariate ($F_{(1,31.7)}$=7.8, p=0.0088, η^2=0.984). Additional significant effects were detected when biological mother was included in the model: group ($F_{(1,15.5)}$=5.9, p=0.0283, η^2=0.972), sex ($F_{(1,16.8)}$=6.0, p=0.0261, η^2=0.973), group x age ($F_{(3,13.5)}$=4.7, p=0.0184, η^2=0.957), group x sex ($F_{(1,31.7)}$=22.9, p=0.00004, η^2=0.998), group x biological mother ($F_{(1,26.3)}$=6.2, p=0.0193, η^2=0.975), sex x biological mother ($F_{(1,25.2)}$=5.0, p=0.0337, η^2=0.962), group x age x biological mother ($F_{(2,26.5)}$=5.4, p=0.0110, η^2=0.967), and group x sex x biological mother ($F_{(1,30.6)}$=20.3, p=0.00009, η^2=0.998) were detected. Post-hoc t-tests showed significantly higher positive FC in Control than MALT animals born to MALT (but not Control) biological moms at 12 months of age ($t_{(1,9.7)}$=3.0, p=0.0139, η^2=),

and across all ages in Control than MALT females born to a MALT biological mother ($t_{(1,16.8)}$=2.1, p=0.0476, η^2=). When biological mother was not included in the model no significant main or interaction effects of group ($F_{(1,22.0)}$=0.4, p=0.5263, η^2=0.138), age ($F_{(3,24.6)}$=0.5, p=0.6672, η^2=0.200), sex ($F_{(1,22.3)}$=0.6, p=0.4394, η^2=0.265), hemisphere ($F_{(1,22.0)}$=0.001, p=0.9733, η^2=<0.0001), group x age ($F_{(3,19.0)}$=0.3, p=0.8138, η^2=0.083), group x sex ($F_{(1,42.5)}$=0.09, p=0.7726, η^2=0.008), group x hemisphere ($F_{(1,22.0)}$=0.03, p=0.8760, η^2=0.0009), age x sex ($F_{(3,18.9)}$=0.2, p=0.9078, η^2=0.039), age x hemisphere ($F_{(3,24.6)}$=1.1, p=0.3765, η^2=0.548), or sex x hemisphere ($F_{(1,22.3)}$=0.6, p=0.4510, η^2=0.265) were detected.

Amygdala- OFC FC

Amygdala-BA 11

A trend effect of hemisphere ($F_{(1,42.6)}$=3.2, p=0.0820, η^2=0.911) was detected, with higher positive FC seen in the right than left hemisphere, which became significant ($F_{(1,36.8)}$=4.7, p=0.0375, η^2=0.957) when biological mother was added as a covariate. Only additional trends toward significance were detected: age x sex ($F_{(3,28.8)}$=2.4, p=0.0842, η^2=0.852), and sex x biological mother ($F_{(1,39.7)}$=3.2, p=0.0830, η^2=0.911) and group x sex x biological mother ($F_{(1)29.5}$=3.5, p=0.0706, η^2=0.925) when biological mother was added as covariate in the models. No other significant main or interaction effects of group ($F_{(1,42.7)}$=0.06, p=0.8095, η^2=0.004), age ($F_{(3,35.4)}$=1.5, p=0.2299, η^2=0.692), sex ($F_{(1,43.3)}$=1.4, p=0.2387, η^2=0.662), group x age ($F_{(3,28.7)}$=0.8, p=0.5024, η^2=0.390), group x sex ($F_{(1,52.9)}$=0.2, p=0.6670, η^2=0.039), group x hemisphere ($F_{(1,42.7)}$=2.4, p=0.1257, η^2=0.852), age x hemisphere ($F_{(3,35.4)}$=0.3, p=0.7977, η^2=0.083), or sex x hemisphere

($F_{(1,43.3)}$=0.6, p=0.4282, η^2=0.265), were detected when biological mother was not included in the model.

Amygdala-BA 13

A significant main effect of group ($F_{(1,60.0)}$=5.0, p=0.0289, η^2=0.962) was detected, with higher positive FC in Control than MALT animals, which remained significant after adding biological mother as a covariate ($F_{(1,50.8)}$=11.3, p=0.0015, η^2=0.992). Additional significant effects detected after adding biological mother as covariate include age ($F_{(3,27.9)}$=4.9, p=0.0071, η^2=0.960),age x sex ($F_{(3,27.3)}$=3.5, p=0.0290, η^2=0.925) and group x age ($F_{(3,32.6)}$=3.4, p=0.0286, η^2=0.920). Post-hoc t-tests revealed significant differences at 3 months ($t_{(1,26.7)}$=3.8, p=0.0009), with Controls showing higher positive FC than MALT animals (which showed either uncoupling or negative FC), but only in infants born to Control Biological mom. Other trends include group x hemisphere ($F_{(1,50.8)}$=3.1, p=0.0854, η^2=0.906), detected when biological mother was added as a covariate. No other significant main or interaction effects of age ($F_{(3,40.6)}$=2.0, p=0.1243, η^2=0.800), sex ($F_{(1,60.8)}$=0.003, p=0.9572, η^2=<0.0001), hemisphere ($F_{(1,59.9)}$=0.2, p=0.6635, η^2=0.039), group x sex ($F_{(1,52.0)}$=0.1, p=0.7109, η^2=0.010), group x hemisphere ($F_{(1,60.0)}$=1.6, p=0.1149, η^2=0.719), age x sex ($F_{(3,41.4)}$=1.6, p=0.2134, η^2=0.719), age x hemisphere ($F_{(3,40.6)}$=0.7, p=0.5812, η^2=0.329), or sex x hemisphere ($F_{(1,60.8)}$=0.2, p=0.6788, η^2=0.039), were detected when biological mother was not included in the model.

Amygdala- dlPFC FC

Amygdala-BA 9

A significant age x group x hemisphere interaction ($F_{(3,36.7)}$=4.3, p=0.0106, η^2=0.949) was detected, , with positive FC in the left and negative in the right hemisphere of Controls at 12 months ($t_{(1,6)}$=4.2, p=0.0056). Adding biological mother as a covariate did not confirm that interaction but uncovered a different group x sex x hemisphere x biological mother interaction ($F_{(1,30.2)}$=4.3,p=0.0467, η^2=0.949) with higher FC right hemisphere compared to the left of MALT males born to a Control Biological mother ($t_{(1,6)}$=-2.6, p=0.0415). An age x sex x hemisphere ($F_{(3,36.4)}$=3.8, p=0.0189, η^2=0.935) was also detected, and remained after adding biological mother as covariate ($F_{(3,23.2)}$=4.1, p=0.0180, η^2=0.944). A trend group x sex was detected ($F_{(1,44.7)}$=3.6, p=0.0654, η^2=0.928). No other significant main or interaction effects of group ($F_{(1,52.5)}$=0.09, p=0.7651, η^2=0.008), age ($F_{(3,32.1)}$=1.1, p=0.3708, η^2=0.548), sex ($F_{(1,53.1)}$=0.9, p=0.3471, η^2=0.448), hemisphere ($F_{(1,52.5)}$=1.5, p=0.2254, η^2=0.692), group x age ($F_{(3,36.7)}$=1.8, p=0.1712, η^2=0.764), group x hemisphere ($F_{(1,52.5)}$=1.0, p=0.3230, η^2=0.500), age x sex ($F_{(3,36.4)}$=0.6, p=0.6467, η^2=0.265), age x hemisphere ($F_{(3,32.1)}$=1.7, p=0.1860, η^2=0.743), or sex x hemisphere ($F_{(1,53.1)}$=0.7, p=0.4037, η^2=0.329), were detected when biological mother was not included in the model.

Amygdala-BA 46

A significant main effect of sex ($F_{(1,74.5)}$=4.8, p=0.0318, η^2=0.958) was found, with higher positive FC in females than males. It did not persist after adding biological mother as a covariate; however a significant main group effect was uncovered ($F_{(1,51.1)}$=4.2, p=0.0428, η^2=0.946), with higher positive FC in Control than MALT animals. No other significant main or interaction effects of group ($F_{(1,74.2)}$=0.003, p=0.9600, η^2=<0.0001), age ($F_{(3,37.8)}$=0.5, p=0.6795, η^2=0.200), hemisphere ($F_{(1,74.3)}$=0.04, p=0.8444, η^2=0.002), group x age ($F_{(3,38.7)}$=1.7, p=0.1931, η^2=0.743), group x sex ($F_{(1,65.8)}$=0.5, p=0.4788,

η^2=0.200), group x hemisphere ($F_{(1,74.2)}$=2.7, p=0.1024, η^2=0.879), age x sex ($F_{(3,38.4)}$=0.9, p=0.4273, η^2=0.448), age x hemisphere ($F_{(3,37.8)}$=0.6, p=0.6299, η^2=0.265), or sex x hemisphere ($F_{(1,74.5)}$=0.7, p=0.3974, η^2=0.329) were detected when biological mother was not included in the model.

4.4.1.2 Associations between Postnatal Cortisol Exposure and MALT effects on PFC-AMY FC

Linear regression models were ran to test whether hair cortisol concentrations at 6 months was associated with PFC-amygdala FC group effects detected in the LMM. Hair cortisol at 6 months significantly predicted AMY-dlPFC (BA9) at 12 months in the right hemisphere (β=0.0036, p=0.0497; adjusted R^2=0.48, $F_{(1,5)}$=6.6, p=0.0498), and at 18 months in both right (β=0.0012, p=0.0478; adjusted R^2=0.49, $F_{(1,5)}$=6.8, p=0.0478) and left hemispheres (β=-0.0015, p=0.0329; adjusted R^2=0.56, $F_{(1,5)}$= 8.5, p=0.0329). At 18 months, 6 month hair cortisol also predicted right AMY-dlPFC (BA 46) FC (β=-0.0013, p= 0.0324; adjusted R^2= 0.56, $F_{(1,5)}$=8.6, p= 0.0324) and left AMY-OFC(BA 11) FC (β=0.0014, p=0.0269; adjusted R^2=0.59, $F_{(1,5)}$=9.6, p=0.0269).

4.4.1.3 Whole-brain Voxel-wise Amygdala FC

Amygdala seed-based FC maps were FDR corrected (q=0.05, t>2.630, p<0.0085), and clusters with ten or more contiguous voxels (faces touching) were selected to increase stringency for the secondary analyses (Figure 2B). The Amygdala FC maps obtained in this infant and juvenile macaque dataset during development closely resemble those published in adult macaques (Amaral and Price, 1984; Grayson*, et al.*, 2016; Reding*, et*

al., 2013; Russchen*, et al.*, 1985). Thus, significant positive Amygdala FC was found throughout regions in the temporal lobe, including bilateral Amygdala and hippocampal regions, and the ACC, subcortical regions (e.g. thalamus, caudate and ventral striatum, including nucleus Accumbens –NAcc-), cerebellum, and brainstem areas. Negative Amygdala FC was found dispersed in the cingulate cortex (anterior and posterior) and cerebellum as well as throughout motor, posterior parietal and occipital (visual) cortices, in contrast to what has been shown in adult macaques (Grayson*, et al.*, 2016), where only positive connectivity was found throughout the brain. In addition, negative FC was not found between amygdalae and hypothalamus, as reported in adult macaques (Reding*, et al.*, 2013). Our results are also consistent with studies in adult humans reporting positive Amygdala FC with regions throughout the temporal lobe, bilateral amygdalae and hippocampal regions, subcortical regions (e.g. thalamus and caudate), insula, and brainstem, and negative FC with cerebellum, and occipital and parietal lobes (Roy*, et al.*, 2009).

Secondary MLM (AFNI 3dLME) analysis restricted to these significant clusters of contiguous voxels selected from the Amygdala FC maps showed a main effect of age that mainly reflected increased positive FC with age between Amygdalae and bilateral corticomedial/medial amygdaloid nucleus, right anterior amygdaloid area; cortical regions, such as bilateral superior temporal sulcus (STS: TE anteroventral, TE anterodorsal, temporo-parieto-occipital junction), left parainsular cortex, left visual cortex (V1), left posterior cingulate, left perirhinal cortex, left entorhinal cortex, right periamygdaloid cortex and bilateral ventral/dorsal tegmental area, bilateral dorsal raphe nucluei, left lateral parabrachial nucleus, bilateral cerebellum, bilateral ld/lv Amygdala, left trigeminus, left abducens/facial nucleus/pontine reticular formation, and left pre/pro/parasubiculum (hippocampus) (FDR corrected ($q=0.05$), $t>5.242$, $p<0.0023$, cluster threshold ≥ 2 voxels; Figures 4.3A, 4.3B & 4.4).

Although there were no main effects of MALT, a caregiving group by age interaction effect was detected in Amygdala FC with two main clusters: the left lateral dorsal tegmental area (LDT)/locus coeruleus (LC) and left periamygdaloid Cortex (PAC)/basal amygdala (BA). MALT effects emerged with age so that, while controls' FC strengthened between 12 and 18 months, MALT's FC with LC/LDTA weakened (became uncoupled) or became negatively coupled with PAC/BA (FDR corrected ($q=0.05$), $t>7.146$, $p<2.5x10^{-4}$, cluster threshold ≥ 2 voxels; Figure 4.5). Post-hoc (t-test) comparison of the means confirmed Controls had stronger FC with both clusters than MALT animals at 18 months (PAC/BA $t_{(5.9)}= 4.47$, $p=0.0043$; LDT/LC $t_{4.3}= 3.7$, $p= 0.0179$) and also at 12 months for the LDT/LC ($t_{(11)}=2.49$, $p=0.0302$) but not PAC/BA ($t_{(10.3)}=- 0.36$, $p=0.7274$). Interestingly, FC LC/LDTA seems higher in MALT than controls initially (at 3 months), followed by gradual decoupling at later ages.

4.4.1.4 Associations between Postnatal Cortisol Exposure and MALT effects on Whole Brain Voxel-wise Amygdala FC

Linear regression models were ran to test whether hair cortisol concentrations at 6 months predicted the MALT effects identified in the MLM (AFNI 3dLME) analysis on Amygdala FC with PAC/BA and LDT/LC clusters at 18 months of age. Hair cortisol at 6 months significantly predicted Amygdala FC with the PAC/BA cluster ($\beta=-0.0027$, $p=0.0351$; adjusted $R^2=.56$, $F_{(1,5)}=8.2$, $p=0.0351$) and with the LDT/LC cluster ($\beta=-0.0028$, $p=0.0003$;adjusted $R^2=0.9$, $F_{(1,5)}=-8.7$, $p=0.0003$) at 18 months (Figure 4.6).

4.4.2 ADOLESCENCE AMYGDALA-PREFRONTAL FUNCTIONAL CONNECTIVITY STUDIES

4.4.2.1 Amygdala-PFC ROI-ROI Functional Connectivity (Figure 4.7)

Left Amygdala-Right Amygdala

No main effect of group ($F_{(1,21)}$=0.5, p=0.4872, η^2=0.02) or sex ($F_{(1,21)}$=0.2, p=0.6535, η^2=0.01), or group x sex ($F_{(1,21)}$=0.1, p=0.7165, η^2=0.006) was detected. No additional main or interaction effects were detected when biological mother was included as a covariate in the statistical model.

Amygdala- mPFC FC

Amygdala-BA 25

No main effects of group ($F_{(1,21)}$=0.4, p=0.5287, η^2=0.02), sex ($F_{(1,21)}$=0.4, p=0.5501, η^2=0.02), or hemisphere ($F_{(1,21)}$=0.2, p=0.6552, η^2=0.01), or group x sex ($F_{(1,21)}$=0.05, p=0.8254, η^2=0.002), group x hemisphere ($F_{(1,21)}$=0.09, p=0.7734, η^2=0.004), or sex x hemisphere ($F_{(1,21)}$=0.3, p=0.6050, η^2=0.01), were detected. Including biological mother as a covariate to the model did not affect the statistical findings.

Amygdala-BA 32 FC

Biological mother had a significant effect when added a covariate in the model ($F_{(1,20)}$=9.0, p=0.0070, η^2=0.31), such that animals born to a Control Biological mother had higher positive FC than those born to a MALT. A main effect of hemisphere ($F_{(1,20)}$=5.0, p=0.0371, η^2=0.2) was also detected when biological mother was included as

covariate, with higher positive FC in the right than left hemisphere. Additionally, there was also a trend of sex ($F_{(1,20)}$=3.9, p=0.0635, η^2=0.16). No main effects of group ($F_{(1,21)}$=0.1, p=0.7392, η^2=0.005), sex ($F_{(1,21)}$=0.2, p=0.6538, η^2=0.01), or hemisphere ($F_{(1,21)}$=2.1, p=0.1595, η^2=0.09), or interaction effects of group x sex ($F_{(1,21)}$=0.03, p=0.8646, η^2=0.001), group x hemisphere ($F_{(1,21)}$=0.6, p=0.4408, η^2=0.03), or sex x hemisphere ($F_{(1,21)}$=0.007, p=0.9319, η^2=0.0004), were detected when biological mother was not included in the model.

Amygdala- ACC(BA 24) FC

A significant sex x hemisphere interaction effect was detected ($F_{(1,21)}$=5.1, p=0.0351, η^2=0.20) with females, but not males, showing lower FC in the left vs. right hemisphere. This effect became a trend ($F_{(1,20)}$=3.5, p=0.0744, η^2=0.15) when biological mother was added as a covariate. A trend in sex effects ($F_{(1,21)}$=3.8, p=0.0650, η^2=0.15) was detected, which remained as a trend ($F_{(1,20)}$=3.3, p=0.0847, η^2=0.14) when biological mother was added as a covariate. No effects of group ($F_{(1,21)}$=0.6, p=0.4504, η^2=0.03), hemisphere ($F_{(1,21)}$=0.09, p=0.7721, η^2=0.004), group x sex ($F_{(1,21)}$=0.4, p=0.5503, η^2=0.02), or group x hemisphere ($F_{(1,21)}$=2.3, p=0.1441, η^2=0.10), were detected when biological mother was not included in the model.

Amygdala- OFC FC

Amygdala-BA 11 FC

A trend of sex ($F_{(1,21)}$=3.7, p=0.0687, η^2=0.15) was detected, suggesting stronger positive FC in males than females. When biological mother was added as a covariate, a main hemisphere ($F_{(1,20)}$=8.0, p=0.0105, η^2=0.29) and hemisphere x biological mother effect ($F_{(1,20)}$=4.9, p=0.0381, η^2=0.20) were detected, with animals born to Control biological mothers showing higher positive FC in the right vs. left hemisphere. No main effects of group ($F_{(1,21)}$=0.3, p=0.6118, η^2=0.01), hemisphere ($F_{(1,21)}$=2.6, p=0.1199, η^2=0.11), group x sex ($F_{(1,21)}$=0.4, p=0.5430, η^2=0.02), group x hemisphere ($F_{(1,21)}$=0.4, p=0.5384, η^2=0.02), or sex x hemisphere ($F_{(1,21)}$=0.005, p=0.9437, η^2=0.0002) were detected when biological mother was not included in the model.

Amygdala-BA 13 FC

When biological mother was included as a covariate, a trend toward a hemisphere effect ($F_{(1,20)}$=3.7, p=0.0690, η^2=0.16) was detected. No main effects of group ($F_{(1,21)}$=0.04, p=0.8530, η^2=0.002), sex ($F_{(1,21)}$=2.8, p=0.1121, η^2=0.12), or hemisphere ($F_{(1,21)}$=2.9, p=0.1020, η^2=0.12), or interaction effects of group x sex ($F_{(1,21)}$=1.4, p=0.2475, η^2=0.06), group x hemisphere ($F_{(1,21)}$=0.1, p=0.7449, η^2=0.005), or sex x hemisphere ($F_{(1,21)}$=1.3, p=0.2610, η^2=0.06), were detected when biological mother was not included in the model.

Amygdala-dlPFC FC

Amygdala-BA 9 FC

When biological mother was included as a covariate, a trend effect of biological mother ($F_{(1,20)}$=4.2, p=0.0527, η^2=0.18) was detected, with Control animals having positive FC , whereas MALT monkeys had negative FC. No main effects of group ($F_{(1,21)}$=0.004,

p=0.9499, η^2=0.0002), sex ($F_{(1,21)}$=0.9, p=0.3421, η^2=0.04), hemisphere ($F_{(1,21)}$=2.5, p=0.1274, η^2=0.11), group x sex ($F_{(1,21)}$=0.04, p=0.8508, η^2=0.002), group x hemisphere ($F_{(1,21)}$=0.4, p=0.5525, η^2=0.02), or sex x hemisphere ($F_{(1,21)}$=0.4, p=0.5272, η^2=0.02), were detected when biological mother was not included in the model.

Amygdala-BA 46 FC

No main effects of group ($F_{(1,21)}$=0.01, p=0.9173, η^2=0.001), sex ($F_{(1,21)}$=0.4, p=0.5541, η^2=0.02), hemisphere ($F_{(1,21)}$=0.04, p=0.8542, η^2=0.002), group x sex ($F_{(1,21)}$=1.1, p=0.3011, η^2=0.05), group x hemisphere ($F_{(1,21)}$=0.8, p=0.3914, η^2=0.04), or sex x hemisphere ($F_{(1,21)}$=0.4, p=0.5562, η^2=0.02) were detected. No additional main or interaction effects were detected when biological mother was included as a covariate in the model.

4.5 Discussion

The goal of the current study was to determine the developmental impact of infant MALT on amygdala FC longitudinally, from macaque infancy through adolescence, which may underlie the enhanced emotional and stress reactivity reported previously by our group in MALT animals as compared to monkeys raised by competent, nurturing mothers. The study utilized a well-established rhesus monkey model of MALT by the mother leading to infant distress, and a crossfostering experimental design with random assignment of infants to either Control or MALT caregiving group at birth. In this model, the highest rates of abuse and rejection take place during the first three months of life, an important period of rapid and drastic cortico-limbic maturation. Resting state fMRI scans were collected longitudinally on these animals during infancy (at 3 and 6 months of age) and again during the juvenile, prepubertal, period (at 12, and 18 months) in order

to examine the impact of MALT on the development of PFC-Amygdala circuits as well as a whole brain, voxel-wise analysis, of Amygdala FC. Results from the AMY FC voxel-wise analysis showed developmental increases with age with many regions, likely reflecting increased processing of socioemotionally relevant stimuli from infancy to the juvenile period, which would affect fear-learning. MALT effects on AMY FC emerged with age; so that, whereas controls' AMY's functional coupling with regions implicated in arousal and stress (i.e. Locus Coeruleus, laterodorsal tegmental area) increased during the juvenile period, it weakened in MALT animals, and it was predicted by higher cortisol exposure during infancy. This uncoupling may be promoting detachment or emotional supression/blunting as a survival mechanism in response to threatening environments. We also performed an ROI analysis to examine specific alterations in AMY-PFC circuits FC that could underlie the higher emotional reactivity, attention bias to threat (chapter 2), and elevated state anxiety and fear/safety discrimination issues (chapter 3) reported in MALT animals. From infancy to the juvenile, prepubertal period, MALT animals showed weaker AMY FC than Controls with subgenual cingulate (BA 25) in females, ACC (BA 24, also in females), OFC (BA 13), and dlPFC (BA 46), but stronger FC between left and right AMY in MALT than Control animals. Interestingly, the general weaker PFC-AMY FC detected in MALT animals during infancy and the early juvenile period seem to have normalized during adolescence, when we uncovered several unexpected effects of biological ancestry or prenatal factors. Together, these findings demonstrate that maternal MALT results in developmental alterations of AMY circuits that result in weakened AMY FC with PFC and brainstem arousal centers, some of them predicted by exposure to elevated cortisol levels during infancy, although these alterations seem to be transient and normalize by adolescence. Since MALT animals show elevated state anxiety and problems with the modulation of fear by safety cues during adolescence, there is a need to understand what neural circuits are responsible for these problems

with emotional regulation as well as the mechanisms underlying the dynamic developmental changes and alterations we have uncovered in theses circuits could generate useful neural biomarkers for future studies testing interventions in individual with childhood MALT and other adverse experiences.

Our exploratory, seed-based voxel-wise analysis showed brain wide strengthening of Amygdala FC with many regions in the brain as infants mature, which may reflect increased processing of socioemotional stimuli, especially related to the emergence of threat/fear-learning as the animals go through weaning (3-6 months) and increase exploration, play and independence from the mother particularly during the juvenile period (12, 18 months) (Sanchez*, et al.*, 2015). Similar changes in Amygdala FC has already been mapped to Amygdala functional maturation during weaning in rat pups (Sullivan*, et al.*, 2000). Of course, the developmental changes in FC with the amygdala were also region-specific, as seen in amygdaloid nuclei, regions along the ventral visual pathway, other temporal sensory areas, and the cerebellum. In contrast to a previous study in adult macaques reporting absence of negative FC (anticorrelation or uncoupling) between Amygdala and other regions of the brain (Grayson*, et al.*, 2016), we found negative FC with the cingulate cortex (anterior –ACC- and posterior), cerebellum, and motor, posterior parietal and occipital (visual) cortices (Grayson*, et al.*, 2016). These discrepancies between macaque studies may be related to developmental changes in Amygdala FC with these regions with age. Among regions showing a main effect of age, AMY-FC increased with age in regions part of the ventral visual pathway (STS, TEav, TEad, TPO), which may support the emergence of face processing and facial expression recognition during the first few months of life (Kuwahata*, et al.*, 2004; Lutz*, et al.*, 1998; Muschinski*, et al.*, 2016; Parr*, et al.*, 2016; Sugita, 2008). Interestingly, prior to 18 months of age, Amygdala FC with Right TPO/STS was close to zero, consistent with previous reports in developmental studies in children (Gabard-Durnam*, et al.*, 2014),

and interpreted by the authors as a functional uncoupling between these regions, which becomes stronger during the juvenile period when higher-order face processing has matured and inputs from the Amygdala are pruned and refined (Webster*, et al.*, 1991a; Webster*, et al.*, 1991b), to better inform on the valence of facial expressions. Amygdala FC with the cerebellum was found to decrease with age, in contrast to what has been reported in adult humans, where a strengthening between centromedial amygdala (CMA) and the cerebellum emerges with age (Qin*, et al.*, 2012), as the CMA and its connections regulate reflexive and defensive behaviors in response to fear (LeDoux, 2007; LeDoux, 2000). Other regions involved in sensory processing and memory (entorhinal cortex, parasubiculum, perirhinal cortex) also showed decreased FC with Amygdala with age in our animals, although it has been reported to increase in adulthood, especially with the BLA, so perhaps this is another circuit that is still developing and may "switch" and become stronger in adulthood (Qin*, et al.*, 2012), as this connectivity will be important for detecting and perceiving fearful stimuli.

Our findings also showed an MALT by age interaction effect in the left lateral dorsal tegmental area LDTA/LC and left PAC/BA, regions implicated in arousal, vigilance, and fear learning, so that the effects of maternal MALT emerged with age. Thus, while Controls' Amygdala FC with LC/LDTA gets stronger between 12 and 18 months, MALT's FC weakens (gets uncoupled) or gets negatively coupled with PAC/BA (left). Interestingly, FC with LC/LDTA is initially higher in MALT than controls (at 3 months), which parallels hyperarousal/reactivity observed in MALT infants, followed by gradual decoupling of the two regions at later ages, which may serve an adaptive role to downregulate the arousal system. By the juvenile period (18 months of age), MALT animals show weaker FC between Amygdala and the left lateral dorsal tegmental area/locus coeruleus and left periamygdala/basal amygdala.

Our finding that cortisol in hair at 6 months is predictive of FC in LC/LDTA and PAC/BA clusters at 18 months of age, is consistent with previous studies examining the effects of cortisol exposure on FC. Exposure to chronic stress and/or glucocorticoids has downstream effects on neuronal density and dendritic arborization, in circuitry vulnerable to the effects of trauma (McEwen, 2004; Roozendaal, et al., 2009; Vyas, et al., 2002). Many of these regions, such as PFC, the amygdala, and hippocampus, have high glucocorticoid receptor densities, which suggest they are very sensitive to the impacts of chronic exposure to cortisol (Anisman, et al., 1998; Sanchez, et al., 2000a; Sanchez, et al., 2000b). FC studies in adult men given a one-time dose of hydrocortisone, have also reported uncoupling of the amygdala, especially a reduction in positive FC with regions involved in initiating and maintaining the stress response, such as the LC, in addition to reducing negative FC with regions involved in executive functions (Henckens*, et al.*, 2012). Exposure to chronic glucocorticoid exposure during early development may have lasting impacts into the juvenile period, as seen in the disconnection between amygdala - LC/LDTA and PAC/BA clusters detected in MALT animals at 18 months of age.

Of particular relevance to the effects of early life adversity/stress experiences (such as infant MALT) on the development of Amygdala circuits is the deleterious impact of chronically elevated glucocorticoid (e.g. cortisol) exposure on dendritic arborization and postsynaptic spine density. As stated earlier, our laboratory has shown that MALT infants were exposed to elevated levels of cortisol during infancy, which was evident in their higher hair cortisol accumulation at six months of age than in Controls (Drury*, et al.*, 2017). Cortisol fluctuations occur normally across the circadian rhythm and during ultradian oscillations on a smaller temporal scale, aiding in the stabilization of spines and pruning of pre-existing synapses, a balance important in learning and plasticity (Liston*, et al.*, 2013). However, exposure to chronically high levels of glucocorticoids, even found in as little as 30 min of increased exposure (Chen*, et al.*, 2008), prevents the

formation of new spines and pruning of synapses during development, a time of enhanced plasticity and remodeling (Liston and Gan, 2011). The elimination of stable spines, and perhaps inability to develop and maintain spines and effectively prune synapses during this period of development may be a factor leading to reduced structural connectivity of Amygdala circuits, potentially resulting in the weaker FC Output projections of the amygdala to the lateral dorsal tegmental area may mediate increased synaptic transmission in thalamic sensory neurons (by way of activating cholinergic projections), during states of fear. In studies of early developmental exposure to glucocorticoids in rodents, increased recruitment of cholinergic neurons in the LDTA can lead to prolonged hyperanxious states (Borges*, et al.*, 2013; Kaufer*, et al.*, 1998). This "programming" of the neurons in the LDTA towards increased activity may be leading to the heightened arousal and vigilance reported in MALT animals despite weakened FC with the amygdala. Rodent studies have found that cholinergic neurons in the LDTA also project to the LC (Jones and Yang, 1985). Activation of these terminals through perfusions of cholinoceptor agonists in the LC increase the firing rate of these neurons and increase arousal, suggesting that the LDTA projections to the LC are bolstering arousal through excitatory connections (Egan and North, 1985; Engberg and Svensson, 1980). In response to stressful stimuli, LC neurons are activated (Abercrombie and Jacobs, 1987; Aston-Jones*, et al.*, 1991; Grant*, et al.*, 1988; Rasmussen*, et al.*, 1986) and facilitate norepinephrine increase in the amygdala through terminals in the basolateral amygdala (BLA) (Asan, 1998). This pathway is thought to support proper neuroendocrine responses to a stressor or fearful stimulus. Reciprocal connections from the Amygdala to the LC (Van Bockstaele*, et al.*, 2001) provide feedback to facilitate an increase in responding to stressful stimuli, and therefore arousal (Goldstein*, et al.*, 1996), which may be implicated in the development of maladaptive responses to stress in fear disorders (Buffalari and Grace, 2007). Norepinephrine neurons projections to the

amygdala also modulates memories of aversive stimuli (McGaugh, 2002) through the consolidation of learning within the BLA (Gallagher*, et al.*, 1977). Perhaps the weakened Amygdala FC (or uncoupling of this pathway) observed in MALT animals, emerging between 12 and 18 months of age is contributing to abnormal regulation of fear learning, which may be contributing to the noise in this circuit during memory consolidation, leading to increased fear and stress responses to generalized stimuli due to a lack of learned fear memories to a specific cue. Corticotropin releasing factor (CRF), a peptide hormone involved in the stress response, also plays a role in the LC norepinephrine (NE) system. In response to chronic stress, there is a postsynaptic sensitization to CRF in the LC, leading to an increased release of NE to downstream targets (Finlay*, et al.*, 1997). This plasticity may result in hyperactivity or hyperresponsiveness of the LC–NE system. Disconnection between Amygdala and LC could be a consequence of exposure to chronic stress-induced increased cortisol, previously shown in human studies (Henckens*, et al.*, 2012). This amygdala uncoupling may be adaptive, promoting emotional blunting, detachment or suppression as a survival mechanism, giving priority to other networks as to not overwhelm the system, or perhaps promoting normalization of the HPA axis through this uncoupling. A similar weaker FC between the amygdala and ACC/mPFC as we report in this study (i.e. weaker Amygdala FC with mPFC regions: BA 25 –subgenual cingulate-, BA 24 –ACC, as well as with dlPFC region BA 46, and OFC –BA 13- in MALT than Control animals during infancy and juvenile development) has also been reported in human populations with early adverse experiences (VanTieghem and Tottenham, 2018), including adolescents and adults with histories of childhood maltreatment (Thomason*, et al.*, 2015) and in humans with PTSD (Fonzo*, et al.*, 2010b), and is thought to contribute to arousal dysregulation through promoting hypervigilance and attentional bias to threat. Even in the case when the LC may be less activated through weaker reciprocal connections with the BLA in MALT animals, cholinergic neurons

originating in the LDTA can send projections to the LC and increase arousal with increased firing rates of the LC (Jones and Yang, 1985). This suggests an alternative pathway to facilitate increased arousal and therefore maladaptive responses to stress, as memory consolidation may be impaired from the altered FC of these regions with the amygdala (Egan and North, 1985; Engberg and Svensson, 1980).

Decreased FC between amygdala and left periamygdala/basal amygdala emerged at the later age (18 months) in the MALT animals. The periamygdaloid cortex receives direct connections from the olfactory bulb and is involved in conditioning to explicit environmental sensory stimuli. The basal amygdala also plays a critical role in fear learning, specifically conditioned fear expression and extinction (Amano*, et al.*, 2011). Previous studies in humans have reported increased activation of the amygdala when subjects have prior awareness of the aversive nature of the stimuli (Morris*, et al.*, 1998). Perhaps the reduced functional connectivity between both amygdala and the left basal amygdala may be interfering with the modulation of the fear response to learned aversion of environmental stimuli in maltreated animals.

In previous studies, negative resting state FC has been shown to reflect competition between brain networks (i.e. default mode and task positive networks) (Kelly*, et al.*, 2008) or a history of regulation on one region over another (i.e. limbic circuits) (Liang*, et al.*, 2012; Roy*, et al.*, 2009). However, the negative correlations between these regions are extremely close to zero, which may in fact not represent a truly negative correlation, but could perhaps be due to the regression of the global signal, which would center all correlations about zero. Additionally, this near-zero correlation could be interpreted as an uncoupling of the amygdala with these regions in maltreated animals. Uncoupling or disconnection between brain regions, especially limbic and regulatory regions, such as the amygdala and insula with the ACC, has been shown in individuals with PTSD when

evaluating fearful/threatening stimuli (Fonzo*, et al.*, 2010a; Rockstroh and Elbert, 2010).

Despite the strengths of this study, there are also limitations, notably the sample size used in this analysis which, although large for a macaque study, it is still small and lacks statistical power to investigate complex interactions between variables. Given a larger dataset, it would be important to do a full statistical comparison study of outcomes of infants that were raised with their biological mothers and did not experience an early shift in environment to determine if the crossfostering paradigm has an effect on any of these functional differences. Another important limitation of the study is that our longitudinal, within subject design, from 3-18 months could not be extended through adolescence, and could only study some of the same animals crossectionally at the adolescence time point. Due to illness or other factors not directly associated with the study, not all animals had imaging data at all 4 infant-juvenile ages (3, 6, 12, 18 months), although the 3dLME model allowed to include animals that had at least two time points in the analysis. Despite limitations inherent to resting state functional connectivity MRI (in particular, the lack of directionality information in FC measures when using rsfMRI) it has strengths and advantages to task fMRI for developmental and translational purposes, and has proven useful to characterize brain function and emotional regulation across many human populations (Britton*, et al.*, 2013; Costa Dias*, et al.*, 2015; Foster and Wilson, 2006; Gee*, et al.*, 2013; Hare*, et al.*, 2008; Matthews and Fair, 2015). Due to the dampening effect of isoflurane on BOLD signal, we standardized levels to the lowest possible in the literature (1% isoflurane). Previous studies show robust and dynamic fluctuations of the BOLD signal and patterns of network FC similar to awake monkeys, even using ≥1% isoflurane anesthesia (Grayson*, et al.*, 2016; Hutchison*, et al.*, 2012; Hutchison*, et al.*, 2013; Li*, et al.*, 2013; Margulies*, et al.*, 2009; Miranda-

Dominguez*, et al.*, 2014b; Palanca*, et al.*, 2015; Sallet*, et al.*, 2011; Tang and Ramani, 2016; Vincent*, et al.*, 2007) and dose-dependent effects of isoflurane on BOLD signal at much higher dosages (Hutchison*, et al.*, 2014).

Clusters in this analysis showing a significant main effect of age are regions that show, in general, a stronger FC with amygdalae over time. This may reflect increased processing of socioemotional stimuli from infancy to the juvenile period, especially related to fear-learning. Decreased coupling or "uncoupling" between the Amygdala and regions implicated in the initiation and regulation of the stress response (i.e. Locus Coeruleus, laterodorsal tegmental area) seen in maltreated juveniles could be a consequence of exposure to chronic stress-induced increased corticosteroids, as shown in human FC studies (Henckens*, et al.*, 2012). This uncoupling of the amygdala may be promoting detachment or super-supression as a survival mechanism, giving more priority to other networks as to not overwhelm the system, or perhaps promoting normalization of the HPA axis through this uncoupling.

In summary, our findings demonstrate that infant MALT alters the development of AMY circuits, resulting in weakened AMY FC with PFC and brainstem arousal centers, and in part predicted by exposure to elevated cortisol levels during infancy. However, these AMY FC alterations seem to normalize by adolescence, despite MALT adolescent macaques showing elevated state anxiety, problems with safety modulation of fear responses and enhanced attention to threat. Although we can only speculate about why this normalization, it is possible that the group differences get diluted as the animals mature and go through other life experiences, including the relocation to the Main Center. Or it can be that Controls go through synaptic remodeling during adolescence earlier than the MALT, which temporarily brings both groups' FC closer together. The latter possibility will need to be tested through additional follow up of the animals during

the adult period, when both groups are fully mature. In any case, we also still need to address what neural circuits or processes underlie problems with emotional regulation during adolescence, if not PFC-Amyg networks. It is possible that the derailed developmental trajectories in AMY circuits have a downstream effect on other brain networks, and we plan to explore this question next. Overall, these seem to be sensitive, promising and useful neural biomarkers for future studies testing interventions in individual with childhood MALT and other adverse experiences. Future work will be necessary to determine the underlying mechanisms responsible for alterations in functional connectivity in maltreated individuals developmentally, and to elucidate what behavioral manifestations they have longitudinally, as well as in adolescence and adulthood. Utilizing fear learning paradigms and stress response assays like the ones we have used in this NHP model, will help determine to what extent these functional differences lead to changes in arousal and fear/stress regulation.

Table 4.1

Groups breakdown based on randomized crossfostering assignment at birth. Crossfostering conditions are denoted in the y-axis (e.g. C→M identifies infants born to a control biological mother, but fostered by a MALT mother). *All animals were crossfostered except for a male control that was raised by his biological mother. From Morin et al, 2019., with permission.

		FEMALE	MALE	
CONTROL	M→C	3	0	**11**
	C→C	3	5*	
MALTREATED	C→M	1	5	**14**
	M→M	5	3	
		12	**13**	**25**

Table 4.2

Summary of Human Connectome Pipeline (HCP) preprocessing pipeline & modifications applied for macaque brains

HCP Pipeline Stages	Original Human Pipeline	Additional Modifications for Macaque Brains
Preliminary Masking	N/A	average multiple anatomical images to improve signal quality, and register to template similar to HCP-style masking with ANTS
PreFreesurfer	normalize structural data to a standard template	ANTS joint-label fusion used to improve delineation of subcortical structures and grey matter with hand-edited macaque labeled data
Freesurfer	segment subcortical structures, reconstruct native surfaces from the normalized structural data, and register the surfaces to the template surface	perform hypernormalization to improve priors for surface reconstruction, input metadata is faked at “1mm”, so that the native images are segmented
DCAN Preproc	N/A	functional connectivity preprocessing: functional signal detrending, denoising (including global signal regression –GSR-), bandpass filtering (temporal low-pass filtering, f < 0.1Hz), motion censoring, and generation of parcellated timeseries for specific atlases
PostFree	convert native surfaces into HCP-compatible format (i.e. CIFTIs).	No modifications
Vol	registers functional data to the volumetric standard template through the normed structural data	preliminary masks used to mask the data before registration
Surf	projects functional data to the template-space surfaces	No modifications
Executive summary	N/A	standard outputs for quality control

Figure 4-1

Development of Amygdala(AMY)-Prefrontal (PFC) Functional Connectivity (FC) from Infancy Through the Juvenile Period (3-18 mos of age). AMY-AMY and AMY FC with PFC subregions across four ages are plotted separately by group (MALT vs. control). ACC: anterior cingulate cortex; BA: Brodmann area; dlPFC: dorsolateral PFC; mPFC: medial PFC; OFC: orbitofrontal cortex. L: left; R: right. Plots represent mean±standard error of the mean (SEM).

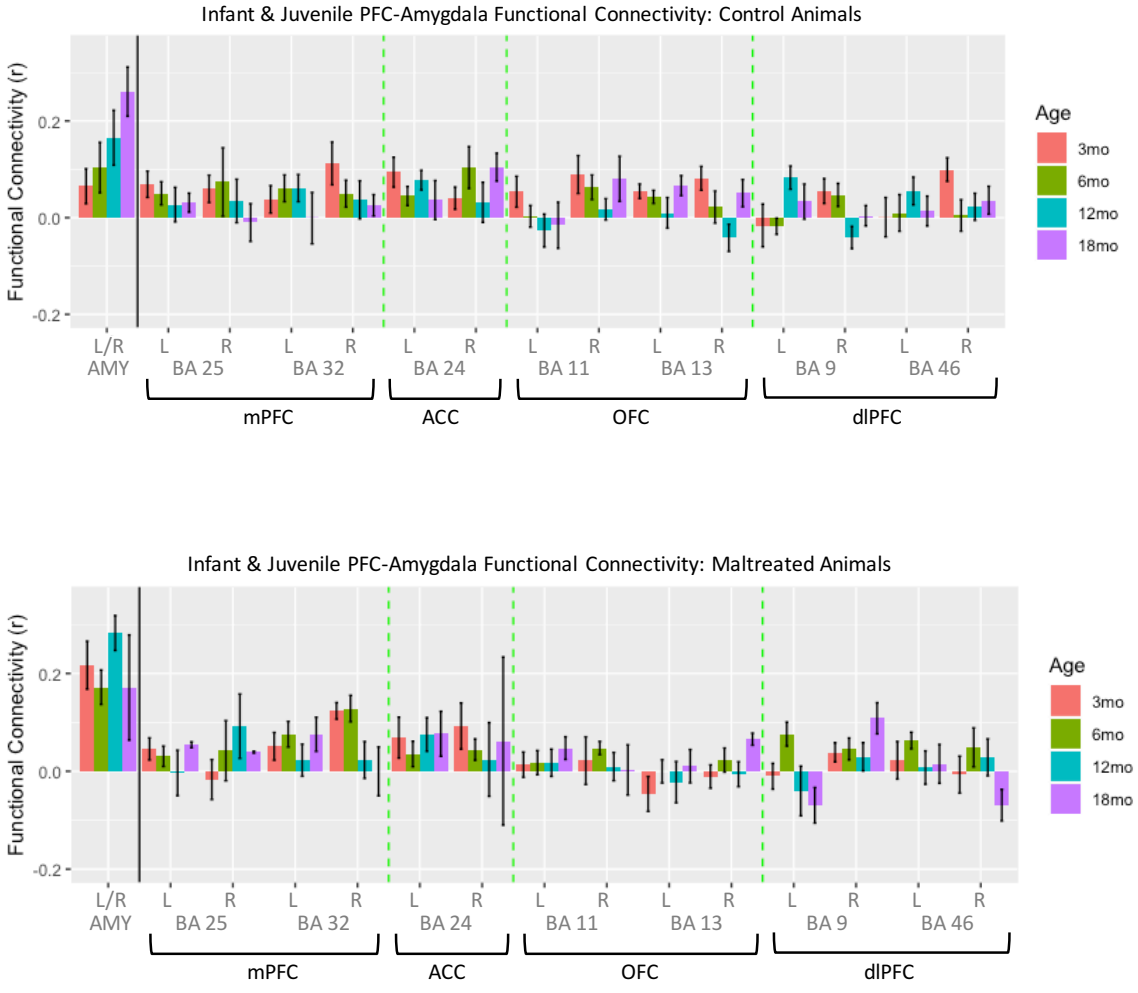


Figure 4-2

Development of Amygdala Functional Connectivity (FC) with the Rest of the Brain from Infancy Through the Juvenile Period (3-18 mos of age). (A) Amygdala region of interest (ROI) with dropout removed (blue); red indicates original ROI. **(B)** Amygdala FC with the rest of the brain obtained from the seed-based whole-brain analysis and collapsed across group, age, sex and hemisphere. L: left; R: right.

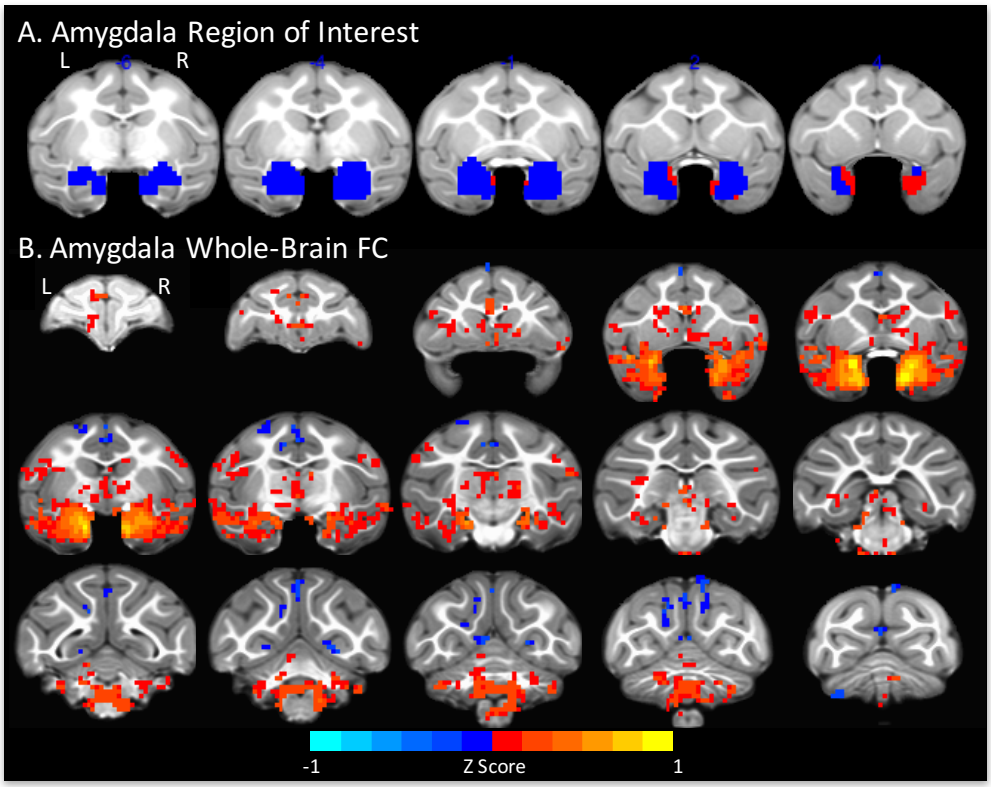


Figure 4-3

Voxelwise Analysis of Developmental Changes in Amygdala Functional Connectivity (FC) with the Rest of the Brain (3-18 mos of age). Amygdala (AMY) FC obtained from the seed-based, whole-brain analysis. Clusters with significant main effect of age, FDR corrected (q=0.05): 1- Parainsular Cortex, 2&3- Perirhinal Cortex,4- Corticomedial/Medial Amygdaloid Nucleus, 5- Anterior Amygdaloid Area, 6- Laterodorsal/Lateroventral Amygda, 7- Laterodorsal Amygdala, 8- Perirhinal Cortex, 9- TPO/STS, 10- TEa, STS VEntral Bank, 11- Teav, Tead, 12- Presubiculum/Prosubiculum, 13- Posterior Cingulate, 14- Parasubiculum, 15- Dorsal & Laterodorsal Tegmental Area, 16- Lateral Parabrachial Complex, 17- Principal Sensory, 18- Dorsoventral Tegmental Area/Dorsal Raphe-caudal nuclei, 19&20- Cerebellum. L: left; R: right.

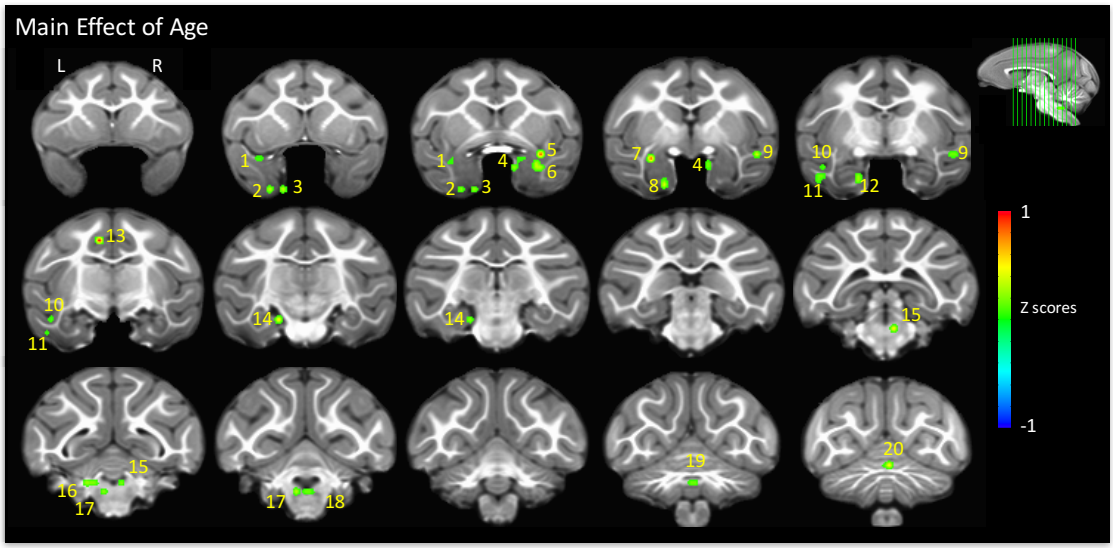


Figure 4-4

Representative Examples of Brain Clusters with Significant Developmental Changes in Functional Connectivity with Amygdala. Functional connectivity between right/left amygdala and four representative clusters showing a significant main effect of age. L: left; R: right.

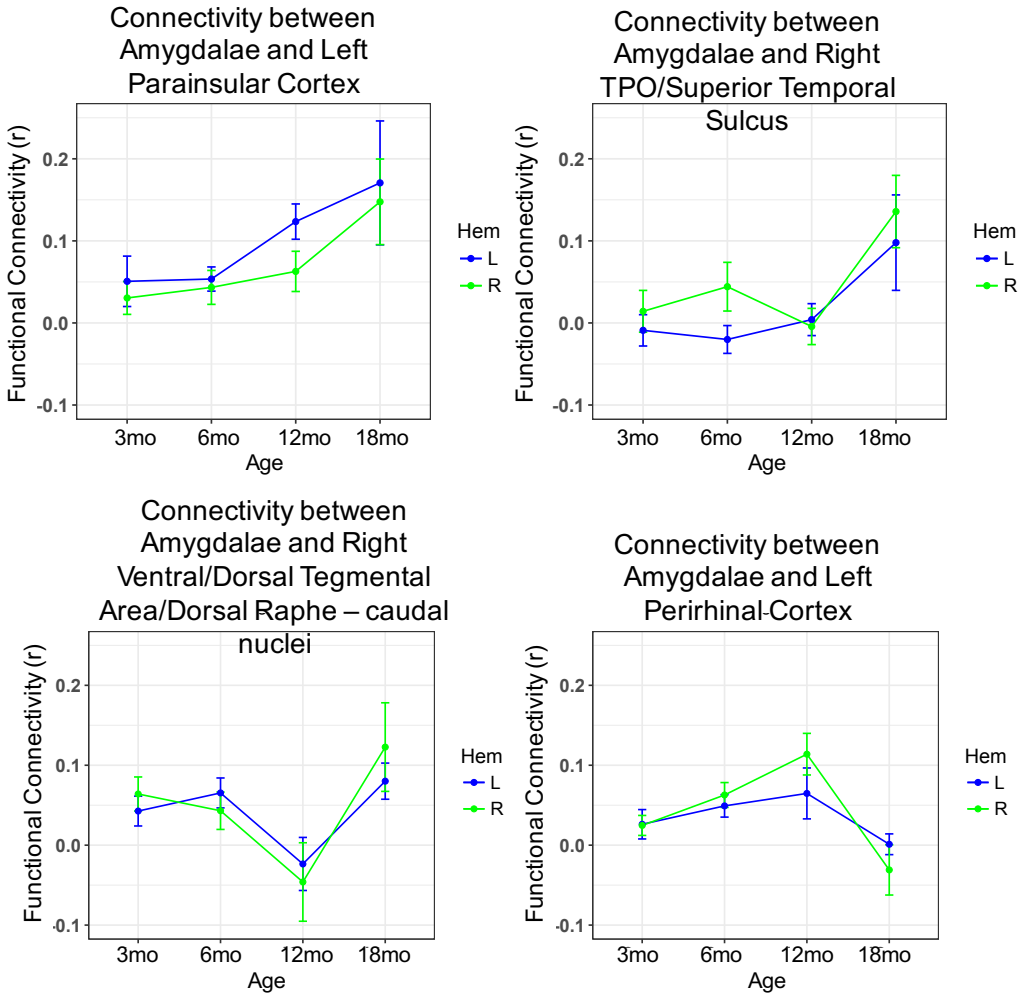
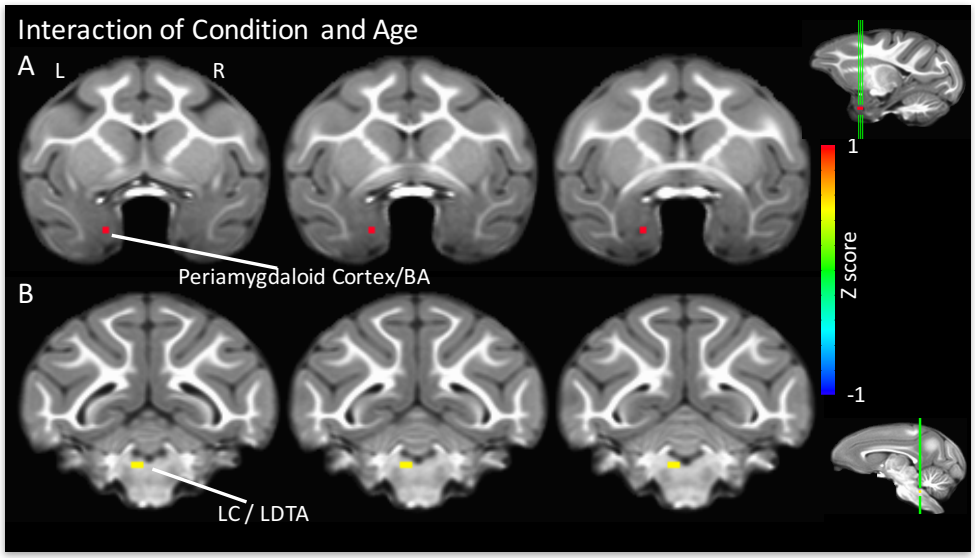


Figure 4-5

Significant Brain Clusters Showing an Interaction Effect of Group (condition) and Age on FC with Amygdala. (A) Periamygdaloid Cortex/Basal Amygdala (PAC/BA) **(B)** Locus Coeruleus (LC)/Laterodorsal Tegmental Area (LDTA). L: left; R: right. **(C)** Developmental trajectories of Amygdala FC with these 2 clusters show MALT effects emerging with age (between 12 and 18 months) so that, while controls' FC strengthens, MALT's FC with LC/LDTA weakens (gets uncoupled) or gets negatively coupled with PAC/BA (left). Interestingly, FC LC/LDTA seems higher in MALT than controls initially (at 3 months), which parallels hyperarousal/reactivity in the MALT infants, followed by gradual decoupling at later ages, which may serve an adaptive role to downregulate the arousal system.



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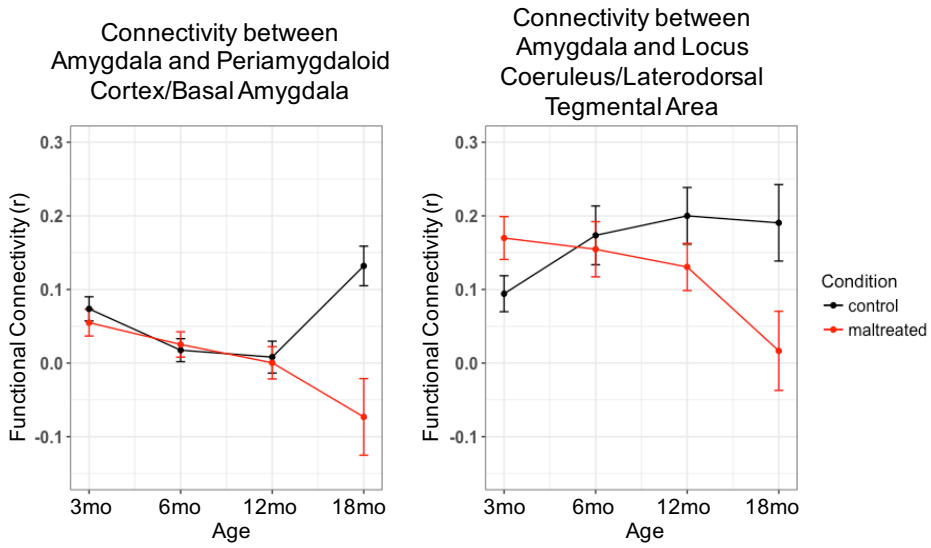


Figure 4-6

Associations between hair cortisol levels at 6 months and Amygdala functional (FC) with PAC/BA and LC/LDT at 18 months. Higher hair cortisol accumulation from birth through 6 months predicts lower FC between these clusters and the amygdala.

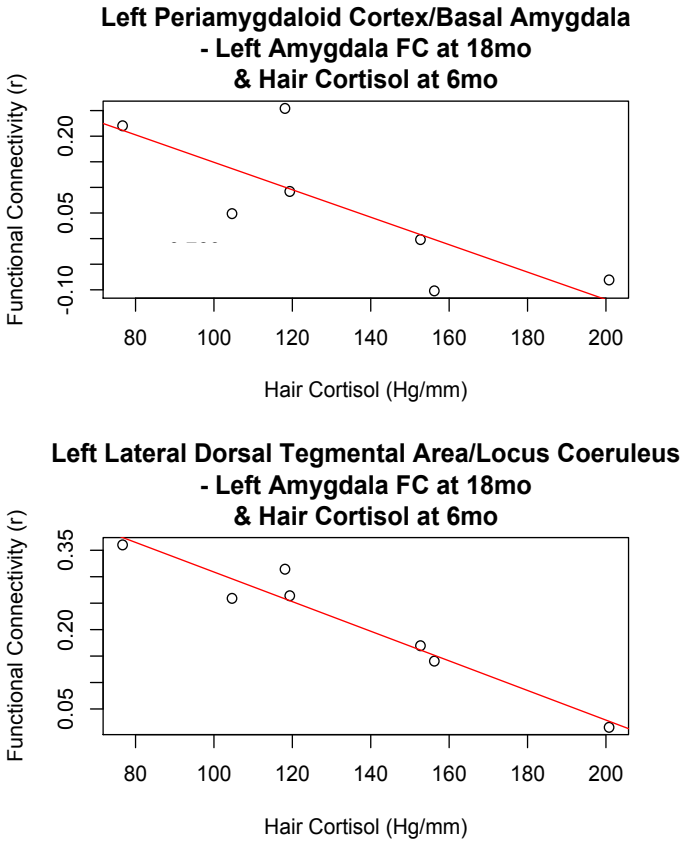
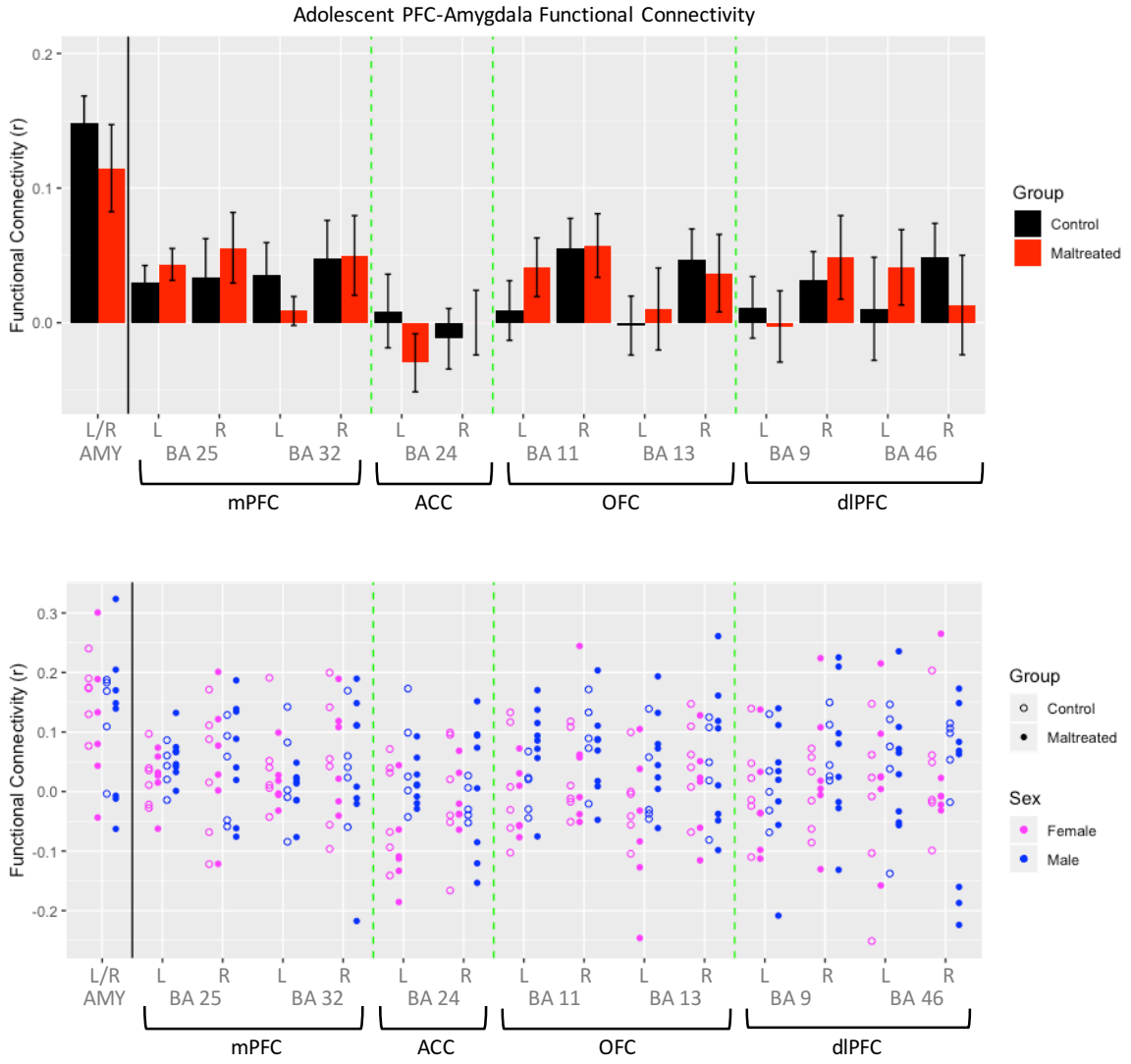


Figure 4-7

Adolescent Amygdala(AMY)-Prefrontal (PFC) Functional Connectivity (FC). AMY-AMY and AMY FC with PFC subregions are plotted by **(Top)** group (MALT vs. control), as well as **(Bottom)** by group by sex. ACC: anterior cingulate cortex; BA: Brodmann area; dlPFC: dorsolateral PFC; mPFC: medial PFC; OFC: orbitofrontal cortex. L: left; R: right. Plots represent mean±SEM **(Top)**, or subjects as dot plots **(Bottom)**.



Chapter 5 Discussion, conclusions, and future directions

5.1 Summary of Results

5.1.1 Effects of early maternal care on adolescent attention bias to threat in nonhuman primates

The goal of chapter 2 was to examine (a) the long-term impact of infant maltreatment on attentional biases toward or away from threat during adolescence; and (b) potential interactions of postnatal adverse care (maltreatment) with other early risk factors (prenatal stress/cortisol exposure and infant's emotional and stress reactivity) that may increase vulnerability to long-term alterations in threat responses during adolescence, and explain individual variability in the outcomes. Numerous studies have reported altered threat responses in children with a history of maltreatment (Pine*, et al.*, 2005; Pollak*, et al.*, 1997; Pollak*, et al.*, 2001; Pollak and Sinha, 2002; Pollak and Tolley-Schell, 2003; Shackman*, et al.*, 2007), as well as improved memory of angry facial expressions in visual tasks (Rieder, 1989), and increased amygdala activity to threat cues (McCrory*, et al.*, 2011; McCrory*, et al.*, 2013), known to mediate rapid attention to threat (Phelps and LeDoux, 2005). However, inconsistent findings have been reported in dot-probe studies of maltreated children, including attentional bias away from threat (Berto*, et al.*, 2017; Kelly*, et al.*, 2015; Pine*, et al.*, 2005), or towards threat during adolescence (Gibb*, et al.*, 2009). Differential attention bias has not been previously studied in maltreated monkeys, particularly during adolescence, which can provide a critical cross-species comparison with findings in human populations of children and adolescents with early adverse experiences (Berto*, et al.*, 2017; Gibb*, et al.*, 2009; Kelly*, et al.*, 2015; Pine*, et al.*, 2005; Pollak*, et al.*, 1997; Pollak*, et al.*, 2001; Pollak and Sinha, 2002; Pollak and Tolley-Schell, 2003; Shackman*, et al.*, 2007). Although this animal model does not span all adverse experiences that human children experience (for instance, sexual abuse), one of its critical strengths lies in its ability to quantify maltreatment during a known postnatal period, providing frequency, duration, and severity of the adverse experience (e.g. abuse

and rejection rates) and the concurrent levels of stress it elicits (e.g. cortisol accumulation in hair during the postnatal ELS exposure), difficult to be accurately determined in studies with children with early adverse caregiving experiences.

We hypothesized that postnatal exposure to adverse caregiving would alter the development of emotional regulation, increasing attention bias towards social threat in adolescence; and that this would be further worsened by prenatal stress/cortisol exposure and infant reactive temperament. To test these hypotheses we examined differences in RT in the dot probe task in adolescent macaques with and without infant maltreatment, presenting threatening and neutral images (social vs. nonsocial). Next, we examined whether RT in the dot probe task was further predicted by prenatal cortisol exposure, and infant emotional reactivity during infancy. We also examined potential interactions of postnatal adverse experience with other early risk factors, particularly measures of emotional and stress reactivity during infancy and prenatal/postnatal cortisol exposure. RT to a cue following the presentation of two social or nonsocial images of different emotional valence (threatening vs. neutral) was measured. Findings indicated group differences in RT during the social, but not the non-social, threat vs. neutral images presentation, with maltreated animals responding slower than controls, both during congruent and incongruent trials. This suggests potential interference of the social threat image in cognitive processing and attentional control. In the nonsocial trials, control, but not MALT, animals' RT became faster over testing days. Higher emotional reactivity (increased rates of distress vocalizations –screams-) during infancy, predicted faster RTs, whereas prenatal cortisol exposure (measured as hair cortisol at birth), was associated with slower RTs. These findings suggest a complex regulation by postnatal experiences, temperament and prenatal biological factors on emotional attention control during adolescence. Our findings suggest altered attentional processing

of threat in maltreated animals, evident in delayed RT in the dot-probe task, which is further modified by emotional reactivity during infancy and prenatal cortisol/stress exposure. We proposed that there may be two subgroups of maltreated animals, one that was more emotionally reactive during infancy, outwardly expressing distress, and another that internalized and developed attention bias away from threat during adolescence. Future work is necessary to determine the relationships between these attentional biases and underlying neurobiological functional mechanisms related to emotional regulation and fear learning circuitry, which may be altered during development.

5.1.2 Maternal Care Controls the Development of Fear Learning in Adolescent Nonhuman Primates

The goal of chapter 3 was to test the hypothesis that ELS results in long-term impacts on state anxiety, fear and safety learning, as well as in fear extinction during adolescence using a translational AX+/BX- fear-potentiated startle paradigm developed for macaques. Consistent with abundant human evidence linking ELS with poor emotional/stress regulation, as seen in anxiety and mood disorders (Cicchetti and Toth, 2005; Douglas*, et al.*, 2010), our group has reported that infant maltreatment also leads to increased emotional reactivity, hyperactivity of stress neuroendocrine systems and impulsive aggression in juvenile (prepubertal) macaques (Howell*, et al.*, 2013; Howell*, et al.*, 2014; Howell BR, 2012; McCormack*, et al.*, 2006; McCormack*, et al.*, 2009; Sanchez, 2006; Sanchez*, et al.*, 2010); and increased attention to threat during adolescence (Morin*, et al.*, 2019). It is unclear whether this is due to enhanced fear learning/conditioning or impaired ability to modulate fear responses by safety cues. And, it would be important to understand the potential long-term, pervasive, impact in fear responses during adolescence in this translational NHP model. The fear-potentiated

acoustic startle paradigm has been used to examine how fear learning processes are affected by not only stress/trauma experience, psychopathology, but also by neurodevelopmental and neuroendocrine changes (Jovanovic*, et al.*, 2013). Using these paradigms, enhanced acoustic startle and inability to modulate the fear response in the presence of a safety cue, have been proposed to be translational biomarkers for anxiety/fear-related disorders, including PTSD, in humans and animal models (Christianson*, et al.*, 2012; Grillon and Baas, 2003; Jovanovic*, et al.*, 2012; Jovanovic*, et al.*, 2014; Norrholm*, et al.*, 2011). While childhood maltreatment has been associated with increased startle reactivity in adulthood (e.g. (Jovanovic*, et al.*, 2009)), there is disagreement among the few studies on the effects of ELS/trauma on startle during adolescence.

We hypothesized that maltreated animals will have higher baseline and fear-conditioned startle, take longer to learn to discriminate fear/safety cues, show impaired attenuation of startle by safety signals, and show impaired extinction –effects that will be more robust in maltreated females than males. Using this rhesus model of MALT, we assessed the long-term effects of ELS on state anxiety and fear (and safety) learning in adolescent macaques (4.5-5.5 yrs, using a translational Pavlovian fear-conditioning paradigm (AX+/BX-)) that uses fear-potentiated startle amplitudes as the peripheral measure. The AX+/BX- paradigm measures baseline startle as an indicator of anxiety, fear-potentiated startle, discrimination of fear/safety conditioned cues, attenuation of startle with safety signals, and extinction. Utilizing macaques allows for more experimental control within this paradigm, including testing over an extended timeline so that we can examine the trajectory of learning during discrimination training and extinction of learned associations with fear, over many months of testing, which would be challenging to do in humans. The main findings from these behavioral tests were that

MALT animals showed higher baseline startle compared to controls (a measure of state anxiety (Brown*, et al.*, 1951)), prior to fear conditioning and fear/safety discrimination training. During fear (AX+) versus safety (BX-) discrimination training, higher fear-potentiated startle to the fear than to the safety cue emerged between early and late acquisition phases, suggesting discrimination learning in both groups of animals. Although no differences in fear learning or expression (fear-potentiated startle) or extinction were detected in MALT animals, the ability to use safety signals to modulate fear-potentiated startle responses seem to be affected in the transfer (AB) test, where MALT animals showed generalized blunted responses to both CS (AX) and the AB cue in comparison control animals. This finding, together with the blunted FPS to both the AX+ and BX- cues in MALT animals during day 1 of discrimination training, are consistent with blunted physiological responses to threat cues during fear conditioning and impaired threat vs. safety discrimination recently reported in maltreated children (McLaughlin*, et al.*, 2016). And we agree with the authors interpretation that those findings as either related to issues of fear generalization –we suggest generalized suppressed fear responses- or deficits in associative learning (McLaughlin*, et al.*, 2016). Thus, blunted emotional and physiological reactivity to threats may be a sequelae of maltreatment, similar to reports in infants raised in impoverished socioemotional environments (Carlson and Earls, 1997). These findings suggest that adverse caregiving experiences have a long-term impact on emotional regulation of macaques during adolescence, leading to elevated state anxiety, as well as impairments in early phases of fear/safety discrimination as well as generalized blunted startle responses when both safety and fear cues are presented together. Our findings suggest MALT animals have increased state anxiety compared to control animals, and may show blunted emotional reactivity, however, MALT animals show no differences to control animals in their ability to begin to discriminate between fear and safety, and to form a new memory of safety to

the previously fearful cue, extinguishing this association. Further studies are necessary to examine the relationships between these attentional biases and underlying neurobiological functional mechanisms related to anxiety, fear learning, and emotional regulation learning circuitry, which may be altered during development.

5.1.3 Developmental Outcomes of Early Adverse Care on Amygdala Functional Connectivity in Nonhuman Primates

The goal of chapter 4 was to determine the developmental impact of infant MALT on amygdala FC longitudinally, from macaque infancy through adolescence, which may underlie the enhanced emotional and stress reactivity reported previously by our group, compared to monkeys raised by competent, nurturing mothers. The study utilized a well-established rhesus monkey model of MALT by the mother leading to infant distress, and a crossfostering experimental design with random assignment of infants to either Control or MALT caregiving group at birth. In this model, the highest rates of abuse and rejection take place during the first three months of life, an important period of rapid and drastic cortico-limbic maturation. Resting state fMRI scans were collected longitudinally on these animals during infancy (at 3 and 6 months of age) and again during the juvenile, prepubertal, period (at 12, and 18 months) in order to examine the impact of MALT on the development of PFC-Amygdala circuits as well as a whole brain, voxel-wise analysis, of Amygdala FC.

Findings from chapter 4 demonstrate that infant MALT altered the development of AMY circuits, resulting in weakened AMY FC with specific PFC subregions (dorsal anterior cingulate cortex –ACC- and subgenual cingulate cortex –BA 24 and BA 25, respectively, as well as orbitofrontal cortex) and brainstem arousal centers, and that these effects were predicted by exposure to elevated cortisol levels during infancy (from birth through 6 months). However, these AMY FC alterations seem to normalize by adolescence, despite

MALT adolescent macaques showing elevated state anxiety, problems with safety modulation of fear responses and enhanced attention to threat. Therefore, we still need to address the question of what neural circuits or processes underlie impaired emotional regulation in MALT animals during adolescence. It is possible that the derailed developmental trajectories in AMY circuits have a downstream effect on other brain networks, and we plan to explore this question next. Future work is needed to determine the underlying mechanisms responsible for alterations in functional connectivity in MALT individuals developmentally, and to elucidate what behavioral manifestations they have longitudinally, as well as in adolescence and adulthood, especially using fear learning paradigms and stress response assays like the ones we have used in this NHP model, which will determine to what extent these functional differences lead to changes in arousal and fear/stress regulation.

5.2 Integration of Findings

Together these findings point towards long-term effects of early life experiences on emotional regulation into adolescence, despite what seems like a normalization of PFC-AMY FC by adolescence. It is tempting to focus the spotlight on alterations to the top-down PFC-AMY circuit, which have been previously reported among trauma-exposed individuals. However, perhaps the long-term alterations in emotional regulation are explained by further downstream circuits from this network. Previous studies that have examined how amygdala disconnection in adult macaques (using DREADD inactivation approaches) impact brain network connectivity have reported that this inactivation is, indeed, associated with large-scale brain network connectivity changes (Grayson*, et al.*, 2016). Perhaps, although early specific alterations in PFC-AMY functional connectivity show recovery through neurodevelopmental changes that occur from the juvenile period throughout adolescence, the weakened AMY functional connectivity throughout infancy

and the juvenile period could have functioned in a similar fashion as the Grayson et al (2016) Amygdala disconnection studies, causing global brain network connectivity changes in the adolescents. Thus, effects of ELS may be present in other regions, reflected in MALT adolescent macaques showing elevated state anxiety, problems with safety modulation of fear responses and enhanced attention to threat. Further studies are necessary (and will be performed) to elucidate developmental FC alterations outside of AMY, which may be perpetuating such behavioral manifestations.

5.3 Conclusions and Future Directions

Overall, these studies provide evidence that (1) early life experience alters attentional processing of threat, which is further modified by emotional reactivity during infancy and prenatal cortisol/stress exposure (2) MALT animals have increased state anxiety compared to control animals, and show blunted emotional responses in situations with ambiguous threat/safety signals (3) infant MALT alters the development of AMY circuits, resulting in weakened AMY FC with PFC and brainstem arousal centers, in part predicted by exposure to elevated cortisol levels during infancy (4) AMY FC alterations normalize by adolescence.

Inconsistencies between these studies and those done in children that have experienced maltreatment may reflect that a spectrum of effects are seen in complex trauma, and that behavioral outcomes, such as increased vigilance and hyperarousal, may not be consistent, as seen in chapter 2. This is despite the adaptations made in our approaches to perform experiments as close as possible as in humans and examine behaviors with translational validity (e.g.attention bias to threat, fear learning, etc). However, species differences may also lie in the type of maltreatment and traumas that human children

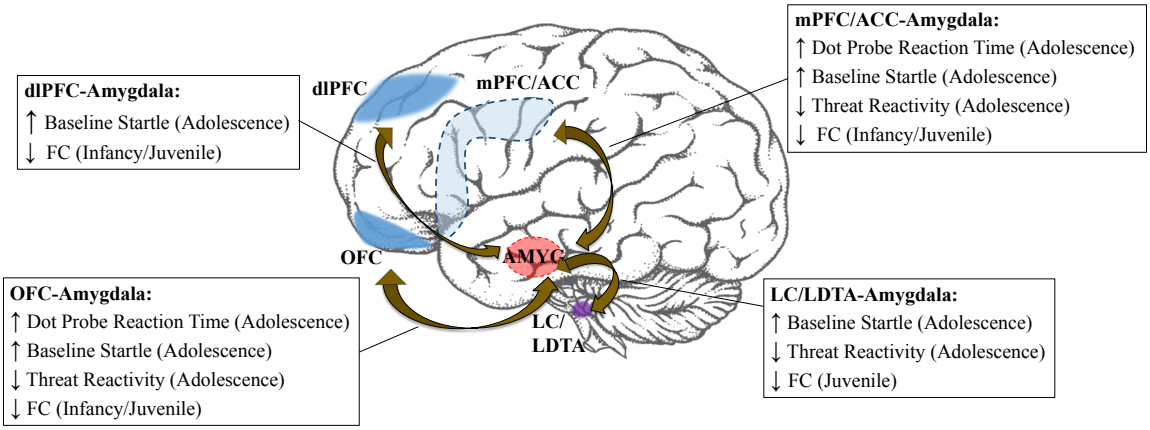
experience in contrast to macaques (e.g. sexual abuse, for which there is no NHP model available). Therefore, although these studies may present alternative findings from those seen in human studies, this may be due to the variation in outcomes that we see as a result of different forms and duration of trauma.

Future studies are necessary to explore additional questions that these studies have brought light to. Although there were no group differences between adolescent MALT and control animals' PFC-AMY circuits, MALT animals showed increased state anxiety – are there other relevant circuits besides those examined here that may show long term alterations from ELS, explain/suggesting a potential mechanism for the maintenance of anxiety in MALT animals? Given adolescents are expected to show fragile extinction recall, will this be exacerbated in MALT animals, leading to spontaneous recovery of fear weeks/months after the completion of startle testing? Will the neutralization of the effects of ELS in these circuits persist into adulthood? Would a 'second hit' of a chronic stressor in adulthood further alter the developmental trajectory of these circuits? How are the effects of common clusters of co-occurring ELS experiences reflected in psychopathology and neurodevelopmental alterations?

Further research examining these questions would help determine the developmental neurobiological mechanisms by functional alterations lead to changes in emotional regulation.

Figure 5-1

Effects of Early Life Maltreatment Summary. Provided are the main findings from Chapter 2-4, with behavioral outcomes described under relevant pathways.



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