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Neonatal Amygdala Lesions in Rhesus Monkeys Living in a Semi-Naturalistic Environment: Effects on Emotional Behavior and Neuroendocrine Stress Response.

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Abstract

Neonatal Amygdala Lesions in Rhesus Monkeys Living in a Semi-Naturalistic Environment: Effects on Emotional Behavior and Neuroendocrine Stress Response.

By Jessica Raper Lawrence

The amygdala plays a critical role in the evaluation of salient and threatening cues from the environment and in the modulation of behavioral, autonomic, and neuroendocrine responses to threats. Much less know of is its potential role on the regulation of the hypothalamic-pituitary-adrenal (HPA) axis basal tone and on the development of emotional and neuroendocrine processes through the lifespan. The current study examined the effects of neonatal amygdala lesions in rhesus monkeys on basal and stress reactive HPA function, as well as emotional reactivity from birth to preadolescence. Neonatal amygdalectomy spared the ability to express emotional behaviors, but altered the modulation of those behaviors based on the contextual information provided by the salience of the threat. Interestingly, the sex of the animal modulated the behavioral effects of neonatal amygdala lesions, leading to different patterns of emotional behaviors depending on the sex and lesion status. Neonatal amygdalectomized animals also had elevated cortisol at 5 and 12 months of age, exaggerated cortisol response to a stressor, and increased corticotropin releasing factor (CRF) in cerebrospinal fluid as compared to controls. Pharmacological challenges used to investigate the neural mechanisms of this exaggerated glucocorticoid secretion revealed blunted response to a corticotropin releasing hormone challenge, suggesting a down-regulation of CRF receptors in the anterior pituitary. Thus, the amygdala plays a critical role in the development of both basal and stress-related HPA axis functions and in the expression of sexually dimorphic behaviors, providing valuable insights into the neural mechanism underlying the symptomatology of many developmental neuropsychiatric disorders.

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General Introduction

Primates live in large complex social groups, characterized by strong hierarchical relationships among group members. Survival within these complex groups requires cohesion and stability within the social group that are maintained by appropriate communication of dynamic patterns of social interactions (Altmann, 1962). Thus, the perception and recognition of social cues from other individuals in the group and the selection of appropriate behavioral responses are critical abilities that facilitate adaptation to an ever-changing social environment. In addition, activation of neuroendocrine responses allows rapid respond to dangers by increasing metabolic states or to maintain homeostatic states during peaceful interactions. Mounting evidence has provided support to the view that these abilities require a set of neural structures that includes the amygdala and its interactions with the hypothalamic-pituitary-adrenal (HPA) axis.

Structure and Connectivity of the Amygdala

The amygdala plays a critical role in evaluating the occurrence of potential dangers and in regulating the behavioral, neuroendocrine, and autonomic responses to threats and social stimuli. The primate amygdala is an almond-shaped structure located in the anterior portion of the medial temporal lobe, consisting of 13 interconnected nuclei that receive projections from overlying temporal lobe cortices (Amaral, 1992; Freese & Amaral, 2009). The intrinsic flow of information through the amygdala is primarily unidirectional, following a lateral-to-medial direction such that the major intra-amygdaloid connections arise in the lateral and basal nuclei and terminate in the nuclei located more medially. The lateral nucleus sends projections to almost all other nuclei of the amygdala, with strong connections to the basal, accessory basal,

paralaminar, anterior cortical, medial and periamygdaloid cortex (PAC) nuclei, and lighter connections to the anterior amygdala area, nucleus of the lateral olfactory tract (NLOT), and central nuclei. The lateral nucleus receives very few inputs from other amygdala nuclei, but instead receives information from all sensory systems from both visual (facial features and expression, gaze direction, body posture and movements) and auditory information (specific vocalizations and intonations) originating in temporal cortical areas, such as area TE, TA, and TG (Amaral, et al., 1992; McDonald, 1998). In turn the amygdala sends feedback projections to the sensory cortical areas (Iwai, & Yukie, 1987; Amaral, et al., 1992; McDonald, 1998; Pitkänen, 2000). The basal nucleus provides sensory specific cortical inputs to the central nucleus (CeA), another output of the amygdala, which relays information to the brainstem and hypothalamus influencing the autonomic and endocrine manifestations of emotions.

The amygdala can modulate motor behaviors via the projections from the basal and basal accessory nuclei to the ventral striatum, subcortical elements of the motor system. Specifically, the amygdala modulates facial and vocal expressions, body postures, and movements important for defensive behaviors, such as freezing to avoid predator detection (Iwai, & Yukie, 1987; Amaral, et al., 1992; Davis, 2000). The amygdala also has a high degree of connectivity with the hippocampal formation, implying that cross-talk between these two structures are critical to build highly integrated representations of sensory stimuli and their context (Amaral, et al., 1992; Suzuki, 1996). The hippocampal formation and amygdala send and receive extensive projections to the prefrontal and temporal cortices, such as the orbital frontal, entorhinal, and perirhinal cortices, which may be involved in the consolidation and retrieval of emotional information (Suzuki, 1996).

In addition to the critical role of the amygdala in regulating the behavioral responses

involved in emotional reactions, such as fear, it is also thought to influence endocrine responses to potential threats through both direct projections to the lateral hypothalamus and indirect projections to the paraventricular nucleus (PVN) of the hypothalamus (Price & Amaral, 1981; Amaral, et al., 1992; Freese & Amaral, 2009). The amygdala has two major extrinsic fiber pathways: the ventral amygdalofugal pathway, and the stria terminalis. The central and basal nuclei of the amygdala project to the bed nucleus of the stria terminalis, which in turn, projects to the PVN creating an indirect pathway by which the amygdala can influence the HPA axis neuroendocrine response to perceived stressors (Price & Amaral, 1981; Amaral, et al., 1992; Pitkänen, 2000; Feldman, et al., 1990, 1995; Herman, et al., 2003, 2005). Studies in adult animals have shown that electrical stimulation of the amygdala results in increased cortisol secretion (Mason, 1959; Ehle, Mason, & Pennington, 1977), whereas lesions of either all amygdala nuclei or only the CeA result in lower HPA axis response to a stressor in both rodents (Knigge, 1961; Knigge & Hays, 1963; Beaulieu, DiPaolo, & Barden, 1986; Feldman, et al., 1994) and primates (Machado & Bachevalier, 2008; Kalin, et al, 2004). Therefore, the amygdala is thought to play an excitatory role on the HPA axis stress response through either direct or indirect connections to the hypothalamus.

The connectivity of the amygdala provides countless number of routes that can modulate information and influence behavior, suggesting that the amygdala is responsible for adaptive social behavior and emotional expression. This idea has been strengthened by neuropsychological studies examining the functions of the amygdala.

Amygdala and Emotional Behavior

In 1939, Klüver and Bucy reported that aspiration removal of the medial temporal lobe,

which included the amygdala and surrounding cortices, resulted in profound alteration of emotional and behavioral responses in adult monkeys. The behavioral changes included in the Klüver Bucy Syndrome were hyperactivity, fearless or unusually tame nature, hypersexuality, "psychic blindness", and excessive examination of objects. Especially striking was the loss of fear as Klüver and Bucy had intentionally selected "wild" and aggressive monkeys for the study.

Klüver and Bucy's observations, although detailed, were only qualitative and anecdotal in nature, lacking direct quantitative measures of behavior. Later researchers employed more systematic observations to examine the effect of temporal lobectomy. Weiskrantz (1956) compared the effects of lesions of the amygdala, inferior temporal convexity, and sham operation in rhesus monkeys. The behavioral effects were apparent immediately following surgery. Amygdalectomized animals allowed researchers to pet and handle them without visible excitement or aggressivity, whereas sham animals and those with inferior temporal convexity continued to display fear and hostility toward humans. Further evidence of the amygdala's involvement in fear-related behavior comes from a split brain study by Downer (1961). Following a midsagittal transection of the optic chiasm and cerebal commissures, rhesus monkeys were trained to discriminate between edible and non-edible objects through visual cues alone to ensure that their visual perceptual ability was intact. Once the visual discrimination was learned, animals underwent unilateral selective aspiration lesions of the amygdala in the right hemisphere. After recovery from surgery, the contralateral eye (left) to the amygdalectomy was sutured to deprive the intact amygdala of visual input. Animal, which were "wild" and aggressive, became "placid and "peaceful" following the suturing of the eye. In contrast, when the ipsilateral eye (right) to the amygdalectomy was sutured and visual information was reaching the intact amygdala, the animal's behavior abruptly returned to the pre-operative level of

aggression to visual threats.

These earlier studies demonstrate that animals, which were previously "wild" and aggressive toward experimenters prior to surgery, became "tame and "peaceful", and did not express fear or hostility toward human handling following amygdalectomy. These results should be viewed with caution because aspiration and radiofrequency lesions not only damage the cell bodies within the amygdala, but also cause additional damage to surrounding cortices and connection fibers from adjacent areas that pass through or nearby the amygdala (Bachevalier, 2000; Amaral, et al., 2003; Bachevalier, & Meunier, 2005). Therefore, it is difficult to ascertain if the behavioral changes are due to damage to the amygdala, the adjacent cortices, or both. Recent technological advancement has made it possible to overcome these problems and to perform selective lesions that only destroy neural cells and spare fibers of passage. Lesions employing MRI-guided stereotaxic techniques to administer neurotoxins (e.g. ibotenic acid, a glutamatergic agonists that opens calcium channels, causing the cell body to become hyperactivated and fire to death) can selectively damage the amygdala.

Meunier and colleagues (1999) used naturally threatening stimuli (e.g. unfamiliar human or conspecific) to compare the effects of aspiration and neurotoxic lesions of the amygdala. Although both lesion types produced similar effects of hypoaggression and reduced fear, the emotional changes of enhanced submission and decreased aggression were more subtle after neurotoxic lesions than after aspiration lesions. These data suggest that some of the affective changes seen after aspiration lesions of the amygdala are likely due to unintentional damage to surrounding cortices. In fact, a study directly investigating the effects of entorhinal and perirhinal damage on emotional reactivity confirms this idea. Rhinal cortical damage produced heightened defensiveness and attenuated submission and approach responses, which are the

opposite of some of the most distinctive symptoms following amygdala damage. Therefore, it is possible that the rhinal cortex and amygdala have distinct, interactive functions in normal behavioral adaptation to affective stimuli (Meunier & Bachevalier, 2002; Meunier, Cirilli, & Bachevalier, 2006).

In addition, early aspiration studies relied on anecdotal observations of subjects' reactivity toward the experimenter as a threatening social stimulus; however, later studies have systematically examined emotional reactivity using the "intruder" paradigm. The "intruder" paradigm investigates the animal's response to being confronted with a social threat of either an unfamiliar conspecific or novel human. Neurotoxic lesions of the amygdala in adult monkeys result in less fearful and less aggressive behaviors toward a taxidermic or live conspecific as compared to controls (Meunier, et al, 1999; Kalin, et al., 2001), and increased willingness to approach an unfamiliar human (Meunier, et al., 1999; Kalin, Shelton, & Davidson, 2004; Mason, et al., 2006; Machado & Bachevalier, 2008). These results from neurotoxic lesions support the key role played by the amygdala in the regulation and expression of fear response and emotional reactivity.

HPA axis Regulation

The role of the amygdala in emotion regulation is also tightly associated with the HPA axis. The HPA axis plays an essential role in an organism's ability to adapt to a homeostatic challenge. Activation of the HPA axis results in the release of glucocorticoids that act at multiple levels to redirect bodily energy resources. Glucocorticoids restore homeostasis by replenishing lost energy through increasing glucose production in the liver, controlling inflammation through inhibiting T-cell proliferation, and reducing energy expense through

suppression of other hormonal systems such as the hypothalamic-pituitary-gonadal axis (de Kloet, et al., 1998; Sapolsky, et al., 2000; Herman, et al., 2003). These homeostatic restorative properties of glucocorticoids can also have deleterious effects if allowed to go unchecked, thus negative feedback control is also essential to the health of the organism. Therefore, the HPA response is generally characterized by an "activation" signal from ACTH release followed by a "stop" signal generated by glucocorticoids as well as neuronal negative feedback (Herman, et al., 2003). Specifically, the HPA response begins with the release of corticotrophin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus into the hypophyseal portal veins and travels to the anterior pituitary, which then stimulates the release of ACTH into the systemic circulation. ACTH binds to the adrenal cortex, which in turn stimulates the synthesis and release of glucocorticoids. The circulating glucocorticoids then complete a negative feedback loop acting back on the hypothalamus to shut off the HPA axis activity.

The HPA axis plays two equally important roles. First, under relatively unstressed conditions, the HPA axis exhibits a circadian rhythm, with a peak at the time of waking, followed by a decline across the day, and finally a trough overnight during rest (Weitzman, et al., 1971; Keller-Wood & Dallman, 1984). This rhythmicity of the HPA axis is under "proactive" negative feedback control of glucocorticoids to maintain basal activity and set the sensitivity and threshold of the organism's stress response. This basal secretory rhythm is essential for the body's homeostasis and normal functioning of other systems in the organism, such as metabolism, growth, immune function, the sleep/wake cycle, and efficient performance of learning and memory tasks (Munck, Guyre, & Holbrook, 1984; de Kloet, et al., 1998; Diamond et al., 1992). The second role of the HPA axis is to promote a reactive response to actual or perceived challenges (threats) to the organism by initiating an increase in ACTH. It is this

reactive stress response that is important for the activation of the "fight or flight" response. This stress response is controlled by "reactive" glucocorticoid negative feedback to prevent the system from overshooting (Sapolsky, et al., 2000) and gives the organism the ability to cope with, adapt to, and recover from stress. Thus, the HPA axis serves two essential roles in the overall function and survival of an organism.

Amygdala and HPA axis interactions

The dramatic effects of amygdalectomy on emotional behavior led researchers to question its effects on HPA axis reactivity. Electrical stimulation of the amygdala in conscious adult monkeys produced a significant increase in cortisol compared to no stimulation or stimulation of a control area, i.e. the putamen (Mason, 1959; Ehle, Mason, & Pennington, 1977). This significant increase in cortisol was even seen with unilateral amygdala stimulation (Mason, 1959). Although this rise in cortisol in response to electrical stimulation could have been due to the animal's behavioral change, this factor was eliminated by examining the effects of electrical amygdala stimulation in unconscious (anaesthetized) monkeys (Frankel, Jenkins, & Wright, 1978). Selective stimulation of either the lateral or basal nuclei of the amygdala was sufficient to create an increase in cortisol in anaesthetized animals. Thus, the amygdala can influence the HPA axis response without directly affecting behavior. Overall, these studies suggest that the activation of the amygdala has an excitatory effect on the HPA axis.

The strong evidence involving the amygdala in emotional behavior led to the design of new studies mostly focusing on its role in the reactive stress response (for review Herman, et al., 2003, 2005) without consideration of its potential contributions on basal cortisol secretion. Consequently, only a few studies have examined the influence of the amygdala on basal HPA

axis functions. In one study, the authors assessed whether the amygdala is necessary to maintain basal tone of the HPA axis (Allen & Allen, 1975). Adrenalectomy (removal of the adrenal glands) eliminates the production of glucocorticoids, and, in turn reduces negative feedback on the HPA axis, resulting in hypersecretion of ACTH. However, adrenalectomy combined with bilateral radiofrequency amygdala lesions yielded significantly lower ACTH release as compared to that of intact animals or those with unilateral lesions of the amygdala (Allen & Allen, 1975). These data suggest that an input from only one amygdala is sufficient to maintain hypersecretion of ACTH after adrenalectomy. Additionally, unilateral amygdala lesion coupled with severing the connection to the hypothalamus from the intact amygdala on the contralateral side also yielded a significant reduction of ACTH hypersecretion after adrenalectomy in rodents (Allen & Allen, 1975). Although other studies in rodents or primates with adult amygdala lesions have found no difference in baseline (pre-stressor) cortisol levels (Sapolsky, et al., 1991; Prewitt & Herman, 1994; Kalin, et al., 2004; Machado & Bachevalier, 2008), these studies did not thoroughly examine the potential effects of amygdalectomy at different points throughout the diurnal rhythm, leaving the role of the amygdala on basal cortisol secretory rhythm largely unknown in primates. In fact, a recent study by Regev and colleagues (2012) showed that CRH knockdown in the CeA of adult mice leads to increases in basal corticosterone secretion, specifically in the later phase of the diurnal rhythm (close to bedtime, when levels of glucocorticoids are low). Lastly, neurotoxic amygdala lesions led to lower CRF expression in the PVN of the hypothalamus under stressed and non-stressed conditions (Prewitt & Herman, 1994) and lower CRF in the median eminence (Beaulieu, et al., 1989). Taken together, these results suggest that the amygdala and its connections with the hypothalamus have an excitatory role during times of stress as well as during tonic (basal) conditions.

These results demonstrate that the amygdala and its connections to the hypothalamus directly influence the HPA axis, such that excitation of the amygdala results in increase glucocorticoid secretions and influences the endocrine response to stress. Additionally, lesion studies of adult rodents and monkeys demonstrate that the amygdala regulates emotional reactivity as well as the neuroendocrine response to stressors and basal HPA function.

The studies outlined above relate to the role of the amygdala in adult animals that have already acquired the social skills necessary for appropriate responses essential to survival and in which both central structures, the amygdala and hypothalamus, are fully mature at the time of the experimental manipulation (typically, stimulation or lesion). Thus, little is known about the influence of the amygdala on normal development of behavioral and neuroendocrine responses.

Behavioral Development and Amygdala Maturation

During primate development there exists critical periods when significant refinements in behavioral repertoire appear to coincide with neural development of the amygdala. The amygdala exhibits a developmental increase in volume from birth to 2 years of age in rhesus monkeys. A longitudinal developmental MRI study in rhesus monkeys demonstrates that between 1 week to 2 years of age the volume of the amygdala increases 86.49% in males and 72.94% in females (Payne, et al., 2010). Histological examination reveals that the postnatal development of the amygdala occurs in two stages (Chareyron, et al., 2012). The first stage occurs between birth and 3 months of age when the lateral, basal, and accessory basal nuclei significantly increase in volume, whereas the second stage is characterized by a slow and continuous increase in most amygdala nuclei volume. This increase in volume is mostly due to an increase in oligodendrocyte number (Chareyron, et al., 2012). The second stage most likely

reflects the postnatal myelination of the amygdala and its connections to other neural systems that help coordinate responses to threats in the environment.

The protracted development of the amygdala described above is associated with refinement in behavior responses. For a period of about three months following birth, infant macaques appear to lack fear and defensive behaviors and do not understand the meaning of social signals (Mendelson, et al., 1982a, 1982b). Between one week and three months of age, projections from higher-order visual cortices (area TE and TEO) to the amygdala develop, giving the amygdala more accurate visual information regarding social signals (Rodman, 1994). At approximately two months of age, the stria treminalis, which connects the amygdala to the hypothalamus, basal ganglia, basal forebrain, and areas of the brainstem, has developed moderate myelination, presumably increasing the effectiveness of amygdala communication to other areas (Amaral, et al., 1992; Gibson, 1991). The emergence of appropriate responses to social signals in infant macaques (Mendelson, et al., 1982a, 1982b) correlates with these refinements of cortical-amygdala projections and increased myelination of amygdala connections. These refinements occur around the time when infant primates begin to leave the security of their mother (only short distances) and enter into social interactions with their peers (Hinde, Rowell, & Spencer-Booth, 1964). Considering how vulnerable infants are when away from their mothers, it would seem important for them to also have acquired appropriate predator avoidance (freezing) and defensive behaviors by three months of age. This was in fact demonstrated by Kalin and colleagues (1991) using the human intruder paradigm. Infants did not respond to the human intruder with distinct fear and predatory avoidance until 9-12 weeks of age. Considering the correlations between neuroanatomical and behavioral development, several important questions emerge. Would early amygdala lesions lead to behavioral deficits comparable to those

seen in adult-onset lesions? Or would further development and brain plasticity reduce or even prevent the appearance of the deficit? Finally, would early-onset lesions appear unnoticeable in early life but result in a cascading effect, causing drastic behavioral changes later in life?

There is little information available to directly answer these questions. In humans, cases of early amygdala damage are rare, and often the damage extends to surrounding cortical areas and other structures (Chutorian & Antues, 1981; Tonsgard, Harwicke, & Levine, 1987; Rossitch & Oakes, 1989; Lanska & Lanska, 1993; Caparros-Lefebvre, et al., 1996; Adolphs, et al., 1998; 2001). There are also very few developmental studies in monkeys (described below). Yet information about early onset condition is vital to explain how social and emotional competencies develop from a neurobiological standpoint, and particularly the role of the amygdala in normal development.

Neonatal Lesions of the Amygdala and Emotional Behavior

In 1967, Kling and Green explored the effects of early damage to the amygdala on emotional behavior of maternally reared and maternally deprived infants. No differences in emotional behavior were found between operated animals and controls. Maternally reared amygdalectomized infants expressed species-typical responses toward human observers (i.e. fear grimace, withdrawal, threat postures, and barking) as compared to their maternally reared unoperated peers. Likewise, amygdalectomized maternally deprived infants reacted toward human observers in the same way as unoperated maternally deprived controls, exhibiting withdrawal, cowering, and rocking. Researchers only observed these subjects through the first two years of life, before the monkeys had reached puberty. Kling and Green suggested that emotional changes after neonatal amygdalectomy may require some degree of sexual maturation

in order to manifest.

A longitudinal study by Thompson and colleagues (1969) examined the effects of neonatal aspiration amygdalectomy on female rhesus monkeys. Emotional reactivity was examined at 6.2 months of age by placing subjects alone in a novel cage and presenting them pictures: a monkey exhibiting a threatening, frightened, or neutral (relaxed) expression, an infant monkey, and a control picture of an inanimate object. Amygdala-operated animals expressed less fear as compared to controls when the pictures were presented. To directly address whether emotional changes after early amygdala damage would become more apparent with further maturation, Thompson and colleagues (1977) re-examined the subjects after they had reached sexual maturity at 6 years of age. Compared to control subjects, neonatal amygdalectomized subjects exhibited increased activity when placed alone in a novel environment for 24 hours. The adult control subjects were re-tested after receiving an amygdala lesion, which allowed for comparison between the behavioral effects of neonatal and adult amygdala lesions (Thompson, et al., 1977). Adult-operated animals exhibited increased activity in the test, similar to subjects with neonatal amygdala damage. These results suggest that, despite the timing of the amygdala lesion, rhesus monkeys exhibit similar blunting of emotional reactions after both neonatal and adult lesions of the amygdala.

These results from Thompson and colleagues may seem contradictory to those of Kling and Green (1967); however, the differences in results may reflect differences in methodology of the measurement of emotional behavior. Kling and Green merely measured subject's reaction to humans during passive observations, whereas Thompson and colleagues designed a specific test to systematically examine reactivity to emotional stimuli. Thompson and colleagues (1969; 1976) found blunted fear response and increased activity as early as 6 months, and these

emotional changes were still present when subjects reached sexual maturity. Thompson also reported that neonatal and adult lesions produced similar blunting of emotional behavior in subjects (Thompson, et al., 1977). Therefore, the amygdala is important for the normal expression of emotional behavior despite the age at which the damage occurs. Although one study reported no effects of early amygdala damage, it appears clear that when affective behavior is tested more systematically, early amygdala damage produced similar emotional changes to those seen in animals with amygdala lesions in adulthood. Conclusions from these studies are limited by the aspiration technique used to create the amygdala damage, since this technique is known to cause unintentional damage to the surrounding cortical areas and fibers of passage.

Recent studies have examined the effects of neonatal neurotoxic amygdala lesions on emotional reactivity in rhesus monkeys (Prather, et al., 2001; Bliss-Moreau, et al., 2010, 2011a, 2011b). Lesions of the amygdala were done at 14 days of age, and emotional reactivity was examined in infancy (Prather, et al., 2001, Bliss-Moreau, 2010), adolescence (Bliss-Moreau, et al., 2011a), and adulthood (Bliss-Moreau, 2011b). Emotional reactivity was assessed by presenting subjects with neutral objects (i.e. luggage tag) and fearful objects (i.e. rubber snake replica). Neonatal amygdala-operated animals engaged in more object manipulation and expressed less fearful behaviors compared to sham-operated controls (Prather, et al., 2001; Bliss-Moreau, et al., 2010, 2011a, 2011b). These findings suggest that emotional changes seen after early amygdala damage are specifically due to the amygdala damage and not damage to surrounding cortical areas or fibers of passage.

A recent study examined the impact of neonatal neurotoxic lesions on emotional reactivity using the Human Intruder paradigm (Raper, et al., 2012a). Neonatal amygdala lesions did not impair or delay the emergence of defensive behaviors in early infancy, but instead altered

their modulation depending on the gaze direction of the intruder. The changes in emotional reactivity persisted into adulthood, becoming more pervasive. Animals with neonatal amygdala lesions exhibited a blunted freezing response during the Profile (No Eye Contact) condition but heightened fearful behaviors in the Alone condition. Unlike controls, neonatal amygdala lesion subjects were unable to modulate their fearful, hostile, or anxious behaviors depending on the presence and gaze direction of the intruder. These results suggest that, despite the potential for greater brain plasticity and compensatory reorganization after early brain damage, neonatal amygdala lesions result in pervasive changes in emotional reactivity. Nevertheless, although the data demonstrated that lesions of the amygdala early in life result in altered emotional reactivity, another question that needs to be answered is whether these behavioral changes were associated with concurrent changes in the HPA axis?

Neonatal Amygdala Lesions and HPA axis Regulation

Goursaud and colleagues (2006) examined the neuroendocrine response to stress in monkeys with neonatal amygdala lesions. Between 3 and 5 months of age, animals were separated from their mothers and relocated to single cages for a 48 hour period of behavioral testing. During this behavioral testing, blood samples were taken from the femoral vein, the first sample was taken 1.5 to 2.5 hours after the initial separation from the mother. The second blood sample was taken at the end of the behavioral testing day, just before an injection of dexamethasone (synthetic glucocorticoid). On day two, a third blood sample was taken to assess the effects of the dexamethasone challenge. Immediately after the third blood draw, ACTH was injected, and a final fourth blood draw was taken 30 minutes after the ACTH challenge. No differences were found between groups. That is, animals with neonatal amygdala lesions

responded to the challenges with similar levels of cortisol as compared to controls. However, these results should be viewed with caution since the first sample was collected after at least 1.5 hours of the stress of being separated from their mother and the baseline sample taken prior to the dexamethasone was after several stressful behavioral tests (e.g. Human Intruder paradigm); therefore, this study lacked a true basal blood sample to accurately compare hormonal reactivity.

A recent study examined the effects of neonatal amygdala lesions on stress reactive HPA response to the Human Intruder paradigm (Raper, et al., 2012a). Since changes in HPA secretions can be detected within 10 minutes of initial disturbance, this study controlled for changes in cortisol secretion by collecting the baseline sample within 5-7 minutes of the experimenter entering the housing room. To ensure that group differences in hormone secretion were due to the effects of the stressor and not merely the effect of handling or blood sampling technique, ACTH and cortisol levels were examined on a baseline day two days prior to the Human Intruder stressor. Neonatal amygdala lesions resulted in lower awakening basal cortisol and blunted stress response (Raper, et al., 2012a). The blunted cortisol response to a stressor was similar to that seen in adult-onset amygdala lesions (Kalin, et al., 2004; Machado & Bachevalier, 2008). However, in this study, the change in HPA axis functioning was observed in adulthood long after the amygdala damage occurred. Thus, the question of whether the amygdala influences basal HPA axis functioning during early development remains to be directly investigated.

There are several issues that remain to be further investigated to characterize the impact of selective amygdala damage early in life on emotional behavior and the HPA axis. The first is the determination of how neonatal amygdala lesions impact emotional and HPA axis functioning during early development. The second is the role of the amygdala in sexually dimorphic

emotional reactivity and HPA functioning. With the exception of Thompson and colleagues (1969, 1976, 1977), the majority of studies examining the effects of amygdala damage, either in adulthood or in infancy, have only examined males. The third is the lack of findings regarding the role of the amygdala on basal HPA functioning in primates. The goal of this project is to directly address these issues. Specifically, the effects of emotional and neuroendocrine behavior will be examined at different time points during development to determine the long-term effects of early amygdala damage on both male and female rhesus monkeys living in large complex social groups.

Specific Aims

HPA axis Basal Rhythm

Basal blood samples will be collected at 2.5, 5, and 12 months of age to investigate the effects of neonatal amygdala damage on the basal tone of the HPA axis. Evidence from adult lesion studies in rodents (Allen, & Allen, 1975; Beaulieu, et al., 1989; Regev, et al., 2012) and neonatal lesions in primates (Raper, et al., 2012a) suggests that diurnal rhythm of the HPA axis will be disrupted by amygdala damage. Therefore, early amygdala damage will result in a flattened and lower diurnal cortisol rhythm compared with normally developing controls.

Emotional Behavior

The Human Intruder paradigm was employed at 2.5-3 months, and 12 months of age to examine the effects of early amygdala damage on the development of emotional behavior. Since the expression of species typical defensive behaviors are fully developed by 3 months of age (Kalin, Shelton, & Takahashi, 1991) and changes in emotional behavior can be detected very

early in development (Raper, et al., 2012a), we predict that alterations in emotional reactivity will become increasingly apparent with age. Specifically, neonatal amygdala-operated subjects will exhibit less freezing behavior and lack modulation of emotional behavior based on the gaze direction of the intruder as compared to controls. Thus, neonatal amygdala damage will alter the ability of subjects to process potentially threatening situations resulting in emotional dysregulation.

HPA axis Reactive Stress Response

The reactive HPA axis response will be investigated with blood samples collected immediately before and after the emotional stressors, Human Intruder paradigm at 12 months of age. Normally developing animals will exhibit a significant increase in ACTH and cortisol from baseline to post-test sample. Given the amygdala's excitatory influence on the HPA axis stress response, subjects with neonatal amygdala lesions will exhibit a significantly lower cortisol response to the stressor compared to controls (Kalin, et al., 2004; Machado & Bachevalier, 2008; Raper, et al., 2012a). Thus, early amygdala damage will disrupt the HPA axis reactive stress response, causing a blunted response to threatening stimuli.

HPA axis Functioning

If the basal rhythm as well as the reactive stress response are negatively impacted by early amygdala damage, then examination of the overall functioning of the HPA axis is necessary to determine at which level of the HPA axis the disruption has occurred. Pharmacological challenges will be employed at 12 months of age to examine the overall responsiveness of the HPA axis. The CRH challenge measures the responsiveness of the

pituitary, whereas an ACTH challenge assesses the responsiveness of the adrenal cortex. Finally, a dexamethasone suppression test is used to investigate glucocorticoid negative feedback on the HPA axis. The impact of early amygdala damage on the hypothalamus will result in altered hormonal response to the CRH and dexamethasone pharmacological challenges. Specifically, neonatal amygdala-operated animals will exhibit an increased stress hormone response to the CRH challenge due to the disconnection of the excitatory influence of the amygdala on the hypothalamus. This disconnection will lead to an inability to unable to escape from dexamethasone suppression as compared to controls. Lastly, there will be no differences between groups' responses to the ACTH challenge because the communication between the pituitary and the adrenal cortex has not been altered.

These four aims have been divided into three chapters. Chapter 1 will focus on how damage to the amygdala early in life impacts the development of the HPA axis, by examining basal cortisol secretions at 2.5 and 5 months of age. Chapter 2 focuses on the impact that neonatal amygdala lesions have on emotional behavior, as examined by the Human Intruder paradigm during infancy and preadolescence. Finally, Chapter 3 will focus on how neonatal amygdala lesions have impacted the normal functioning of the HPA axis during preadolescence, specifically examining the basal, stress reactivity, as well as response to neuropeptide challenges.

Manuscript I. Neonatal amygdala lesions alter basal cortisol levels in infant rhesus monkeys.

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Abstract

The amygdala is mostly thought to exert an excitatory influence on the hypothalamic-pituitary-adrenal (HPA) axis, although its role regulating HPA basal tone is less clear, particularly during primate development. The current study examined the effects of neonatal amygdala lesions on basal HPA function and the postnatal testosterone (T) surge of rhesus monkeys reared with their mothers in large outdoor social groups. An early morning basal blood sample was collected at 2.5 months of age, whereas at 5 months samples were collected not only at sunrise, but also at mid-day and sunset to examine the diurnal rhythm of cortisol. At 2.5 months of age sham-operated males exhibited higher cortisol than females, but this sex difference was abolished by neonatal amygdalectomy, with lesioned males also showing lower basal cortisol than controls. Although neonatal amygdalectomy did not alter the postnatal T surge, there was a positive relationship between T and basal cortisol levels. At 5 months of age, neither the sex difference in cortisol, nor its correlation with T levels were apparent any longer. Instead, the diurnal cortisol rhythm of both males and females with amygdalectomy showed a blunted decline from mid-day to sunset compared to controls. These results indicate that neonatal amygdala damage alters basal HPA function in infant rhesus monkeys, affecting males only at early ages (at 2.5 months), while leaving the postnatal T surge intact, and resulting in a flattened diurnal rhythm in both genders at the later ages. Thus, the primate amygdala has a critical influence on the HPA axis in the first few months of life.

Keywords amygdala, HPA axis, testosterone, sex difference

Introduction

The amygdala is anatomically positioned to play a critical role in the evaluation of salient and threatening cues from the environment and in the modulation of behavioral, autonomic, and neuroendocrine responses to potential threats (e.g., Freese & Amaral, 2009). The amygdala influences neuroendocrine stress responses through indirect inputs to the hypothalamic paraventricular nucleus (PVN), via direct projections to the bed nucleus of the stria terminalis (Herman, et al., 2003; Freese & Amaral, 2009). Thus, in response to a perceived threat, stressor-specific pathways from the amygdala activate the PVN, yielding a cascade of events beginning with the secretion of corticotrophin releasing hormone (CRH) into the hypophyseal portal blood followed by the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into the systemic circulation. ACTH then binds to receptors in adrenocortical cells, which in turn increases the synthesis and release of glucocorticoids, such as cortisol in primates (Herman, et al., 2003). A stimulatory role of the amygdala on these neuroendocrine responses has mostly been demonstrated in adult animals. Thus, electrical stimulation of the primate amygdala increases cortisol secretion (Mason, 1959), whereas either complete bilateral lesions of the amygdala nuclei or selective lesions of the central nucleus (CeA) of the amygdala reduces glucocorticoid secretion in response to a stressor in both rodents (Herman, et al., 2003) and primates (Kalin, et al., 2004; Machado & Bachevalier, 2008). Most of these studies, however, have focused on the role of the amygdala regulating HPA axis stress reactivity (for review see Herman, et al., 2003) with little consideration of its role on basal cortisol secretion and its circadian secretory rhythm. Allen and Allen (1975) reported that the rodent amygdala was necessary to maintain ACTH secretion after adrenalectomy, suggesting a potential stimulatory role of the amygdala for tonic (basal) control of the HPA axis in the absence of glucocorticoid

regulation. However, a more recent study showed that CRH knockdown in the CeA of adult mice actually increases basal corticosterone (Regev, et al., 2012). In primates, no effects on baseline (pre-stressor) cortisol levels have been reported after adult amygdala lesions in monkeys (Sapolsky, et al., 1991; Kalin, et al., 2004; Machado & Bachevalier, 2008). Even less studied is the role of the amygdala in the regulation of basal HPA axis function during prepubertal development.

Human studies suggest that the basal HPA axis secretory rhythm emerges between 8–12 weeks of age (see review Tarullo & Gunnar, 2006). The few studies that have examined the ontogeny of basal HPA function in monkeys indicated either stable or slight decreases in basal cortisol secretion between 2 and 24 weeks of age (Champoux, et al., 1989; Higley, et al., 1992; Clarke, 1993) with an adult-like diurnal pattern of cortisol secretion already present by one year of age (Sanchez, et al., 2005; Barrett, et al, 2009). Thus, the available developmental data point to a progressive maturation of HPA axis function throughout infancy and raise the question of whether the amygdala plays a critical role in this maturation. Few studies have examined the effects of amygdala lesions on HPA axis function in developing rhesus monkeys, either during the juvenile period (Norman & Spies, 1981) or in infancy (Goursaud, et al., 2006; Raper, et al., 2012 submitted). Two have reported no effects on basal activity (Norman & Spies, 1981; Goursaud, et al., 2006). However, these negative results may have resulted either from studying the juvenile rather than the infant developmental period (Norman & Spies, 1981) or from the lack of true baseline samples in the experimental design (Goursaud, et al., 2006). In the most recent study (Raper, et al., 2012 submitted), lower basal cortisol was found in adult animals with neonatal amygdala lesions, although cortisol was not measured in infancy. Thus, the amygdala's influence on basal HPA axis functioning during early primate development remains to be

directly investigated.

The current study had two main aims: 1) to examine the effects of neonatal neurotoxic amygdala lesions on basal HPA function of rhesus monkeys during infancy, and 2) to determine whether these effects are sexually dimorphic. In adulthood, gonadal hormones modulate the HPA axis activity. Thus, estrogens in females have mostly a stimulatory effect on the basal HPA axis (Burgess & Handa, 1992; Stavisky, et al., 2003), whereas testosterone appears to inhibit corticosteroid secretion, at least in rodents (Seale, et al., 2004). Although the relationship between gonadal hormones and the HPA axis in adults is complex and not clearly understood, a few studies have also reported sex differences in basal cortisol levels during childhood, prior to the pubertal increases in gonadal hormones. Thus, boys have higher basal cortisol levels than girls in some studies (Davis & Emory, 1995; Elmlinger, et al., 2002; Ouellet-Morin, et al., 2010); but opposite findings have also been reported (Essex, et al., 2002; Koupil, et al., 2005; Sondeijker, et al., 2007). The differing results may reflect differences in the age at which boys and girls were sampled. Importantly, Davis and Emory (1995) reported higher cortisol levels in boys than girls during a developmental phase when the hypothalamic-pituitary-gonadal (HPG) axis is temporarily activated in boys, resulting in a transient postnatal testosterone (T) surge (Forest, 1979). A similar HPG activation occurs neonatally in male rhesus monkeys, with elevated T levels from birth through 4 months of age followed by HPG inactivation until puberty (Robinson & Bridson, 1978; Mann, et al., 1989). Furthermore, amygdala androgen receptors (AR; Choate, et al., 1998) are present in higher concentrations in males than females (Pomerantz & Sholl, 1987), suggesting that the amygdala may be an important site for the regulation of the HPG axis and for interactions between the HPG and HPA axes. To investigate this potential regulatory role of the amygdala on HPA and HPG activity during infancy, we measured both

basal cortisol and T at 2.5 months (during the postnatal T surge) and at 5 months of age (after the surge, when T was expected to be low) in male and female infant monkeys with and without neonatal amygdala lesions.

Methods

Subjects: Twenty-eight infant rhesus monkeys (*Macaca mulatta*) were selected from middle-ranking multiparous mothers living in large social groups at the Yerkes National Primate Research Center (YNPRC) Field Station (Lawrenceville, GA), Emory University. The social groups were housed in 38 x 38 m outdoor compounds with indoor housing and capture area. Social groups consisted of 20-30 adult females with their immature offspring and two unrelated adult males. Infants were divided into two treatment groups; neonatal amygdala lesion (Neo-A; males = 9, females = 7) and sham-operated controls (Neo-C; male = 6, females = 6). Infants in group Neo-A received MRI-guided bilateral neurotoxic lesion of the amygdala at an average of 25.6 ± 0.8 days of age (range: 20 – 30 days). Infants in group Neo-C received a sham surgery (see below) at an average of 24 ± 1.6 days of age (range: 12 – 33 days). All neuroimaging and surgical procedures were performed at the YNPRC Main Station (Atlanta, GA).

To minimize the risk of maternal rejection, four days prior to surgery, mother-infant pairs received a one hour separation-trial that simulated the manipulations that the mother and infant would receive prior to the imaging and surgical procedures (see below). The separation-trial required isolation of the mother-infant pair away from their social group and separating the infant from the mother, shaving the infant's head, cleaning the area with alcohol and betadine, and placing the infant away from the mother in a temperature-controlled isolette incubator for one hour. The infant was then reunited with the mother and the mother-infant pair was returned

to their social group. Two days prior to surgery, the mother-infant pair was transported from the YNPRC Field Station to the YNPRC Main Station. Once all surgical and post-surgical procedures were completed, the mother-infant pairs were transported back to the YNPRC Field Station where they were reintroduced to their social group using a staged procedure.

All procedures were approved by the Animal Care and Use Committee of Emory University in Atlanta, GA and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Imaging and surgical procedures:

Magnetic Resonance Imaging (MRI) Procedure: The neurotoxic surgical technique utilized MR images of each animal for pre-surgical location of injection sites and pre-surgical calculations of stereotaxic coordinates of each injection site. On the day of the surgery, the infant was removed from their mother, anesthetized (Ketamine hydrochloride, 100mg/ml), intubated and given isoflurane (1-2% to effect). An intravenous drip of dextrose and 0.45% sodium chloride was placed to maintain normal hydration during MRI and surgery. The animal's head was shaved and secured in a nonferromagnetic stereotaxic apparatus. After the animal was aligned in the center of the magnet, the brain was imaged in each of two stereotaxic planes (sagittal and coronal) using a Siemens 3.0T/90 cm whole body scanner and a 3" circular surface coil. Following a sagittal localizer, a high resolution T1-weighted scan (spin-echo sequence, echo time [TE] = 11ms, repetition time [TR] = 450ms, 12cm field of view [FOV], 256x256 matrix) was acquired throughout the brain at 1 mm in the coronal plane. This series was used to estimate the coordinates of injection sites. Additionally, three fluid attenuated inversion recovery (FLAIR) scans (3D T2-weighted fast spoiled gradient [FSPGR]-echo sequence, TE = 2.6ms, TR

= 10.2ms, 25 flip angle, 12 cm FOV, 256x256 matrix) were obtained in the coronal plane at 3.0 mm (each offset of 1 mm posteriorly) throughout the brain. The FLAIR images reveal tissue T2 prolongation with cerebrospinal fluid suppression and were used with the post-surgical FLAIR images to estimate the lesion extent.

Injection coordinates: A T1 coronal image representing the largest body of the amygdala, roughly at the level of the chiasm and the middle portion of the anterior commissure, was selected. From this image, four injection sites were located 1 mm dorsal, 1 mm ventral, 1 mm lateral, and 1 mm medial to the center point of the amygdala, allowing diffusion of ibotenic acid through the entire amygdala. Two additional images, one immediately anterior and one immediately posterior to the central image, were used to determine 2-3 additional injection sites spaced 2-mm apart depending on the size of the amygdala at these levels for each subject. Coordinates for each injection site were determined by measuring the distance of the target site to each of three referents for each monkey: Anterior/Posterior Coordinates were calculated from the zero point determined from the tips of the ear bars; Medial/Lateral Coordinates were calculated from the midline of the brain; and Dorsal/Ventral Coordinates were calculated from the dorsal/ventral coordinates of the earbars. These MRI coordinates were then translated into stereotaxic coordinates.

Surgical Procedure: After the imaging procedure, the animals were kept anesthetized and immediately transported to the surgical suite. The scalp was disinfected with Nolvasan Solution and a local anesthetic (Bupivicaine 0.25% concentration, 1.5ml) was injected subcutaneously along the midline (beginning at the supra-orbital ridge and ending at the occipital notch) to

reduce the pain during skin incision. Under aseptic conditions, the skin was opened and connective tissue was gently displaced laterally to expose the skull. Two small craniotomies were made bilaterally, in front of bregma and above the amygdala, and the dura was cut and retracted to expose the brain. Injections were made simultaneously in the two hemispheres using 30-gauge needles attached to 10 µl Hamilton syringes. The needles were lowered slowly at each injection site, and 0.6-0.8 µl of ibotenic acid (PH 7.8-7.9, 10 mg/ml concentration) was manually injected at a 0.2 µl/min rate. After injection, the needles were left in place for an additional 3-minute period to allow complete diffusion of the neurotoxin at the tip of the needle and minimize its spread in the needle track during retraction of the needles. After the last injection, the dura was closed with silk sutures, the bone opening was covered with Surgicel NU-KNIT (absorbable hemostat), and connective tissues and skin were sutured at the midline. Neo-C animals receiving sham-surgeries were treated in the same way as the experimental animals except that no needles were lowered and no injections were administered.

Postoperative care: Upon complete recovery from anesthesia, the infants were taken to the nursery and placed in an isolette incubator ventilated with oxygen. They were given banamine (1mg/kg for 3 days), dexamethasone (0.5mg/kg for 3 days) and antibiotic (rocephin, 25mg/kg for 7 days) after surgery to prevent pain, edema, and infection, respectively.

Mother-infant Reunions and Cross-fostering: The day after surgery, after ensuring that the animal was alert, it was returned to the mother. The mother-infant reunions were monitored constantly via a secured internet web camera. In most of the cases, the mothers immediately retrieved their infants and the infants were observed nursing on the mother that same day.

However, for a few infants that demonstrated difficulty nursing on the mother during the first reunion, the mother-infant pairs received additional overnight separations for nutrition and monitoring purposes followed by morning reunions until the infant was able to nurse on the mother adequately.

In three cases (one Neo-C male and two Neo-A animals, one male and female), the mother refused to take the infant back after surgery. Repeated attempts were made to reunite those infants with their mother at the YNPRC Main Station, but in each case the mother refused to cradle and nurse the infant. In two cases (Neo-A female and Neo-C male), another reunion was attempted at the YNPRC Field Station upon reintroduction of the mother-infant pairs to their social group. However, in both cases the mother still rejected its infant, but the "abandoned" infant was adopted and raised by another adult female that already had an infant during the birth season and, thus, raised two infants that year. In these two cases of mother-infant 'twin' pairs, the infants' growth patterns were not different from other subjects of the study. The third case, Neo-A male, was successfully cross-fostered to a mother that had recently lost its infant.

Lesion Assessment: The extent of lesion was assessed in vivo using postsurgical neuroimaging procedures, following previously published protocols (Nemanic, et al, 2002). Six to eight days following the surgical procedures, Neo-A infants were again separated from their mother and given a second MRI session for acquisition of both high resolution T1 and FLAIR images, using the same protocols as described for the pre-surgical MRI (see above). Sham-operated animals did not receive this follow-up MRI, but were separated from their mothers and placed in an isolette incubator for the same amount of time. All infants were then re-united with their mothers.

The extent of lesion was estimated using pre- and post-surgery FLAIR images. Presurgical T1 images were also used to help identify the borders of each structure. The pre- and post-surgery FLAIR images were matched to digital drawings of coronal sections from a normalized rhesus monkey template brain (J. Bachevalier, unpublished atlas). Hypersignals were identified on the post-surgical FLAIR images and plotted onto corresponding coronal drawings from the template brain using Adobe Photoshop CS5 software. These drawings were imported into image analysis program Image-J® (version 1.44) to measure the surface area (in pixels squared) of damage for intended targets, as well as all areas sustaining unintended damage (entorhinal and perirhinal cortex and hippocampus). The surface area of hypersignal on each section through each hemisphere were summed and then multiplied by image thickness (i.e. 1mm) to calculate a total volume of damage. The volume of amygdala damage was then divided by the normal volume of the amygdala (obtained from the template brain in a similar manner) and multiplied by 100 to estimate a percentage of the total volume damage.

Procedures for Assessment of Basal HPA Axis Function

Training and capture procedures: Due to the subjects' young age (2.5 and 5 months) they depend on their mothers, which carry the infants during procedures. Thus, all mother-infant pairs were trained to quickly separate from their social group and enter an indoor area. Once inside, mothers were trained to enter a transfer box with their infant and, then, the pair was placed into a cage from which the infant could be separated from the mother. Mothers learned quickly to voluntarily remove their infant off their ventrum, leaving it in the cage while returning to the transfer box. Infants were then carefully removed from the cage, wrapped in a fleece cloth and gently held while a second researcher took a blood sample via femoral venipuncture from

the unanesthetized infant. Blood samples were collected within 10 minutes from the initial disturbance (i.e., researchers approaching the social group). Blood samples collected under these training/habituation conditions and within 10 minutes reflect basal hormone levels since elevations in plasma cortisol concentrations are minimal/undetectable, as demonstrated by previous studies of rhesus monkeys done at the YNPRC, including infants (McCormack, et al., 2009; Sanchez, et al., 2010). In addition, infants experiencing these procedures exhibit normal development (Wilson, Gordon, & Collins, 1986).

Blood Sampling: At 2.5 months of age, an early morning basal blood sample was collected within 30 minutes from sunrise to assess basal cortisol plasma concentrations. No additional afternoon or evening samples were collected at this early age to avoid multiple disturbances to the mother-infant pair. At 5 months of age, the diurnal rhythm of cortisol secretion was examined by collecting blood samples at sunrise, mid-day (afternoon), and sunset using time charts published by the United States Naval Meteorology and Oceanography Command (http://aa.usno.navy.mil/data/docs/RS_OneYear.php) to determine exact daylight times (Sanchez et al., 2005). These daylight diurnal time points were selected instead of clock times because the animals live under natural lighting conditions that affect their circadian cortisol secretory rhythms.

All blood samples were collected in pre-chilled plastic 2 ml vacutainer tubes containing EDTA (3.6mg) and immediately placed on ice. Samples were centrifuged at 3,000 rpm for 15 minutes in a refrigerated centrifuge (at 4ºC). Plasma was pipetted into sterile eppendorfs and stored at -80ºC until assayed.

Plasma Hormonal Assays: All assays were performed by the YNPRC Biomarker Core Laboratory. Plasma concentrations of cortisol were assayed in duplicate by R.I.A. using commercially available kits (DSL kit: Diagnostic Systems Laboratories, Webster, TX). The sensitivity of the DSL assay was 1.25μg/dl and intra- and interassay coefficients of variation were <10%. Plasma T levels were also assayed in duplicate by R.I.A using commercially available kits (DSL kit: Diagnostic Systems Laboratories, Webster, TX). The sensitivity of the DSL assay was 0.05ng/ml and intra- and interassay coefficients of variation were <7%.

Data Analysis

Of a total of 112 blood samples collected, only seven took more than 10 minutes to collect (five samples were collected within 11-12 minutes of initial disturbance; 2.5 months: n=3 samples; 5 months: n=2 samples) and were kept in the analysis; the other two were collected within 15 and 16 minutes from disturbance (2.5 months) and were excluded from the cortisol analysis. A Neo-C male exhibited T levels two standard deviations higher than the group mean; thus, this sample was excluded from the T analysis as an outlier to avoid bias in any group or sex differences in T. At 5 months, diurnal blood samples could not be collected from one Neo-C male due to illness. Lastly, one Neo-A male had a history of chronic illness (unrelated to the lesion) and thus its data were excluded from the analysis at both 2.5 and 5 months.

Preliminary analyses were performed to ascertain if surgical, research manipulations factors, or the time from disturbance until collection of blood samples had any influence on basal cortisol levels. Two Hierarchical Linear Model (HLM) Regression analyses were performed on basal cortisol levels at 2.5 months and 5 months of age separately. Any factor that explained a significant portion of the variance in cortisol levels was used as a covariate in subsequent

analyses of group differences.

Early morning cortisol levels at 2.5 months were analyzed using a General Linear Model (GLM) ANOVA with Group (2; Neo-C, Neo-A) and Sex (2; male, female) as the between subjects factors, cortisol levels as the dependent variable and time to collect the sample as covariate. Interaction effects were examined with post-hoc comparisons using independent t-tests. Testosterone levels at 2.5 and 5 months of age were analyzed using a GLM ANOVA with Group (2) and Sex (2) as the between subjects factors and T level in the morning as the dependent variable. Lastly, the relationship between cortisol and testosterone levels were examined for each sex (male, female) and age (2.5, 5 months) separately using a HLM Regression analyses accounting for "time to collect the cortisol sample" and group differences in cortisol level.

Diurnal rhythm at 5 months of age was analyzed using a Repeated Measures ANOVA with between subjects factors Group (2) and Sex (2), within subjects factor Time of the Day (3; sunrise, mid-day, sunset), and time to collect the sample as covariate. Comparisons of Time of Day across the between subjects factors were assessed by repeated measures contrasts. The percent change of cortisol concentrations throughout the day was calculated as percent decline between sunrise and mid-day or between mid-day and sunset ([sunrise – mid-day]/sunrise*100 or [mid-day - sunset]/mid-day*100, respectively) for each subject. The percent decline was analyzed by GLM ANOVA with Group (2) and Sex (2) as the between subjects factors and percent decline in cortisol level as the dependent variables. Significance level was set at $p < 0.05$ for all analyses and effect sizes were eta squared for ANOVA's and cohen's d for t-tests.

Lastly, we investigated potential correlations between the extent of amygdala lesion and cortisol levels at 2.5 and 5 months of age. Partial correlations were performed for cortisol and

extent of amygdala damage correcting for the "amount of time it took to collect the blood sample".

Results

Lesion Assessment: Table 1 displays the volume of damage estimated from the FLAIR MR-images for each monkey in group Neo-A. The extent of bilateral amygdala damage in twelve cases was substantial and bilateral (mean: 84.82% and 92.80% on the right and left, respectively). Case Neo-A-F5 had more moderate but bilateral damage (Neo-A-F5: right: 61.6%, left: 58.4%) and cases Neo-A-F3 and Neo-A-M4 had asymmetrical amygdala damage (right vs left: 100% vs 32.2% and 50.5% vs 84.9%, respectively). Only in one case (Neo-A-F1) was the damage restricted to the right hemisphere (right: 82.3%). The extent of unintended damage to the perirhinal and entorhinal cortices, temporal cortical areas TE, TEO, TG, anterior portion of the hippocampus, and tail of the putamen were negligible in 13 cases. In two cases, Neo-A-F1 and Neo-A-F3, moderate damage to the entorhinal cortex was found in the right hemisphere (18.3% and 21.8%, respectively). Lastly, moderate damage to the tail of the putamen was noted in two cases (Neo-A-F5 and Neo-A-F6). Figure 1 illustrates an example of the extent of bilateral amygdala damage in two cases (Neo-A-F4 and Neo-A-M7) as reflected by the location and extent of hypersignals seen in post-surgical FLAIR images.

Basal Cortisol: Preliminary analyses were performed to ascertain if surgical, research manipulation factors, or the time from disturbance until collection of the blood sample had any influence on basal cortisol levels. As shown in Table 2, none of the surgical or research manipulation factors accounted for a significant amount of the variance in cortisol level at either

age. Therefore, since cortisol levels were not influenced by factors such as surgical procedure, handling, or amount of time infants were separated from their mother, these variables were not included as covariates in the analysis of group differences. However, because a significant portion of the variance in cortisol levels at each age could be explained by "time to collect the sample", this variable was used as a covariate in subsequent analysis of group differences.

At 2.5 months of age, Neo-C males had higher basal cortisol levels than did females. However, this sex difference was not evident in animals with amygdala lesions as comparable basal cortisol levels were seen in males and females (Figure 2a). This was confirmed by a significant Group by Sex interaction effect ($F[1,25]=4.9$, $p=0.039$, $\eta^2=0.20$). Bonferroni corrected post-hoc comparisons indicated that Neo-C males differed significantly from Neo-C females ($t[8]=2.58$, $p=0.032$, $d=1.63$), Neo-A males ($t[11]=2.89$, $p=0.015$, $d=1.65$), and Neo-A females ($t[9]=2.9$, $p=0.018$, $d=1.70$), whereas Neo-A males and females had comparable cortisol levels ($t[13]=0.473$, $p=0.64$, $d=0.24$).

Analysis of the diurnal cortisol rhythm at 5 months revealed no Group ($F[1,19]=0.001$, $p=0.986$, $\eta^2=0.001$) or Sex effect ($F[1,19]=2.51$, $p=0.13$, $\eta^2=0.12$), but a significant Group by Time of day interaction ($F[2,38]=3.43$, $p=0.043$, $\eta^2=0.15$), reflecting a flattened diurnal cortisol rhythm in Neo-A animals, as compared to Neo-C animals (Figure 2b). Repeated contrasts showed no group differences in cortisol decline from sunrise to mid-day ($F[1,19]=1.13$, $p=0.30$), but a steeper decline from mid-day to sunset in Neo-C than Neo-A animals ($F[1,19]=6.98$, $p=0.009$). This group effect was most evident when the percent decline in cortisol levels (mid-day to sunset) was calculated ($F[1,26]=5.56$, $p=0.028$, $\eta^2=0.20$; Figure 2c).

To further investigate whether the Sex difference found at 2.5 months of age was truly no longer present at 5 months, an additional *a priori* planned GLM ANOVA was conducted only for

sunrise cortisol at 5 months. Results revealed no Group ($F[1,26]=0.08$, $p=0.78$, $\eta^2=0.004$), or Sex ($F[1,26]=0.01$, $p=0.94$, $\eta^2=0.001$), or Group by Sex interaction effects ($F[1,26]=1.79$, $p=0.20$, $\eta^2=0.08$) on sunrise cortisol at 5 months. These results confirm the lack of Sex effects at this age, when the postnatal T surge is over (as shown below).

Testosterone: As shown in Figure 3a, Neo-A and Neo-C males both demonstrated a normal "postnatal T surge" at 2.5 months of age with comparable levels of T ($F[1,26]=0.062$, $p=0.806$, $\eta^2=0.003$). In addition, all males had significantly higher T levels than did females ($F[1,26]=23.32$, $p<0.001$, $\eta^2=0.52$). Unlike cortisol, there was no Group or Group by Sex interaction for T levels ($F[1,26]=0.37$, $p=0.55$, $\eta^2=0.02$). When time to collect the sample and group differences in cortisol were corrected for, regression analyses revealed that T levels during the postnatal T surge accounted for a significant percentage of the variance in basal cortisol among male subjects (R^2 change=0.34, $F(1,8)=9.3$, $p=0.016$; Figure 3b). Such relationship between cortisol and T levels was not found in female subjects (R^2 change=0.02, $F(1,8)=0.38$, $p=0.55$).

The "postnatal T surge" was over by 5 months of age, as indicated by the low T levels in both male groups, Neo-C (M=0.18 ng/ml±0.03) and Neo-A (M=0.19 ng/ml±0.02) and a significant Age effect ($F[1,23]=66.55$, $p<0.001$, $\eta^2=0.74$). Neonatal amygdala lesions did not affect T levels at this age, as shown by the lack of a Group effect ($F[1,26]=0.16$, $p=0.70$, $\eta^2=0.01$; Figure 3a). The end of the "postnatal T surge" is also evident by the lack of a sex differences in T levels at 5 months of age ($F[1,26]=0.07$, $p=0.79$, $\eta^2=0.003$). Lastly, T level no longer accounted for the variance in morning cortisol level among males (R^2 change=0.03, $F(1,9)=0.3$, $p=0.60$), and the same nonsignificant relationship was found among females (R^2

change=0.05, $F(1,8)=0.86$, $p=0.38$).

Correlations between extent of amygdala damage and cortisol: There were no significant correlations between cortisol levels and the amount of amygdala damage at either age (2.5 or 5 months of age) in Neo-A animals.

Discussion

The present results demonstrate that neonatal amygdala lesions yielded alterations in basal cortisol secretion in infant rhesus monkeys, affecting early morning levels in males only at the early age (2.5 months) at the time of the postnatal T surge. At 5 months of age, after the postnatal T surge, neonatal amygdala lesions did not alter early morning basal cortisol levels, but resulted in a flattened diurnal rhythm driven by higher cortisol levels secreted at night in the amygdala-lesioned animals. This later effect of neonatal amygdalectomy was evident in both males and females.

As with previous reports of blunted cortisol response to stressors after amygdala lesions in adulthood (Herman, et al., 2003; Kalin, et al., 2004; Machado & Bachevalier, 2008), the lower basal cortisol levels in amygdalectomized males at 2.5 months of age suggest that the amygdala has an excitatory effect on the HPA axis in early infancy, at least on its basal activity. These results are consistent with those of an earlier study indicating that neonatal amygdala lesions reduced basal cortisol levels in adulthood (Raper, et al., 2012, submitted), and extend these findings by showing that the effects of neonatal amygdala lesions on basal cortisol levels are already present in infancy. Nonetheless, these findings contrast with the lack of effects on basal glucocorticoid secretion reported in other studies, including adult-onset amygdala lesions in

rodents or primates (Sapolsky, et al., 1991; Herman, et al., 2003; Kalin, et al., 2004; Machado & Bachevalier, 2008), juvenile rhesus monkeys (Norman & Spies, 1981), or in a previous study of infant rhesus with neonatal lesions (Goursaud, et al., 2006). These contrasting results may reflect differences in age at lesion and procedures between studies, including time of day for sample collection. Previous primate studies examined basal cortisol samples taken around mid-morning or mid-day (Kalin, et al., 2004; Goursaud, et al., 2006; Machado & Bachevalier, 2008), but we found that neonatal amygdalectomy affected basal cortisol levels during the early morning (at 2.5 months) or at sunset (at 5 months; see discussion below).

Interestingly, a significant sex difference in basal cortisol levels was detected in Neo-C animals at 2.5 months (i.e., males exhibited higher cortisol levels than females) that was eliminated by the neonatal amygdala lesions. Thus, at 2.5 months, Neo-A males showed lower basal cortisol levels than Neo-C males. Although such a sex dimorphism in basal cortisol during infancy has not been previously reported in human or non-human primates (Champoux, et al., 1989; Higley, et al., 1992; Clarke, 1993; Jonetz-Mentzel & Wiedemann, 1993; Knutsson, et al, 1997; Netherton, et al, 2004), our findings indicate that it may be associated with the T surge in male rhesus infants at this early age given the strong correlation found between cortisol and T levels.

After birth, when infants' HPG-axis is abruptly released from the inhibitory effect of the mothers' high estrogen levels, male infants experience a rise in T levels occurring around birth until approximately day 5 in rodents (Forest, 1979; Weisz & Ward, 1980), 4 months in monkeys (Robinson & Bridson, 1978; Mann, et al., 1989), and 7-9 months in humans (Forest, 1979; Bouvattier, et al., 2002), followed by a quiescent phase until puberty. This "postnatal T surge" is known to affect the normal development of male genitalia and reproductive function later in life

(Mann, et al., 1989; Bouvattier, et al., 2002; Boas, et al., 2006). The positive relationship between T and cortisol occurring during the T surge at 2.5 months together with its absence at 5 months when T levels are low suggests that the elevated postnatal androgen levels stimulate the HPA axis in male infants whose amygdala is intact, but is not present in males with amygdala damage. A similar positive relationship between T and cortisol levels was found in castrated adult male rhesus monkeys that exhibited lower morning cortisol levels than intact males (Smith & Norman, 1987). These findings are in line with those of a study in newborn human infants reporting sex differences in HPA axis activity, with boys exhibiting higher salivary cortisol response to a mild examination stressor than girls (Davis & Emory, 1995), although this study did not focus on basal HPA function nor directly assessed T levels. Although these findings seem contrary to the inhibitory role of T previously described in adult male rodents (Seale, et al., 2004) and the lower basal cortisol reported in post-pubertal boys than in girls (Jonetz-Mentzel & Wiedemann, 1993; Knutsson, et al., 1997; Netherton, et al., 2004), at least for these latter studies we have to consider that after puberty, the sex differences are also influenced by increases in estrogen, and boys show lower cortisol than girls only when estrogen is highest in the menstrual cycle (Wolfram, Bellingrath, & Kudielka, 2011). Altogether, these findings suggest that gonadal hormones in both males and females play an important role when investigating sexual differences in HPA axis function, and that circulating testosterone levels could represent an important factor modulating its activity prior to puberty.

Although neonatal amygdalectomy eliminated sex differences in basal cortisol seen at 2.5 months, it did not affect the postnatal T surge. This suggests that the amygdala's role in the development of the HPA axis and its sexual dimorphism is not due to a direct effect on T secretion, but possibly by mediating the stimulatory effect of T on basal cortisol release.

Although the specific mechanism of these interactions is unknown, they could be potentially mediated via AR found in high density in the amygdala (Choate, et al., 1998) and whose expression is higher in males than females (Pomerantz, & Sholl, 1987). Although, the reduced basal cortisol levels in amygdalectomized males could be due to a lack of normal T binding to ARs in the amygdala, there are other mechanisms by which the lesions could affect HPG axis on HPA function. Follow up studies are needed to address this important question about the mechanisms involved.

The diurnal rhythm of cortisol secretion has not previously been fully characterized in socially-housed, mother-reared, infant rhesus monkeys. Previous research in rhesus monkeys has mostly focused on the impact of early experience on HPA stress reactivity (Champoux, et al., 1989; Clarke, 1993; Capitanio, et al., 2005; Barrett, et al., 2009). Of the few studies that have characterized the diurnal cortisol rhythm during rhesus development (Boyce, et al., 1995; Sanchez, et al., 2005; Barrett, et al., 2009), most have studied juveniles and reported a steep decline in cortisol level between early morning and afternoon, and between afternoon and night (Sanchez, et al., 2005; Barrett, et al., 2009). Our results show that although the diurnal rhythm is already present at 5 months of age, characterized by high cortisol levels in the early morning (at sunrise), there is a nonsignificant decline from sunrise to afternoon (mid-day), followed by a steeper cortisol decline from afternoon to bedtime (sunset). These results suggest that the rhythm is still immature at this age, consistent with the lack of clear cortisol decline from mid-morning to mid-afternoon reported in human infants and toddlers (Larson, et al, 1998; Watamura, et al., 2004). In humans, this immature pattern of cortisol secretion has been attributed to napping (Larson, et al., 1998) and immature self-regulation (e.g., low effortful control and behavioral inhibition: Watamura, et al, 2004; Geoffroy, et al, 2006; Tarullo &

Gunnar, 2006; Gunnar, et al, 2011). With age, children's ability to self-regulate increases and napping periods decrease, resulting in behavioral changes that parallel a steeper decline in cortisol secretion from mid-morning to mid-afternoon (Watamura, et al., 2004). It is possible that similar mechanisms influence the immature pattern of cortisol secretion seen in infant macaques in this study, although this possibility will need to be addressed in future studies.

Although the effects of neonatal amygdala lesions on early morning cortisol seen at 2.5 months were not detected at 5 months, the lesions resulted in a blunted cortisol decline from mid-day to sunset, driven by increased cortisol levels at sunset, in Neo-A as compared to the Neo-C group. Of the few previous studies that have examined the effects of adult, juvenile, or neonatal amygdala lesions on primate basal HPA function (Norman & Spies, 1981; Kalin, et al, 2004; Goursaud, et al., 2006; Machado & Bachevalier, 2008; Raper, et al., 2012 submitted) only one has previously reported effects, in particular lower early morning cortisol compared to controls (Raper, et al., 2012 submitted), in adults with neonatal lesions. One important difference with the studies that did not detect differences in basal cortisol is that the levels were measured during mid-morning or afternoon, where we did not detect changes, either. Regardless of the direction of changes in the diurnal cortisol pattern, both studies point to an important influence of the amygdala on basal HPA function in primates. This evidence supports reports of amygdala regulation of tonic (basal) HPA axis activity in rodents (Allen & Allen, 1975; Regev, et al., 2012). Consistent with our findings, Regev and colleagues (2012) recently showed that CRF knockdown in CeA of mice leads to increased basal corticosterone secretion specifically close to the bedtime/sleep phase of the diurnal rhythm, ("lights on" for rodents, when glucocorticoid levels are low). Altogether, these findings suggest that either disruption of just the CeA or the complete amygdala early in development leads to alterations in HPA axis daytime

rhythm, in addition to the more widely reported stimulatory role of this region on HPA stress reactivity.

In addition to developmental factors, the different effects of the lesions on early morning cortisol at 2.5 and 5 months of age could be explained by the low T levels exhibited by all groups at 5 months. These low T levels could, in fact, explain the lack of a sex difference in cortisol secretion at this older age, and the lack of a positive relationship between T and cortisol. Thus, our findings at 2.5 months suggest, that when T levels are high, the amygdala mediates their stimulatory action on the HPA axis function, so that amygdala lesions lead to lower cortisol in males. However, at 5 months, when T levels are low in all groups, the amygdala seems to have an inhibitory role on cortisol secretion instead, at least during the trough of diurnal activity. Altogether, our findings suggest a different (opposite) mechanism of amygdala regulation of basal cortisol secretion, under a low versus a high gonadal hormone context. Future examination of the effects of neonatal amygdalectomy on HPA axis functioning during the juvenile, adolescence and adulthood periods will further help elucidate some of the relationships between amygdala, gonadal hormones, and basal HPA activity.

In summary, results from the current study demonstrate the importance of normal amygdala development and its influence on basal HPA functioning. For example, damage to the amygdala early in life leads to changes in basal cortisol secretion in infant rhesus monkeys, eliminating the normative sex difference seen during the postnatal T surge and leading to a flattened diurnal rhythm at 5 months of age. The long-term effects of neonatal amygdala lesions on this neuroendocrine axis and its interactions with the HPG axis are currently being evaluated and will shed light on the developmental consequences during the juvenile, adolescent, and adult periods.

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Figure Captions

Figure 1. Four coronal MR images through the amygdala: T1-weighted images in one sham-operated control (Neo-C-1) and Fluid Attenuated Inversion Reversal (FLAIR) images in two representative cases with neonatal amygdala lesions (Neo-A-F4 and Neo-A-M7). The numerals to the left of each coronal section indicate the distance in millimeters from the interaural plane. Black arrows point to the hypersignal resulting from the cell death from neurotoxic injections.

Figure 2. Basal plasma cortisol levels during early morning at 2.5 months (a), diurnal rhythm at 5 months (b), and Percent Decline in the diurnal rhythm at 5 months (c). Sham-operated controls (Neo-C) is represented with open bars or open squares with dashed lines, neonatal amygdala lesion animals (Neo-A) are represented with black bars or black circles with solid lines. * indicates a significance of $p < 0.05$.

Figure 3. Early morning testosterone levels 2.5 and 5 months of age (a) and correlation of testosterone and cortisol at 2.5 months of age in the males only (b). All abbreviations as in Figure 2.

Figure 1. Representative cases of neonatal amygdala lesions.

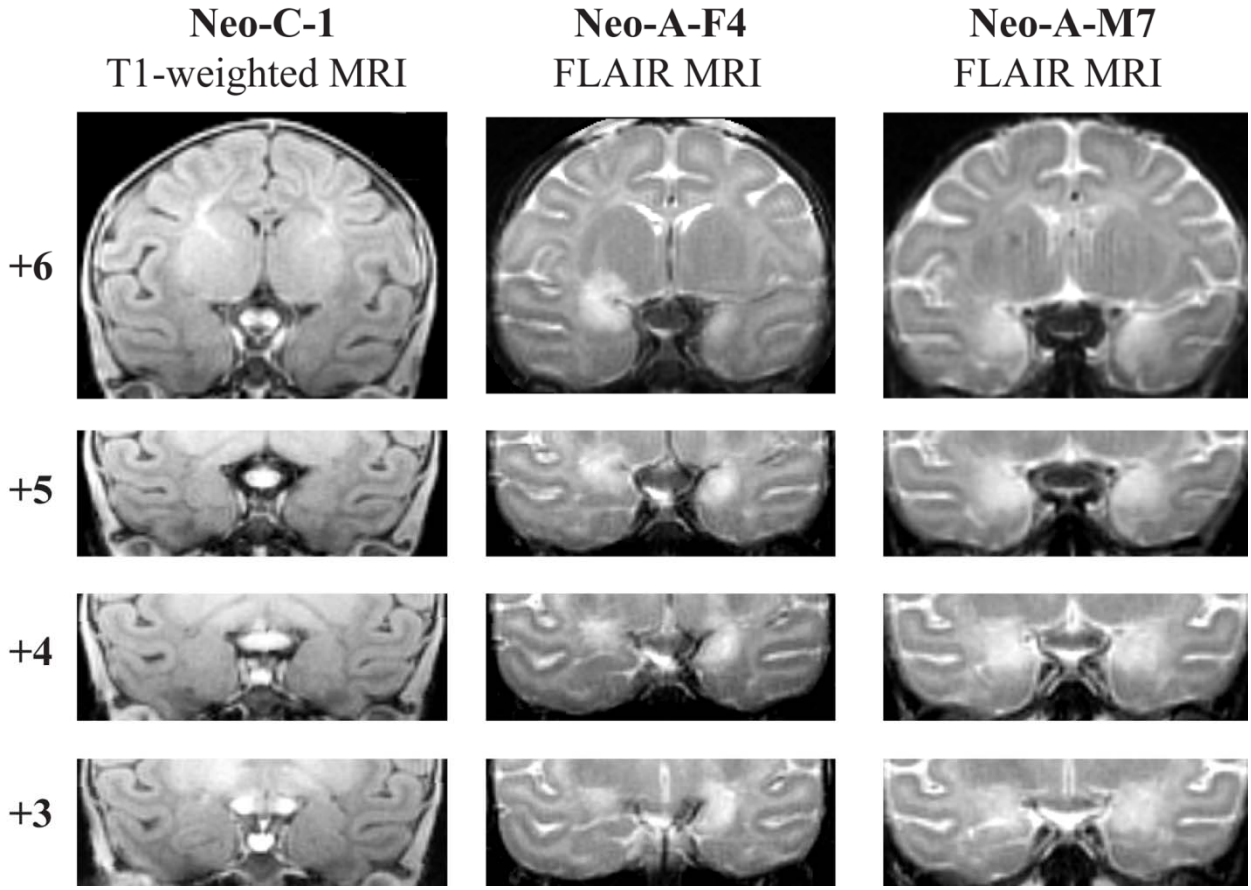

Figure 2. Basal cortisol levels.

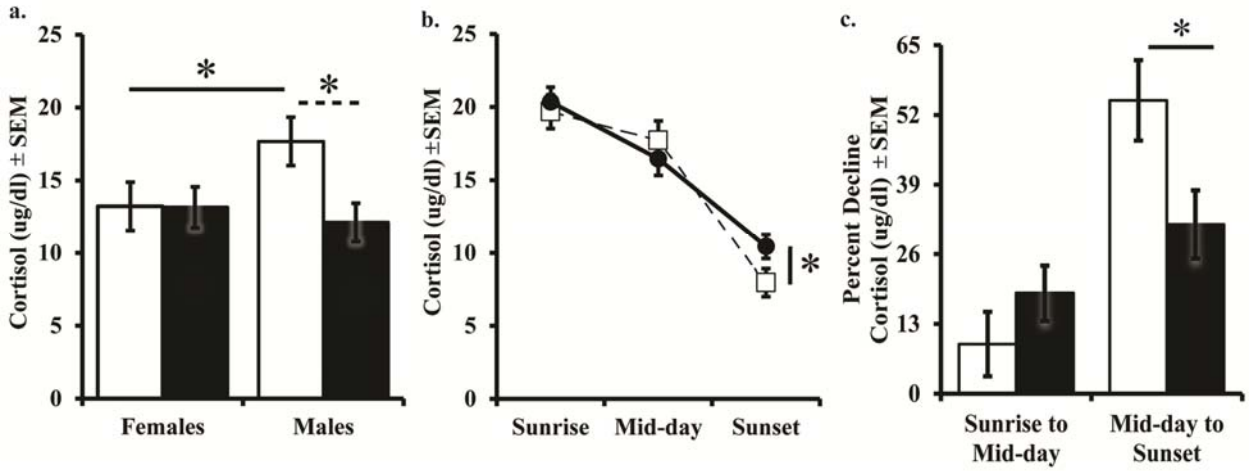

Figure 3. Testosterone levels.

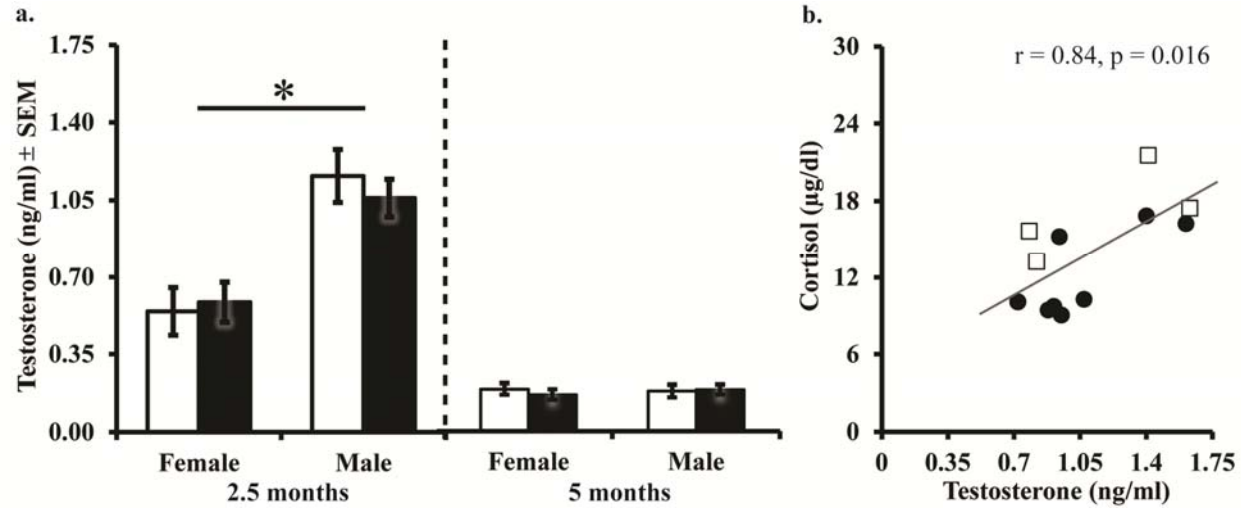

Table 1. Intended and unintended damage after neurotoxic lesions of the amygdala

	Intended Damage				Unintended Damage							
	Amygdala				Hippocampus				Entorhinal			
Subjects	Rt%	Lf%	X%	W%	Rt%	Lf%	X%	W%	Rt%	Lf%	X%	W%
Neo-A-F1	82.3	0.0	41.2	0.0	0.0	0.0	0.0	0.0	18.3	0.0	9.2	0.0
Neo-A-F2	65.7	98.7	82.2	64.8	0.0	5.7	2.9	0.0	0.6	5.5	3.0	0.03
Neo-A-F3	100	32.2	66.1	32.2	2.5	0.0	1.2	0.0	21.8	2.9	12.4	0.6
Neo-A-F4	90.9	89.3	90.1	81.1	1.9	0.0	1.0	0.0	12.3	0.0	6.2	0.0
Neo-A-F5	61.6	58.4	60.0	36.0	0.0	0.0	0.0	0.0	1.6	0.0	0.8	0.0
Neo-A-F6	100	97.7	98.8	97.7	2.4	7.9	5.1	0.2	1.3	2.0	1.6	0.03
Neo-A-F7	98.3	99.0	98.6	97.3	4.3	2.1	3.2	0.1	7.3	2.5	4.9	0.2
Mean	**85.5**	**67.9**	**76.7**	**58.4**	**1.6**	**2.2**	**1.9**	**0.04**	**9.0**	**1.8**	**5.4**	**0.12**
Neo-A-M1	100	80.6	90.3	80.6	8.9	9.0	9.0	0.8	8.3	15.8	12.0	1.3
Neo-A-M2	66.8	89.1	77.9	59.5	0.0	2.7	1.4	0.0	0.1	0.0	0.1	0.0
Neo-A-M3	70.3	90.8	80.6	63.9	3.1	5.3	4.2	0.2	2.1	31.4	16.8	0.7
Neo-A-M4	50.5	84.9	67.7	42.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neo-A-M5	95.9	97.1	96.5	93.1	0.0	12.1	6.1	0.0	5.0	4.4	4.7	0.2
Neo-A-M6	77.3	92.3	84.8	71.3	4.4	0.0	2.2	0.0	0.0	0.3	0.2	0.0
Neo-A-M7	90.9	98.9	94.9	90.0	5.1	0.6	2.9	0.03	1.4	0.0	0.7	0.0
Neo-A-M8	100	87.0	93.5	87.0	6.4	0.4	3.4	0.03	7.1	0.0	3.6	0.0
Neo-A-M9	61.8	93.2	77.5	57.6	0.0	0.0	0.0	0.0	0.0	0.7	0.4	0.0
Mean	**79.3**	**90.4**	**84.9**	**71.8**	**3.1**	**3.4**	**3.2**	**0.1**	**2.7**	**5.8**	**4.3**	**0.2**

L%: percent damage in the left hemisphere; R%: percent damage in the right hemisphere; X%: average damage to both hemispheres; W%: weighted average damage to both hemispheres (W%=(L%×R%)/100). Neo-A-F: female amygdala lesion subject, Neo-A-M: male amygdala lesion subject.

Table 2. Surgical and Research Manipulation Factors

			Interaction with Cortisol Levels	
Factor	Definition	Measured	2.5 months	5 months
Separation from mother	Total time separated from mother from birth until reunion after surgery	Hours	R2 = 0.138 F(1,25)=0.04, p=0.9	R2 = 0.077 F(1,25)=1.71, p=0.2
Handling	Number of times subjects were handled for research (e.g. blood draw), veterinary (e.g. physical exam), and general care (e.g. weight) procedures	Frequency	R2 = 0.167 F(1,24)=0.84, p=0.4	R2 = 0.101 F(1,24)=0.6, p=0.5
Surgical Stress Score*	Measures of stressors during surgery	Cumulative Score	R2 = 0.169 F(1,23)=0.05, p=0.8	R2 = 0.101 F(1,23)=0.4, p=0.9
Length of Surgery	Total time it took to complete the surgical procedure	Hours	R2 = 0.262 F(1,22)=2.8, p=0.11	R2 = 0.101 F(1,22)=0.5, p=0.9
Rate of Isoflurane	Total amount of isoflurane per hour of procedure (pre- & post-surgery MRI, surgery)	% Iso/hr	R2 = 0.297 F(1,21)=1.0, p=0.32	R2 = 0.132 F(1,21)=0.7, p=0.5
Time to collect the blood sample	Time from group disturbance until the blood sample was obtained	Minutes	R2 = 0.718 F(1,20)=30, p<0.001	R2 = 0.345 F(1,20)=6, p=0.02

* Adapted from Anand & Aynsley-Green (1988)

Manuscript II. Sex-dependent role of the amygdala in the development of emotional reactivity to threatening stimuli in infant rhesus monkeys.

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Abstract

Amygdala dysfunction and abnormal fear reactivity are common features of several developmental neuropsychiatric disorders. Yet, little is known about the exact role the amygdala plays in the development of normal fear detection and emotional modulation. The current study examined the effects of neonatal amygdala lesions on defensive and emotional reactivity of infant rhesus monkeys reared with their mothers in large social groups. Monkeys received either bilateral MRI-guided ibotenic acid amygdala (Neo-A; n = 16) or sham (Neo-C; n = 14) lesions at 24.8 ± 1.2 days of age. Defensive and emotional reactivity was assessed using the Human Intruder Paradigm during infancy and preadolescence (2.5 and 12 months of age, respectively). Neonatal amygdalectomy spared the ability to exhibit defensive and emotional behaviors, but did alter the modulation of these responses based on the presence and gaze direction of the human intruder. Interestingly, the sex of the infant modulated the behavioral effects of neonatal amygdalectomy, leading to different patterns of behavior depending on the sex and lesion status of the infant. These changes are also correlated with hormonal changes at this age, and share similarities with the characteristics of some neuropsychiatric disorders such as autism, schizophrenia, and anxiety disorders.

Key words: sex difference, amygdala, emotional, cortisol, testosterone

Introduction

Amygdala dysfunction results in abnormal fear reactivity or affective dysregulation that are common features of several developmental neuropsychiatric disorders, such as autism, William's Syndrome, schizophrenia, and mood disorders. Although the core symptoms of these disorders are similar in males and females, the prevalence and behavioral symptomatology varies in both sex. For example, the prevalence of autism, schizophrenia, and conduct disorder is higher in men, whereas anxiety, depression, and post-traumatic stress disorder are more preponderant in females (Häfner, 2003; Zahn-Waxler, Shirtcliff, & Marceau, 2008; Rinehart, Cornish, & Tonge, 2011). These differences in the clinical profile are consistent with gender differences in behavior of the normal population. For example, typically developing males exhibit more externalizing behaviors (e.g., hyperactivity, aggression, less behavioral inhibition), whereas females exhibit more internalizing behaviors (e.g., shyness, social withdrawal). Thus, children with neuropsychiatric disorders exhibit an exacerbated developmental pattern of these typical sex differences (Häfner, 2003; Rinehart, Cornish, & Tonge, 2011). Although the specific neural mechanisms mediating the expression of these behavioral sex differences are still largely unknown, the amygdala has been thought to be a key structure given the sexual dimorphism reported in its size and androgen receptors (Pomerantz & Sholl, 1987; Micheal, Rees, Bonsall, 1989; Abdelgardir, et al., 1999). In a recent review Schumann and colleagues (2011) have proposed that people with neuropsychiatric disorders can be categorized according to two types of amygdala functioning: 1) those having a low threshold for detecting danger (e.g., amygdala hyperactivity), for whom typically benign environmental cues are being perceived as dangerous; and 2) those having a high threshold for detecting danger (e.g., amygdala hypoactivity), for whom a lack of appropriate danger appraisal from the environment results in increased risk

taking. Therefore, both hyper- and hypo- activity in the amygdala alter an individual's response to potentially threatening stimuli.

Given the clinical evidence reviewed above, it becomes important to investigate the specific role played by the amygdala in the development of normal fear detection and emotional modulation. To date, only a few studies have shown that early amygdala damage yields abnormal threat detection and leads to inappropriate reactivity to objects and social partners (Thompson, 1981; Prather, et al., 2001; Bliss-Moreau, et al., 2010; Raper, et al., 2012a). In one recent study using the Human Intruder paradigm to assess the modulation of emotional reactivity, surrogate peer-reared rhesus monkeys with neonatal amygdala lesions exhibited pervasive alterations in defensive and emotional reactivity (Raper, et al., 2012a). This investigation showed that, although animals with neonatal amygdalectomies were capable of expressing the species-typical defensive behaviors (i.e., freezing or hostility), they lacked the ability to appropriately modulate those behaviors based on the level of threat posed by the human intruder. Therefore, in early infancy, the amygdala is essential to adequately assess the level of threat or danger and to adjust animals' behavioral responses accordingly. In addition, few animal studies have investigated sex differences in threat detection (Kalin, et al., 1998), and none have examined how sex may modulate the effects of early amygdala damage. Thus, the current study aimed to examine the effects of neonatal neurotoxic amygdala lesions on threat detection and modulation of defensive behaviors in both male and female rhesus monkeys. In addition, during the time of testing in infancy, male monkeys are undergoing the postnatal testosterone (T) surge (Robinson & Bridson, 1978; Mann, et al., 1989), thus we also assessed how this surge in gonadal hormones may impact sex-dimorphisms in emotional responses by measuring T levels in circulation.

Methods

Subjects

Thirty-one infant rhesus monkeys (*Macaca mulatta*) from middle-ranking multiparous mothers were used in this study. Mother-infant pairs lived in large social groups (38m x 39 m outdoor compunds) at the Yerkes National Primate Research Center (YNPRC) Field Station (Lawrenceville, GA), Emory University. Infants received neonatal neurotoxic lesions of the amygdala (Neo-A; males = 9, females = 7), and sham operations (Neo-C; males = 6, females = 6) at an average of 24.8 ± 1.2 days of age, or served as behavioral control (Neo-BC; males =2, females = 1). All neuroimaging and surgical procedures were performed at the YNPRC Main Station (Atlanta, GA). Following surgeries, their emotional reactivity to a Human Intruder was assessed during infancy and preadolescence. All procedures were approved by the Animal Care and Use Committee of Emory University and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Procedures for neuroimaging, surgical, estimation of lesion extent, as well as reunions with the mother after surgery have previously been described in detail elsewhere (Raper, et al., 2012b submitted) and will be briefly summarized below.

Imaging and surgical procedures:

Magnetic Resonance Imaging (MRI) Procedure: The day of surgery, infants were removed from their mother, sedated (Ketamine hydrochloride, 100mg/ml), intubated, anesthetized with isoflurane (1-2% to effect), their head was shaved, and secured in a nonferromagnetic stereotaxic apparatus. Infants received an intravenous drip of 0.45% dextrose and sodium chloride to maintain hydration and vital signs (heart rate, respirations, blood pressure, expired CO2) were

monitored throughout the procedures. Two MRI sequences were obtained using a Siemens 3.0T/90 cm whole body scanner and a 3" circular surface coil. First, a T1-weighted scan (spin-echo sequence, echo time [TE] = 11ms, repetition time [TR] = 450ms, contiguous 1mm section, 12cm field of view [FOV], 256x256 matrix) was acquired in the coronal plane and used to determine the coordinates of injection sites in the amygdala. Additionally, three fluid attenuated inversion recovery (FLAIR) scans (3D T2-weighted fast spoiled gradient [FSPGR]-echo sequence, TE = 2.6ms, TR = 10.2ms, 25 flip angle, 12 cm FOV, 256x256 matrix) were obtained in the coronal plane at 3.0 mm (each offset of 1 mm posteriorly) throughout the brain. These MR sequences were repeated for Neo-A animals 7-10 days after the surgical procedure, to accurately localize the areas of edema and estimate the extent of lesion.

Surgical Procedure: After imaging, the infants were kept anesthetized and brought to the surgical suite where they were prepared for aseptic surgical procedures. The scalp was disinfected with Nolvasan solution and a local anesthetic (Bupivicaine 0.25% concentration, 1.5ml) was injected subcutaneously along the midline to reduce the pain during skin incision. The skin and connective tissue were gently displaced laterally, two small bilateral craniotomies were made in front of bregma and above the amygdala, and the dura was cut and retracted to expose the brain.

Neo-A animals received injections of ibotenic acid (PH 7.8-7.9, 10 mg/ml concentration) in 6-8 sites within the center of the amygdala using 10µl Hamilton syringes. Needles were lowered simultaneously in both hemispheres and a total of 0.6-0.8 µl of ibotenic acid was manually injected at a rate of 0.2µl/minute. After each injection, needles were left in place for a 3-minute period to minimize the spread of neurotoxin during needle retraction.

At the completion of the injections, the dura was closed with silk sutures, the craniotomies were covered with Surgicel NU-KNIT (absorbable hemostat), and connective tissues and skin were closed. The animal was placed in a temperature controlled incubator ventilated with oxygen until fully recovered from anesthesia. All animals received banamine (1mg/kg for 3 days), dexamethasone (0.5mg/kg for 3 days) and antibiotic (rocephin, 25mg/kg for 7 days) after surgery to prevent pain, edema, and infection, respectively.

Neo-C animals received the same surgical procedures except no needle was lowered and no injections were given. Animals in the behavioral control group (Neo-BC) received separations from their mother, sedation (Ketamine hydrochloride, 100mg/ml), their head was shaved, scalp was disinfected with Nolvasan Solution, but no surgery was performed. They also received the same "post-surgical" medications.

Lesion Verificaiton: Estimation of the extent of intended and unintended damage for Neo-A animals was made using pre- and post-surgical MR images (Malkova, et al, 2001; Nemanic, et al, 2002). The T1 images were used to identify the borders of each structure and FLAIR images were used to identify extent of hypersignals, which were then plotted onto corresponding coronal drawings from a normalized infant rhesus monkey brain (J. Bachevalier, unpublished atlas) using Adobe Photoshop software. Image-J® (version 1.44) program was used to measure the surface area (in pixels squared) containing hypersignals in amygdala and surrounding structures (entorhinal and perirhinal cortex and hippocampus). The volume of the amygdala damage was then divided by the normal volume of the amygdala (obtained from the template brain in the same manner) and multiplied by 100 to estimate a percentage of the total damage volume. The same procedure was applies to estimate potential damage to structures adjacent to the amygdala.

Lesion Extent

An average of 81.3% damage across both hemispheres was obtained, but varied from case to case. Twelve cases received substantial damage to the amygdala in both hemispheres (right: M = 84.82%; left: M = 92.80%). Three cases received only moderate bilateral damage (Neo-A-F5: right: 61.6%, left: 58.4%) or more asymmetrical amygdala damage (Neo-A-F3: right 100%, left 32.2%; Neo-A-M4: right 50.5%, left 84.9%). One case (Neo-A-F1), the damage was restricted to the right hemisphere (right 82.3%, left 0%). The extent of unintended damage to the perirhinal and entorhinal cortices, anterior portion of the hippocampus, and tail of the putamen were negligible in 13 cases. In two cases, there was moderate damage to the entorhinal cortex in the right hemisphere (Neo-A-F1: 18.3%; Neo-A-F3: 21.8%). Moderate damage to the tail of the putamen was seen in two cases (Neo-A-F5 and Neo-A-F6). Figure 1 illustrates the extent of bilateral amygdala damage in a representative case (Neo-A-M5) as shown by the location and extent of hypersignals seen in the post-surgical FLAIR images.

Human Intruder Paradigm

Thirty subjects were tested as infants at 81 ± 10 days of age and given their dependence on their mother at this early age, all mother-infant pairs were trained for quick capture from the social group (see Raper, et al., 2012b) and, then the infants were separated from their mother for behavioral testing. All subjects were re-tested again at 12 months of age, except 4 subjects that were not tested due to illness unrelated to their experimental procedures. Thus, only 26 subjects were tested as juveniles. Juvenile monkeys were independently trained to quickly separate from their social group without their mothers.

For behavioral testing, at both ages, animals were transported to a novel testing room,

and transferred to a stainless steel cage in which one side wall was made of clear lexan plastic to allow video recording. The Human Intruder paradigm lasted 30 minutes and consisted of three conditions (Alone, Profile [No Eye Contact in other publications], Stare) presented in the same order for all animals. Thus, the animal first remained alone in the cage for 9 minutes (Alone condition), then the intruder (experimenter wearing a rubber mask) entered the room, and sat two meters from the test cage for 9 minutes while presenting his/her profile to the animal (Profile condition). After the Profile, the intruder left the room and the animal remained in the cage alone for a 3-minute period, after which the intruder re-entered the room and made direct eye contact with the animal for 9 minutes (Stare condition). In infancy, an additional condition was added to the end of the Stare condition: the Novel Fruit test. Once the Stare condition was completed, the intruder approached the test cage and placed a slice of novel fruit (kiwi) on a foraging board attached to the outside of the test cage. The intruder then left the room, giving the infant 5 minutes alone to explore and eat the kiwi. Immediately following the test, monkeys were reunited with their mother and/or social group.

Animals' emotional reactivity during the Human Intruder paradigm was video recorded and later coded using a detailed ethogram (described in Table 2). Digital videos were coded using the Observer XT program (Noldus, Inc., Netherlands) by one experimenter (JR with an average intra-rater reliability of Cohens Kappa = 0.98; and an average intra-rater reliability of Cohen's Kappa = 0.845 with other trained experimenters who coded samples of the videos).

Hormone assessment

At both ages, blood samples were collected on all animals, immediately after being removed from their social group and prior to testing, to assess basal hormonal levels. As

described above, animals were trained for quick capture from the social group and blood samples were collected within 10 minutes of initial disturbance, when the experimenters first enter the social group (see Sanchez, et al., 2010; Raper, et al., 2012a). All blood samples were collected in pre-chilled plastic 2 ml vacutainer tubes containing EDTA (3.6mg) and immediately placed on ice. Samples were centrifuged at 3,000 rpm for 15 minutes in a refrigerated centrifuge (at 4ºC). Plasma was pipetted into sterile eppendorfs and stored at -80ºC until assayed.

Testosterone assay was performed by the YNPRC Biomarker Core Laboratory (Atlanta, GA). Plasma testosterone levels were also assayed in duplicate by R.I.A using commercially available kits (DSL kit: Diagnostic Systems Laboratories, Webster, TX). The sensitivity of the DSL assay was 0.05ng/ml and intra- and interassay coefficients of variation were <7%.

Data Analysis

At each age, preliminary analyses were first performed to compare the two groups of control animals (i.e., behavioral control group [Neo-BC] and the sham-operated group [Neo-C]). Repeated measures ANOVA (Group X Condition) revealed no significant main effects or interactions. Therefore, data from the Neo-BC group was combined with those of the animals in the Neo-C group to create a single control group (Neo-C) for all subsequent analyses. There were 14 Neo-C animals (males = 7; females = 7) and 16 Neo-A animals (males = 9; females = 7) tested as infants and 13 Neo-C animals (males = 6; females = 7) and 13 Neo-A animals (males = 7; females = 6) tested as juveniles.

Prior to analysis, the behavioral data were transformed using a natural log plus constant to obtain normal distribution. The impact of early amygdala damage on the expression of defensive and emotional behaviors toward the Human Intruder was examined separately at each

age using a Repeated Measures ANOVAs with Group (Neo-C, Neo-A) and Sex as between subjects factors, and Condition (Alone, Profile, Stare) as the within subjects factor. Interactions were examined with post-hoc one-way ANOVAs.

Finally, we investigated potential relationships between basal testosterone levels and behavior during the Human Intruder paradigm at each age. Partial correlations were performed for testosterone levels and behavior correcting for the amount of time it took to collect the blood sample. The relationship between the extent of amygdala damage and behavior during the Human Intruder paradigm was also examined using Pearson correlations. Significance level was set at $p < 0.05$ for all statistical analyses.

Results

Neonatal amygdala lesions and emotional reactivity in infancy:

Normally developing infant rhesus monkeys modulated their behavioral responses depending on the presence and gaze direction of the intruder, whereas neonatal amygdalectomized monkeys did not (see Table 3). As compared to conditions when the intruder was present (Figure 2), infant monkeys of both groups exhibited significantly greater coo vocalizations during the Alone condition (Condition: $F[2,52]=4.3$, $p=0.019$, $\eta^2=0.14$). However, Neo-A infants emitted more coos throughout all conditions compared to Neo-C infants (Group: $F[1,26]=3.9$, $p=0.05$, $\eta^2=0.13$). Again, as compared to the conditions in which the intruder was present, during the Alone condition, Neo-C infants engaged in more cage exploration than Neo-A animals (Group X Condition: $F[2,52]=3.9$, $p=0.03$, $\eta^2=0.13$; see Figure 2). Additionally, females of both groups explored the cage more compared to males (Sex: $F[1,26]=5.1$, $p=0.032$, $\eta^2=0.17$; Figure 2).

When the intruder presented his/her profile, both groups displayed an increase in freezing behavior (Condition: $F[2,52]=35.8$, $p<0.001$, $\eta^2=0.58$) and there were no differences between groups (see Table 3). However, the pattern of fearful defensive behaviors varied according to the infants' group, sex, and the condition (Group X Sex X Condition: $F[2,52]=3.4$, $p=0.04$, $\eta^2=0.12$; see Figure 2). Neo-C males exhibited a linear decline from Alone to Profile to Stare condition, but Neo-C females had more fearful defensive behaviors during the Profile condition than in the other conditions (see Figure 2). Thus, Neo-C animals displayed a clear modulation of fearful defensive behaviors across conditions, but this modulation was not seen in Neo-A animals. Unlike Neo-C males, Neo-A males exhibited an exaggerated pattern of fearful defensive behaviors and expressed significantly more fearful defensive behaviors during the Profile and Stare conditions ($F[1,15]=5.98$, $p=0.028$, $\eta^2=0.30$; $F[1,15]=8.15$, $p=0.013$, $\eta^2=0.37$, respectively). Similarly, unlike Neo-C females, Neo-A females expressed more fearful defensive behaviors during the Alone and a trend in the Stare conditions ($F[1,13]=5.07$, $p=0.044$, $\eta^2=0.30$; $F[1,13]=4.10$, $p=0.06$, $\eta^2=0.25$, respectively). Taken together, these data suggest that neonatal amygdala lesions altered the ability to modulate fearful defensive behaviors based on the presence or gaze direction of the intruder.

The most salient threat is provided when the intruder stares directly at the monkey, as a result both groups exhibited an increase in hostility (Condition: $F[2,52]=23.4$, $p<0.001$, $\eta^2=0.47$) and did not differ from each other (see Table 3). In contrast, the amount of scream vocalizations emitted by infants varied depending on their group, sex, and condition (Group X Sex X Condition interaction: $F[2,52]=528$, $p=0.009$, $\eta^2=0.17$). As illustrated in Figure 2, Neo-C males and females emitted more screams during the Stare condition as compared to the other conditions, but this pattern was seen in the Neo-A males but not in Neo-A females. Unlike Neo-

C females, Neo-A females emitted significantly more screams during the Alone condition ($F[1,13]=6.47$, $p=0.026$, $\eta^2=0.35$; see Figure 2).

Infant monkeys of both groups expressed more anxious behaviors, tooth grinding, and yawning during the Stare condition (Condition: $F[2,52]=84.7$, $p<0.001$, $\eta^2=0.77$; $F[2,52]=106$, $p<0.001$, $\eta^2=0.80$; $F[2,52]=15.5$, $p<0.001$, $\eta^2=0.37$, respectively). Interestingly, the expression of anxious and tooth grinding behaviors differed depending on the infants' sex and group (Group X Sex interaction: $F[1,26]=5.0$, $p=0.034$, $\eta^2=0.16$; $F[1,26]=4.2$, $p=0.05$, $\eta^2=0.14$), such that Neo-C males exhibited more anxiety and tooth grinding compared to Neo-A animals or Neo-C females (see Figure 2). There was also a significant increase in yawning during the Stare condition that differed according to the sex of the animals (Sex: $F[1,26]=4.1$, $p=0.05$, $\eta^2=0.14$), such that males expressed more yawns than females in both groups (see Table 3). Lastly, self-soothing (e.g., self-grooming) behaviors increased as the salience of the threat increased (Condition: $F[2,52]=9.01$, $p<0.001$, $\eta^2=0.26$). Again, these behaviors tended to differ according to the sex of the infant (Sex X Condition: $F[2,52]=2.88$, $p=0.06$, $\eta^2=0.10$). Females from both groups exhibited more self-soothing behaviors during the Stare condition ($F[1,13]=6.04$, $p=0.03$, $\eta^2=0.34$; Table 3). No significant differences were found between groups for affiliative or stereotypic behaviors.

The Novel Fruit test examines the infants' reactivity (approach or avoidance) toward an ecologically relevant nonsocial stimulus that has a reward value. Analysis of the Novel Fruit test revealed that Neo-A animals touched the fruit as rapidly as Neo-C animals (Group: $F[1,30] = 0.53$, $p = 0.47$, $\eta^2 = 0.02$), although Neo-A males tended to spend slightly less time manipulating the fruit (Group X Sex: $F[1,30] = 4.09$, $p = 0.054$, $\eta^2 = 0.14$; see Figure 3). Although none of the infant monkeys ate all of the novel fruit, the amount of time spent eating the fruit differed

between males and females of both groups (Group X Sex: $F[1,30] = 6.98$, $p = 0.014$, $\eta^2 = 0.21$; Figure 3), such that Neo-A males spent significantly less time eating the fruit compared to Neo-C animals and Neo-A females.

Neonatal amygdala lesions effects on emotional reactivity during preadolescence:

At twelve months of age, normally developing juvenile monkeys continued to modulate their behavioral responses depending on the presence and gaze direction of the intruder, whereas juveniles with neonatal amygdala lesions again showed impairments in modulating their behavior (see Figure 4). Unlike infancy, during this preadolescent stage of development, there were fewer sex differences in the production of emotional response in the two groups (Table 3).

First, both groups exhibited more cage exploration and cooing during the Alone condition compared to conditions when the intruder was present (Condition: $F[2,44]=6.86$, $p=0.003$, $\eta^2=0.24$; $F[2,44]=5.87$, $p=0.006$, $\eta^2=0.21$, respectively). Although the groups did not differ in the amount of cage exploration (Table 3), Neo-A animals continue to express more coos throughout all conditions compared to controls (Group: $F[1,22]=4.43$, $p=0.047$, $\eta^2=0.17$; see Figure 4). Preadolescent monkeys of both groups also exhibited more stereotypies (e.g. pacing; Condition: $F[2,44]=6.75$, $p=0.003$, $\eta^2=0.24$; Table 3).

Interestingly, at this preadolescent period, the impact of neonatal amygdala lesions became more apparent in condition when the intruder presented his/her profile. Thus, as compared to infancy during which both groups exhibited the same amount of freezing, in preadolescence Neo-A animals now exhibited significantly less freezing as compared to Neo-C animals (Group X Condition: $F[2,44]=4.51$, $p=0.017$, $\eta^2=0.17$; see Figure 4). Additionally, both groups exhibited an increase in fearful defensive behaviors depending on the presence and gaze

direction of the intruder (Condition: $F[2,44]=16.11$, $p<0.001$, $\eta^2=0.42$; see Figure 4). However, whereas Neo-C juveniles exhibited more fearful defensive behaviors in the Profile as compared to the other two conditions (Alone vs Profile: $F[1,11]=15.09$, $p=0.003$, $\eta^2=0.58$; Profile vs Stare: $F[1,11]=18.44$, $p=0.001$, $\eta^2=0.63$), Neo-A juveniles did not modulate their fearful defensive behaviors, exhibiting similar levels of fearful defensive behaviors between the Alone and Profile conditions (Alone vs Profile: $F[1,11]=3.84$, $p=0.076$, $\eta^2=0.25$; Profile vs Stare: $F[1,11]=7.06$, $p=0.022$, $\eta^2=0.39$). Thus, damage to the amygdala early in life has impacted anti-predator detection behaviors during preadolescence.

During the Stare condition, juvenile monkeys of both groups exhibited an increase in hostility, although the magnitude of this behavioral modulation was greater in Neo-C animals than in Neo-A animals (Group X Condition interaction: $F[2,44]=4.83$, $p=0.013$, $\eta^2=0.18$; see Figure 4). Additionally, both groups expressed more scream vocalizations, yawning, and anxiety during the Stare condition as compared to the other conditions (Condition: $F[2,44]=8.14$, $p=0.001$, $\eta^2=0.27$; $F[2,44]=15.3$, $p<0.001$, $\eta^2=0.41$; $F[2,44]=34.7$, $p<0.001$, $\eta^2=0.61$, respectively; Table 3). The sex difference in yawning found in infancy was no longer present during preadolescence (see Table 3). However, Neo-A animals expressed significantly less tooth grinding, a specific type of anxious behavior as compared to Neo-C animals (Group: $F[1,22]=8.14$, $p=0.009$, $\eta^2=0.27$; see Figure 4).

Interestingly, the pattern of affiliative behaviors varied depending on the juveniles' group, sex, and condition (Group X Sex X Condition interaction: $F[2,44]=4.29$, $p=0.02$, $\eta^2=0.16$). Neo-C males exhibited more affiliative behaviors during the Alone condition compared to the other conditions, but Neo-C females expressed more affiliative behaviors during the Stare than in the other conditions (see Figure 4). Neo-A males, unlike Neo-C males,

expressed more affilitative behaviors during the Stare condition ($F[1,12]=4.0$, $p=0.05$, $\eta^2=0.27$), whereas Neo-A females, unlike Neo-C females, exhibited a trend toward more affiliative behaviors in the Alone condition ($F[1,12]=3.8$, $p=0.07$, $\eta^2=0.24$). Similar to infancy, juvenile monkeys' exhibited increased self-soothing behaviors during the Stare condition compared to the other conditions (Condition: $F[2,44]=3.22$, $p=0.05$, $\eta^2=0.13$), and females of both groups exhibit more self-soothing compared to the males (Sex: $F[1,22]=9.18$, $p=0.006$, $\eta^2=0.29$; see Figure 4). Again, as in infancy, this sex difference in self-soothing was present in juvenile monkeys with neonatal amygdala lesions as well.

Correlations between Behavior and Testosterone and extent of amygdala damage:

Relationship between testosterone levels and total amount of yawning were examined using partial correlations to control for the "amount of time to collect the blood sample." There was a moderate positive relationship ($pr[22] = 0.37$, $p = 0.038$; see Figure 5) between yawning and testosterone levels in infancy, but this relationship disappeared by preadolescence ($pr[22] = 0.07$, $p = 0.37$; Figure 5), at a time when the postnatal testosterone surge had subsided and sex differences in yawning had disappeared. Thus, during infancy, sex differences in yawning appeared to be linked to circulating testosterone levels. Lastly, examination of potential correlations between the extent of amygdala damage and any of the behaviors examined revealed no significant relationships either during infancy or preadolescence.

Discussion

This study examined the effects of early amygdala damage on emotional reactivity across infancy and pre-adolescence. There were four main findings. First, the data confirmed previous

findings that neonatal amygdala lesions did not impair the expression of emotional or defensive behaviors; rather, these early lesions did impact the modulation of these behaviors depending on the presence and gaze direction of the intruder (Raper, et al., 2012a). They also extended the earlier findings by showing that the effects of the neonatal amygdala lesions on emotional reactivity became more pronounced as the animals matured. Second, unlike previous studies (Kalin, et al., 1998), the data also demonstrated the presence of sex differences in the expression and modulation of emotional reactivity, which were more clear-cut in infancy than in preadolescence. These results indicate intriguing influences of gonadal hormones on expression and modulation of behavior as exemplified by the influence of the postnatal testosterone surge on behavioral sex differenced in yawning during infancy. Together, these results suggest that the amygdala plays a critical role in the expression of sexually-dimorphic emotional responses during primate early development.

Amygdala and Modulation of Fear Reactivity

As shown in previous studies (Kalin, et al., 1989, 1991; Raper, et al., 2012a submitted), infant rhesus monkeys modulated their defensive and emotional behaviors depending on the gaze direction and magnitude of the threat provided by the human intruder in the first few months after birth. Importantly, early damage to the amygdala did not disrupt the development of species typical behaviors. During infancy, both sham-operated controls and animals with neonatal amygdala lesions exhibited more freezing during the Profile condition and more hostility during the Stare condition. However, when reaching preadolescence, the impact of early amygdala damage became more apparent as indicated by a reduction in the production of these behaviors in animals with neonatal amygdala lesions as compared to controls. The current

results are directly in line with those of a recent study indicating that neonatal amygdala lesions yielded no changes in freezing at 4.5 months of age, but decreased freezing in adulthood (Raper, et al., 2012a). This pattern of findings demonstrates that the effects of neonatal amygdalectomy become more apparent with development. There are numerous neural developmental changes occurring between infancy through adolescence. Amygdala volume significantly increased in normal rhesus monkeys from 1 week to 2 years of age (86.49% in males and 72.94% in females; Payne, et al., 2010) and this volume increases are reflected by important cytoarchitectonic changes within the different amygdala nuclei (Chareyron, et al., 2012). There are also developmental changes in the neural pathways involved in the coordination of the reactive stress response (Lidow, et al., 1991; Andersen, 2003). Glucocorticoid receptors increase from infancy to adolescence (Perlman, et al., 2007), whereas neurotransmitter receptor density steadily declines and tapers off at puberty (Lidow, et al., 1991). In addition, the impact of the amygdala on emotional reactivity also involves interactions of the amygdala with other neural systems, such as the orbital and ventromedial prefrontal cortex, which are known to have a more protracted development than the amygdala (Machado & Bachevalier, 2004). Therefore, the protracted effects of amygdala damage on emotional reactivity may have resulted from an impact of the amygdala damage on the normal development of other structures and pathways involved in assessing threat level and coordinating an appropriate behavioral and neuroendocrine response.

Although neonatal amygdala lesions did not disrupt the development of species typical defensive and emotional behaviors, it did impair the animals' ability to modulate the expression of those behaviors. As infants and as juveniles, monkeys with early amygdala damage expressed more coo vocalizations throughout the test and exhibited less cage exploration during the Alone

condition in infancy but not in preadolescence. The alterations in cooing and cage exploration are in line with the role of the amygdala in detecting environmental danger and adapting an appropriate behavioral strategy according to the salience of that threat (Davis & Whalen, 2001). The willingness to emit coo vocalizations regardless of the presence or gaze direction of the intruder suggests that amygdalectomized animals could not discern the difference in threat level between conditions and thus could not modulate their vocalizations accordingly.

Sex-dependent Role of the Amygdala in Emotional Reactivity:

For some emotional responses, the effects of the amygdala lesions varied according to the sex of the animal, indicating an important role of the amygdala in the expression of sexually-dimorphic behaviors. In the case of fearful defensive and scream vocalizations, infant females with neonatal amygdalectomy showed a different pattern compared to sham-operated females, exhibiting more of those behaviors in the Alone condition. Given that the typical sex difference in normal monkeys is for female rhesus monkeys to exhibit more fear grimaces and hostility toward social stimuli (Mason, et al., 1960), the pattern seen in Neo-A females reflects an exaggeration of the normal sex difference. Similarly, neonatal amygdalectomized infant males and control males expressed the same linearly increasing pattern of fearful defensive behaviors across conditions, but Neo-A males exhibited an exaggeration of this behavior pattern. For some other behaviors, such as anxious behaviors and tooth grinding, the typical sex difference was eliminated by early amygdala damage. Normally developing male infant monkeys exhibited increased anxious behaviors and tooth grinding compared to females, neonatal amygdalectomy eliminated this sex difference in infancy.

Neonatal amygdalectomy did not abolish all sex differences, as shown by the presence of

overall sex differences in yawning during infancy and self-soothing behaviors during preadolescence. This is the first report of a sex difference in yawning behavior during infancy. Previous studies have shown that the sex difference in yawning emerges after puberty (Hadidian, 1980; Troisi, et al., 1990) and is influenced by testosterone level in old world monkey (Phoenix, et al., 1973; Graves & Wallen, 2005). This sex difference occurred during a developmental stage when males are experiencing the postnatal testosterone surge, a period when gonadal hormones are temporarily active in male infants only (Forest, et al., 1974, 1979; Robinson & Bridson, 1978; Mann, et al., 1989). Thus, the increased yawning in males was positively related with testosterone levels in males, suggesting that this behavioral sex difference is present at birth and under the activational influence of circulating testosterone levels. Support for this hypothesis was further provided by in the absence of sex difference in yawning during preadolescence, at a time when the postnatal testosterone surge has ended and both sexes are in a period of gonadal quiescence prior to puberty. Finally, the fact that early amygdala damage did not impact the sex difference in yawning during infancy suggests that the amygdala is not involved in all sexually dimorphic behavioral expressions.

Another example of a lack of sex-dependent role of the amygdala is provided with self-soothing behaviors during infancy and preadolescence. Juvenile rhesus females exhibited significantly more grooming with partners in their social group (Hassett, et al., 2010), thus the increased self-soothing (e.g. self-grooming) was expected. Interestingly, early damage to the amygdala did not disrupt this typical sex difference in juvenile rhesus monkey females. Nevertheless, additional sex differences in behavior may emerge after puberty among these animals; therefore continued monitoring of these animals will shed light on the long-term effects of early amygdala damage and its potential to impact normal development of sexually dimorphic

behaviors.

Amygdala and Reactivity to Novelty:

The Novel Fruit test examined the infants' reactivity (approach or avoidance) toward a nonsocial stimulus with a reward value. Although groups did not differ in their latency to first approach the kiwi, neonatal amygdala lesion males spent less time manipulating and eating the kiwi. Since neonatal amygdala lesion males approached the kiwi at the same rate compared to other groups, it seems unlikely that their decreased manipulation and eating is caused by inattention, lack of interest, or increased avoidance of novelty. Alternatively, neonatal amygdalectomized males decreased eating may be due to an alteration in food preference, which has been found in previous studies of amygdala lesions (Machado, et al., 2010). Future studies should systematically examine food preference in these animals to determine whether the early amygdala damage has altered food preferences.

Amygdala and Neuropsychiatric Disorders in Humans:

In summary, the present findings inform our understanding on the role of the amygdala on the development of the expression and regulation of emotion. Early amygdala damage leads to an inability to modulate behavioral responses based on the level of threat presented by the human intruder. Furthermore, the amygdala plays a critical role in the expression of sexually-dimorphic emotional response during primate early development. These sex-dependent alterations in defensive and emotional reactivity after neonatal amygdala lesions bare some similarities with sex-dependent symptomatology of developmental neuropsychiatric disorders.

Developmental neuropsychiatric disorders, such as autism, schizophrenia, Williams

Syndrome, and mood disorders, share common characteristics of amygdala dysfunction, alterations in fear reactivity, and sex differences in prevalence and behavioral profile (Häfner, 2003; Zahn-Waxler, et al., 2008; Rinehart, et al., 2011; Schumann, et al., 2011). There is growing evidence of aberrant amygdala and brain development in children with neuropsychiatric disorders (see review Schumann, et al., 2011). Previous studies have focused on overall amygdala volume among patients with autism or schizophrenia; however, more recent studies point to an abnormal growth trajectory instead of overall volume changes. For example, among patients with either autism or schizophrenia, the amygdala volume is enlarged early in development compared to typically developing children, whereas examination of older patients (adolescents or adults) reveals smaller amygdala volumes (Aylward, et al., 1999; Pierce, et al., 2001; Sparks, et al., 2002; Schumann, et al., 2004, 2009, 2011; Nacewicz, et al., 2006; Mosconi, et al., 2009). This abnormal brain development has been associated with many of the behavioral characteristics of autism, such as gaze avoidance, unusual affect, anxiety, and impaired social behavior (Nacewicz, et al., 2006; Schumann, et al., 2009). Results from the current developmental primate study may shed light on how disruption of normal amygdala function can directly impact emotional behavior in infancy and can manifest through development.

The results provide three examples of impaired emotionality that could related to some of the symptomatology characterizing neuropsychiatric disorders. First, we found that neonatal amygdala-lesioned animals exhibited impaired threat appraisal and reduced avoidance of threatening stimuli (less hostility and freezing). Similar inappropriate appraisal of threat level in stimuli and reduced avoidance is generally found in individuals with Williams Syndrome who exhibit hypersociality, increased approach, and reduced amygdala activation to social threatening stimuli, together with increased anxiety (Einfeld, et al., 1997), although amygdala activation in

these individuals was found for non-social threatening stimuli (Meyer-Lindenberg, et al., 2005).

Second, we also found that neonatal amygdala-lesioned animals exhibited an exaggerated pattern of the typical sex differences in fearful defensive behaviors. Similarly, although the core symptoms of neuropsychiatric disorders are similar between the sexes, the behavioral profile differs between males and females in a way that is consistent with gender-related differences in the normal population (Häfner, 2003; Rinehart, Cornish, & Tonge, 2011).

Finally, during the Novel Fruit test, neonatal amygdala-lesioned males spent less time manipulating and eating the fruit compared to the other animals. This avoidance or withdrawal from exploring novel foods or objects has consistently been reported in autistic children who also exhibit decreased approach of novel toys as compared to typically developing and developmentally delayed children (Brock, et al., 2012), and are frequently reported to be picky eaters, mouth nonfood items, and resist new foods (Provost, et al., 2010). Overall, neonatal amygdala lesions in rhesus monkeys appear to model some of the aspects of neuropsychiatric disorders and continued examination of these animals may give valuable insight into the mechanisms underlying the behavioral profiles of these developmental disorders.

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Figure captions

Figure 1. Two coronal MR images through the amygdala: T1-weighted images in one sham-operated control (Neo-C-1) and Fluid Attenuated Inversion Reversal (FLAIR) images in a representative case with neonatal amygdala lesions (Neo-A-M5). The numerals to the left of each coronal section indicate the distance in millimeters from the interaural plane. Black arrows point to the hypersignal resulting from the cell death from neurotoxic injections.

Figure 2. Average Coo vocalizations, Freezing, Hostile, Cage Exploration, Fearful defensive, Tooth Grinding, and Scream vocalizations during the Alone (A), Profile (P), and Stare (S) conditions of the Human Intruder paradigm for animals with sham operations (Neo-C, open bars) and animals with neonatal amygdala lesions (Neo-A, black bars). * indicates a significant difference from same sex controls ($p < 0.05$). ǂ indicates a trend toward a difference from same sex controls ($p < 0.06$). § indicates a significant sex difference.

Figure 3. Animals' investigation of the novel fruit (kiwi), average Latency, Manipulation, and Eating behaviors. All other abbreviations as in Figure 2.

Figure 4. Average Coo vocalizations, Freezing, Hostile, Affiliative, Tooth Grinding, and Self-soothing behaviors during the three conditions of the Human Intruder paradigm.

Figure 5. Correlation between Testosterone and Total Yawning expressed across all three conditions for all animals. Sham operated controls (Neo-C) are represented by squares (males =

open squares, females = grey squares), neonatal amgydala lesion animals (Neo-A) are represented by circles (males = black circles, females = grey circles).

Figure 1. Representative case of a neonatal amygdala lesion.

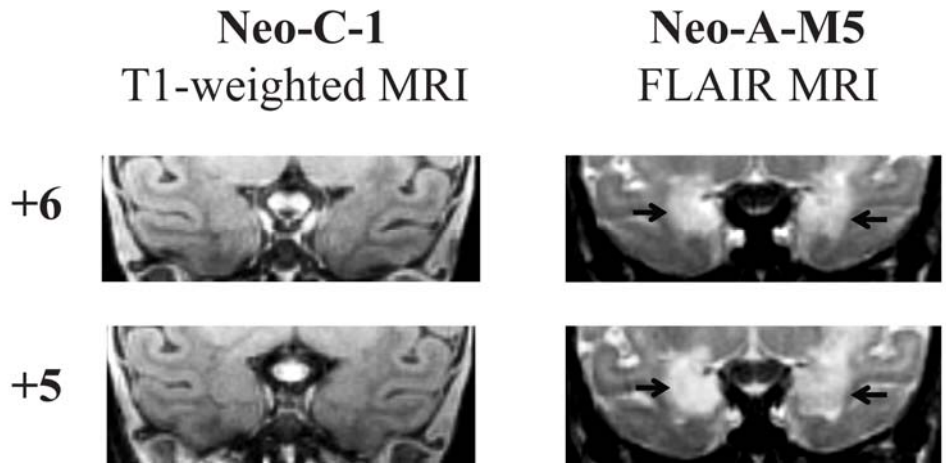

Figure 2. Behavioral responses during the Human Intruder paradigm in Infancy

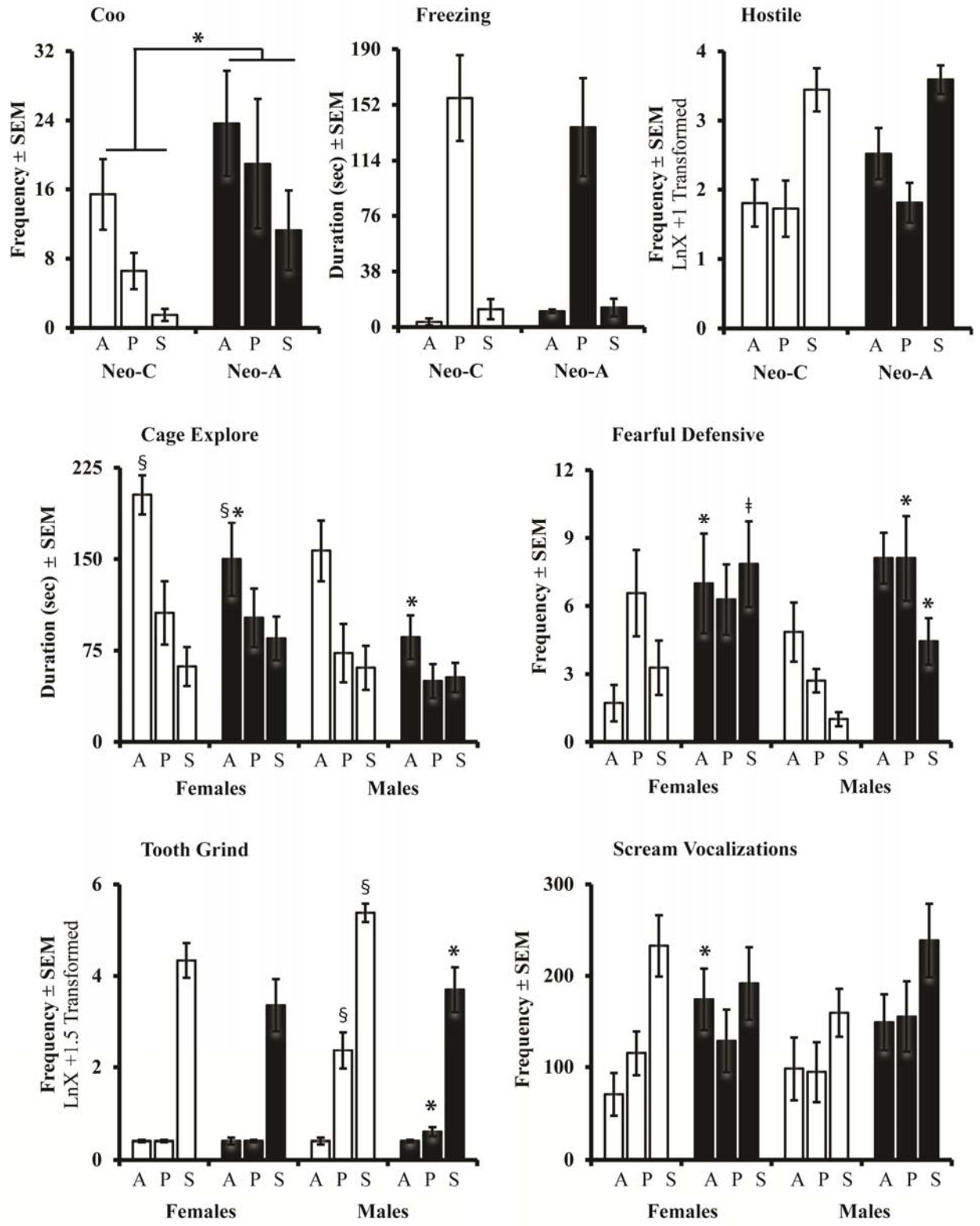

Figure 3. Behavioral responses toward the Novel Fruit Test

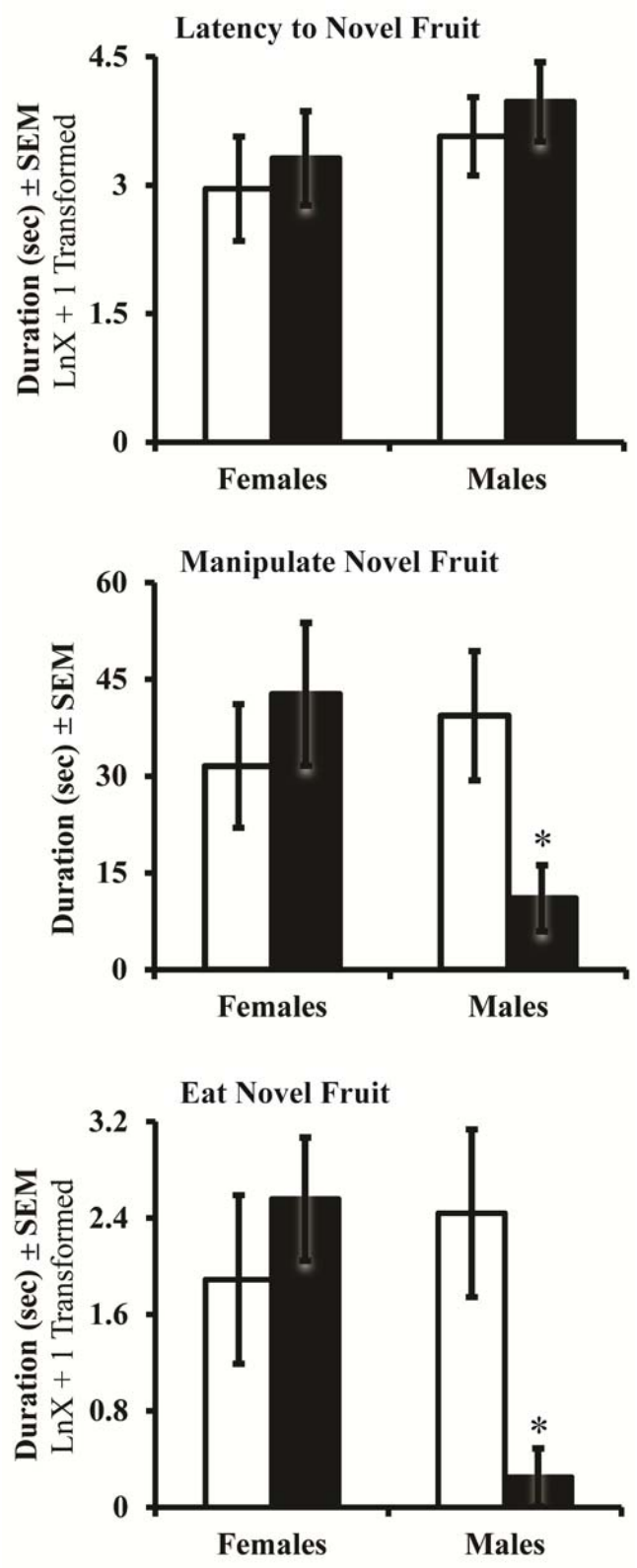

Figure 4. Behavioral responses during the Human Intruder paradigm in Preadolescence

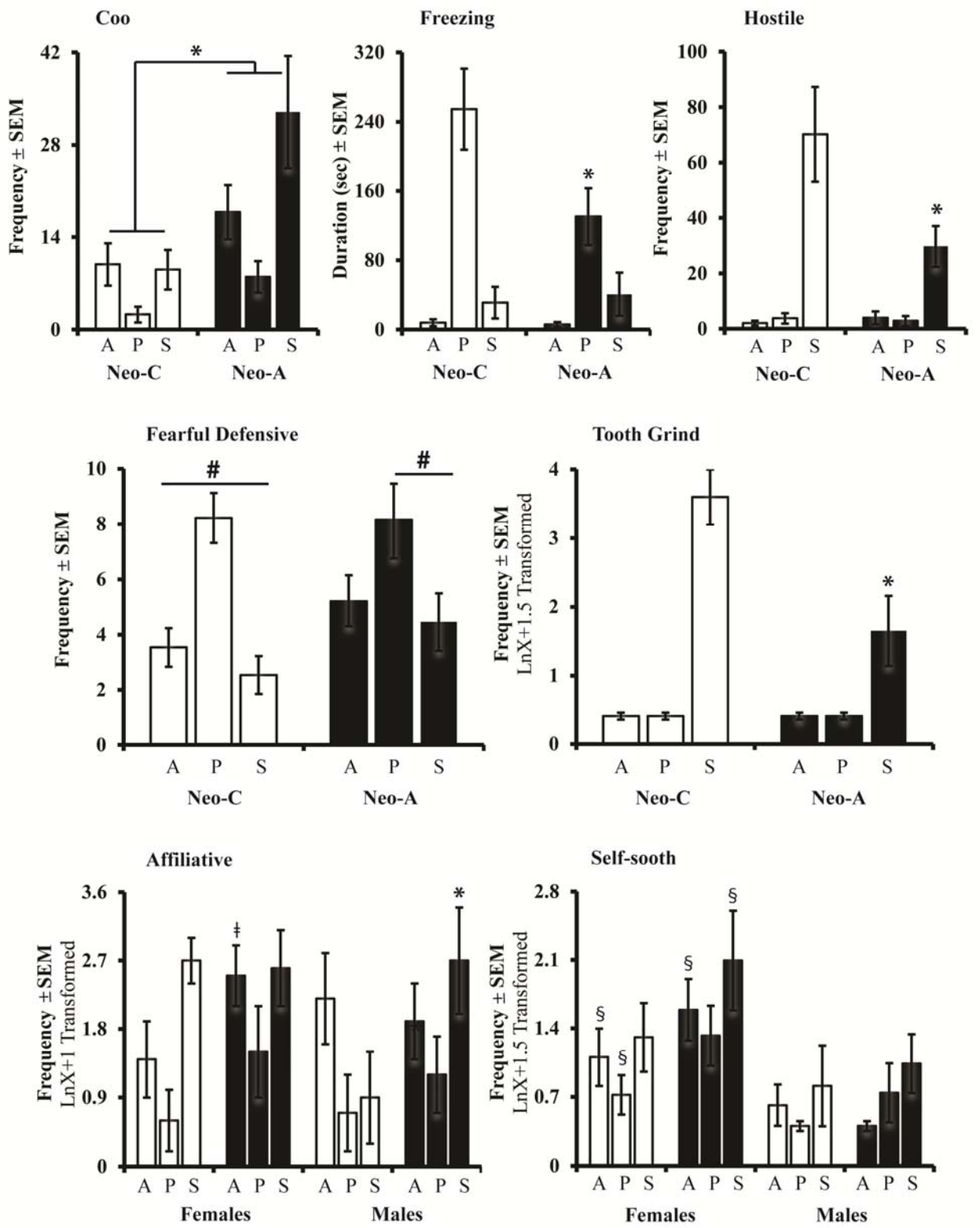

Figure 5. Correlations between testosterone levels and yawning

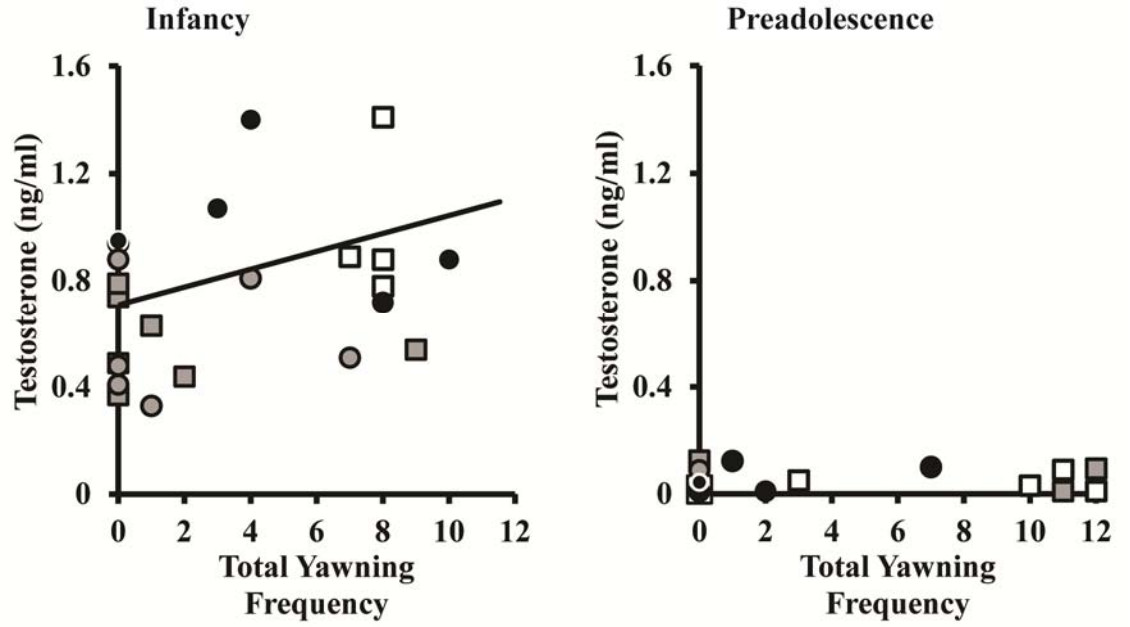

Table 1. Extent of damage after neurotoxic amygdala lesions

Male	Amygdala Damage				Female	Amygdala Damage			
Subjects	Rt%	Lf%	X%	W%	Subjects	Rt%	Lf%	X%	W%
Neo-A-M1	100	80.6	90.3	80.6	Neo-A-F1	82.3	0.0	41.2	0.0
Neo-A-M2	66.8	89.1	77.9	59.5	Neo-A-F2	65.7	98.7	82.2	64.8
Neo-A-M3	70.3	90.8	80.6	63.9	Neo-A-F3	100	32.2	66.1	32.2
Neo-A-M4	50.5	84.9	67.7	42.8	Neo-A-F4	90.9	89.3	90.1	81.1
Neo-A-M5	95.9	97.1	96.5	93.1	Neo-A-F5	61.6	58.4	60.0	36.0
Neo-A-M6	77.3	92.3	84.8	71.3	Neo-A-F6	100	97.7	98.8	97.7
Neo-A-M7	90.9	98.9	94.9	90.0	Neo-A-F7	98.3	99.0	98.6	97.3
Neo-A-M8	100	87.0	93.5	87.0					
Neo-A-M9	61.8	93.2	77.5	57.6					
Mean	**79.3**	**90.4**	**84.9**	**71.8**	**Mean**	**85.5**	**67.9**	**76.7**	**58.4**

L%: percent damage in the left hemisphere; R%: percent damage in the right hemisphere; X%: average damage to both hemispheres; W%: weighted average damage to both hemispheres (W%=(L%×R%)/100). Neo-A-F: female amygdala lesion subject, Neo-A-M: male amygdala lesion subject.

Table 2. Behavioral ethogram

Category and specific behavior	Measurement	Brief Definition
Fearful Defensive Behaviors	Cumulative Frequency	
Freeze	frequency[a]	Rigid, tense, motionless posture except slight head movement
Crouch	frequency[a]	Whole body or just front limbs bent with head near floor
Withdrawal	frequency	Quick, jerky motion away from intruder (jump back)
Fear Grimace	frequency	Refracted lips, exposed clenched teeth (exaggerated grin)
Hostile Defensive Behaviors	Cumulative Frequency	
Threat Bark Vocalization	frequency	Low pitch, high intensity, rasping, guttural
Threat (facial expression)	frequency	Any of the following: open mouth (no teeth exposed), head-bobbing, or ear flapping
Cage Aggression	frequency	Vigorously slaps, shakes or slams body against cage
Lunge	frequency	A quick, jerky movement toward the intruder
Anxious Behaviors	Cumulative Frequency	
Scratch	frequency	Rapid scratching of body with hands or feet
Body Shake	frequency	Whole body or just head and shoulder region shakes
Tooth Grind	frequency[a]	Repetitive, audible rubbing of upper & lower teeth
Yawn	frequency	Open mouth widely, exposing teeth
Stereotypies	Cumulative Duration	
Pacing	duration	Repetitive motor pattern around the test cage
Motor stereotypy	duration	Repetitive, abnormal voluntary or involuntary motor patterns (swinging, twirling, floating limb)
Self-directed	duration	Sucking thumb, eye poke, self-bite
Affiliative Behaviors	Cumulative Frequency	
Coo Vocalization	frequency	Clear soft, moderate in pitch and intensity, usually "oooooh" sounding
Grunt Vocalization	frequency	Deep, muffled, low intensity, almost gurgling sound
Lipsmack	frequency	Rapid movement of pursed lips, accompanied by a smacking sound
Present	frequency	Rigid posture (knees locked) with tail elevated and rump oriented toward intruder
Scream Vocalization	Frequency	High pitch, high intensity screech or loud chirp
Cage Explore	Duration	Calm and inquisitive inspections of cage either by tactile, oral, or visual means
Self-Sooth	Cumulative Duration	
Self-grooming	Duration	Use of hands or mouth to smooth or pick through fur
Self-clasping	Duration	Non-manipulatory enclosing or holding of a limb or body part with arms

List of all behaviors scored, how they are measured and a brief definitions. a Behavior for which total duration was also measured

Table 3. Human intruder behavioral responses during infancy and preadolescence

Behavior	Infancy	Preadolescence
Fearful defensive		
Condition:	$F_{(2,52)}$=2.58, p=0.08, η^2=.09	**$F_{(2,44)}$=16.11, p<0.001, η^2=.42**
Group:	$F_{(1,26)}$=10.61, p=0.003, η^2=.29	$F_{(1,22)}$=2.05, p=0.16, η^2=.09
Sex:	$F_{(1,26)}$=0.27, p=0.61, η^2=.01	$F_{(1,22)}$=2.72, p=0.11, η^2=.11
Group X Sex:	$F_{(1,26)}$=0.14, p=0.71, η^2=.006	$F_{(1,22)}$=0.23, p=0.64, η^2=.01
Condition X Group:	$F_{(2,52)}$=0.66, p=0.52, η^2=.02	$F_{(2,44)}$=0.64, p=0.53, η^2=.03
Condition X Sex:	$F_{(2,52)}$=4.88, p=0.011, η^2=.16	$F_{(2,44)}$=0.89, p=0.41, η^2=.04
Condition X Group X Sex	**$F_{(2,52)}$=3.4, p=0.04, η^2=.12**	$F_{(2,44)}$=1.53, p=0.23, η^2=.06
Freezing		
Condition:	**$F_{(2,52)}$=35.8, p<0.001, η^2=.58**	$F_{(2,44)}$=32.39, p<0.001, η^2=.59
Group:	$F_{(1,26)}$=0.04, p=0.84, η^2=.002	$F_{(1,22)}$=2.64, p=0.12, η^2=.11
Sex:	$F_{(1,26)}$=0.03, p=0.85, η^2=.001	$F_{(1,22)}$=1.99, p=0.17, η^2=.08
Group X Sex:	$F_{(1,26)}$=0.37, p=0.55, η^2=.01	$F_{(1,22)}$=0.01, p=0.92, η^2=.001
Condition X Group:	$F_{(2,52)}$=0.29, p=0.75, η^2=.011	**$F_{(2,44)}$=4.51, p=0.017, η^2=.17**
Condition X Sex:	$F_{(2,52)}$=0.02, p=0.97, η^2=.001	$F_{(2,44)}$=0.47, p=0.62, η^2=.02
Condition X Group X Sex:	$F_{(2,52)}$=0.02, p=0.98, η^2=.001	$F_{(2,44)}$=0.05, p=0.95, η^2=.002
Hostile defensive		
Condition:	**$F_{(2,52)}$=23.4, p<0.001, η^2=.47**	$F_{(2,44)}$=26.39, p<0.001, η^2=.55
Group:	$F_{(1,26)}$=0.75, p=0.39, η^2=.03	$F_{(1,22)}$=3.79, p=0.06, η^2=.15
Sex:	$F_{(1,26)}$=0.02, p=0.90, η^2=.001	$F_{(1,22)}$=1.92, p=0.18, η^2=.08
Group X Sex:	$F_{(1,26)}$=0.69, p=0.42, η^2=.03	$F_{(1,22)}$=1.09, p=0.31, η^2=.05
Condition X Group:	$F_{(2,52)}$=0.84, p=0.44, η^2=.03	**$F_{(2,44)}$=4.83, p=0.013, η^2=.18**
Condition X Sex:	$F_{(2,52)}$=1.23, p=0.30, η^2=.05	$F_{(2,44)}$=1.89, p=0.16, η^2=.08
Condition X Group X Sex:	$F_{(2,52)}$=0.38, p=0.69, η^2=.01	$F_{(2,44)}$=2.57, p=0.09, η^2=.10
Scream		
Condition:	$F_{(2,52)}$=24.62, p<0.001, η^2=.49	**$F_{(2,44)}$=8.14, p=0.001, η^2=.27**
Group:	$F_{(1,26)}$=2.15, p=0.15, η^2=.08	$F_{(1,22)}$=0.01, p=0.93, η^2=.001
Sex:	$F_{(1,26)}$=0.01, p=0.92, η^2=.001	$F_{(1,22)}$=0.01, p=0.94, η^2=.001
Group X Sex:	$F_{(1,26)}$=0.39, p=0.54, η^2=.01	$F_{(1,22)}$=0.08, p=0.78, η^2=.004
Condition X Group:	$F_{(2,52)}$=2.43, p=0.09, η^2=.08	$F_{(2,44)}$=1.14, p=0.33, η^2=.05
Condition X Sex:	$F_{(2,52)}$=0.214, p=0.81, η^2=.008	$F_{(2,44)}$=0.25, p=0.78, η^2=.01
Condition X Group X Sex	**$F_{(2,52)}$=5.2, p=0.009, η^2=.17**	$F_{(2,44)}$=0.95, p=0.39, η^2=.04
Stereotypy		
Condition:	$F_{(2,52)}$=2.67, p=0.08, η^2=.09	**$F_{(2,44)}$=6.75, p=0.003, η^2=.24**
Group:	$F_{(1,26)}$=0.37, p=0.55, η^2=.01	$F_{(1,22)}$=1.30, p=0.27, η^2=.06
Sex:	$F_{(1,26)}$=0.45, p=0.51, η^2=.02	$F_{(1,22)}$=0.13, p=0.72, η^2=.006
Group X Sex:	$F_{(1,26)}$=0.95, p=0.34, η^2=.03	$F_{(1,22)}$=0.49, p=0.48, η^2=.02
Condition X Group:	$F_{(2,52)}$=2.30, p=0.11, η^2=.08	$F_{(2,44)}$=1.05, p=0.36, η^2=.05
Condition X Sex:	$F_{(2,52)}$=0.16, p=0.85, η^2=.006	$F_{(2,44)}$=0.09, p=0.91, η^2=.004
Condition X Group X Sex:	$F_{(2,52)}$=0.31, p=0.74, η^2=.01	$F_{(2,44)}$=0.92, p=0.41, η^2=.04
Cage Explore		
Condition:	$F_{(2,52)}$=23.9, p<0.001, η^2=.48	**$F_{(2,44)}$=6.86, p=0.003, η^2=.24**
Group:	$F_{(1,26)}$=3.9, p=0.05, η^2=.13	$F_{(1,22)}$=1.46, p=0.24, η^2=.06
Sex:	**$F_{(1,26)}$=5.1, p=0.032, η^2=.17**	$F_{(1,22)}$=0.98, p=0.33, η^2=.04
Group X Sex:	$F_{(1,26)}$=0.16, p=0.69, η^2=.006	$F_{(1,22)}$=0.06, p=0.81, η^2=.003
Condition X Group:	**$F_{(2,52)}$=3.9, p=0.03, η^2=.13**	$F_{(2,44)}$=1.79, p=0.18, η^2=.08
Condition X Sex:	$F_{(2,52)}$=1.18, p=0.32, η^2=.04	$F_{(2,44)}$=0.27, p=0.76, η^2=.01
Condition X Group X Sex:	$F_{(2,52)}$=0.04, p=0.96, η^2=.001	$F_{(2,44)}$=0.15, p=0.86, η^2=.007

Table 3. (continued)

Behavior	Infancy	Preadolescence
Anxious		
Condition:	**$F_{(2,52)}$=84.7, p<0.001, η^2=.77**	**$F_{(2,44)}$=34.7, p<0.001, η^2=.61**
Group:	$F_{(1,26)}$=4.68, p=0.04, η^2=.15	$F_{(1,22)}$=2.72, p=0.11, η^2=.11
Sex:	$F_{(1,26)}$=7.42, p=0.011, η^2=.22	$F_{(1,22)}$=0.83, p=0.37, η^2=.04
Group X Sex:	**$F_{(1,26)}$=5.0, p=0.034, η^2=.16**	$F_{(1,22)}$=0.46, p=0.51, η^2=.02
Condition X Group:	$F_{(2,52)}$=1.19, p=0.31, η^2=.04	$F_{(2,44)}$=1.66, p=0.20, η^2=.07
Condition X Sex:	$F_{(2,52)}$=1.77, p=0.18, η^2=.06	$F_{(2,44)}$=0.67, p=0.94, η^2=.003
Condition X Group X Sex:	$F_{(2,52)}$=0.80, p=0.46, η^2=.03	$F_{(2,44)}$=1.12, p=0.34, η^2=.05
Tooth Grind		
Condition:	**$F_{(2,52)}$=106, p<0.001, η^2=.80**	**$F_{(2,44)}$=42.2, p<0.001, η^2=.66**
Group:	$F_{(1,26)}$=9.59, p=0.005, η^2=.27	**$F_{(1,22)}$=8.14, p=0.009, η^2=.27**
Sex:	$F_{(1,26)}$=5.8, p=0.023, η^2=.18	$F_{(1,22)}$=0.05, p=0.82, η^2=.002
Group X Sex:	**$F_{(1,26)}$=4.2, p=0.05, η^2=.14**	$F_{(1,22)}$=0.51, p=0.48, η^2=.02
Condition X Group:	$F_{(2,52)}$=2.36, p=0.11, η^2=.08	$F_{(2,44)}$=8.14, p=0.001, η^2=.27
Condition X Sex:	$F_{(2,52)}$=1.75, p=0.18, η^2=.06	$F_{(2,44)}$=0.05, p=0.95, η^2=.002
Condition X Group X Sex:	$F_{(2,52)}$=1.22, p=0.30, η^2=.04	$F_{(2,44)}$=0.51, p=0.60, η^2=.02
Yawns		
Condition:	**$F_{(2,52)}$=15.5, p<0.001, η^2=.37**	**$F_{(2,44)}$=15.3, p<0.001, η^2=.41**
Group:	$F_{(1,26)}$=0.001, p=0.99, η^2=.001	$F_{(1,22)}$=0.90, p=0.35, η^2=.04
Sex:	**$F_{(1,26)}$=4.1, p=0.05, η^2=.14**	$F_{(1,22)}$=0.001, p=0.98, η^2=.001
Group X Sex:	$F_{(1,26)}$=1.75, p=0.19, η^2=.06	$F_{(1,22)}$=0.74, p=0.39, η^2=.03
Condition X Group:	$F_{(2,52)}$=0.33, p=0.72, η^2=.01	$F_{(2,44)}$=0.90, p=0.41, η^2=.04
Condition X Sex:	$F_{(2,52)}$=1.98, p=0.15, η^2=.07	$F_{(2,44)}$=0.001, p=0.99, η^2=.001
Condition X Group X Sex:	$F_{(2,52)}$=0.60, p=0.55, η^2=.02	$F_{(2,44)}$=0.74, p=0.48, η^2=.03
Self-sooth		
Condition:	**$F_{(2,52)}$=9.01, p<0.001, η^2=.26**	**$F_{(2,44)}$=3.22, p=0.05, η^2=.13**
Group:	$F_{(1,26)}$=2.41, p=0.13, η^2=.09	$F_{(1,22)}$=2.68, p=0.12, η^2=.11
Sex:	$F_{(1,26)}$=0.14, p=0.71, η^2=.005	**$F_{(1,22)}$=9.18, p=0.006, η^2=.29**
Group X Sex:	$F_{(1,26)}$=2.68, p=0.11, η^2=.09	$F_{(1,22)}$=1.25, p=0.28, η^2=.05
Condition X Group:	$F_{(2,52)}$=1.92, p=0.15, η^2=.07	$F_{(2,44)}$=0.47, p=0.63, η^2=.02
Condition X Sex:	**$F_{(2,52)}$=2.88, p=0.06, η^2=.10**	$F_{(2,44)}$=0.48, p=0.62, η^2=.02
Condition X Group X Sex:	$F_{(2,52)}$=0.69, p=0.51, η^2=.02	$F_{(2,44)}$=0.14, p=0.87, η^2=.006
Allifiative		
Condition:	**$F_{(2,52)}$=7.9, p=0.001, η^2=.23**	$F_{(2,44)}$=10.16, p<0.001, η^2=.32
Group:	$F_{(1,26)}$=3.27, p=0.08, η^2=.11	$F_{(1,22)}$=3.11, p=0.09, η^2=.12
Sex:	$F_{(1,26)}$=2.01, p=0.17, η^2=.07	$F_{(1,22)}$=0.47, p=0.50, η^2=.02
Group X Sex:	$F_{(1,26)}$=0.05, p=0.83, η^2=.002	$F_{(1,22)}$=0.001, p=0.98, η^2=.001
Condition X Group:	$F_{(2,52)}$=0.65, p=0.53, η^2=.02	$F_{(2,44)}$=0.36, p=0.69, η^2=.02
Condition X Sex:	$F_{(2,52)}$=0.36, p=0.70, η^2=.01	$F_{(2,44)}$=1.69, p=0.19, η^2=.07
Condition X Group X Sex:	$F_{(2,52)}$=0.28, p=0.76, η^2=.01	**$F_{(2,44)}$=4.29, p=0.02, η^2=.16**
Coo		
Condition:	**$F_{(2,52)}$=4.3, p=0.019, η^2=.14**	**$F_{(2,44)}$=5.87, p=0.006, η^2=.21**
Group:	**$F_{(1,26)}$=3.9, p=0.05, η^2=.13**	**$F_{(1,22)}$=4.43, p=0.047, η^2=.17**
Sex:	$F_{(1,26)}$=0.7, p=0.41, η^2=.03	$F_{(1,22)}$=0.06, p=0.81, η^2=.003
Group X Sex:	$F_{(1,26)}$=0.16, p=0.69, η^2=.006	$F_{(1,22)}$=0.04, p=0.84, η^2=.002
Condition X Group:	$F_{(2,52)}$=0.11, p=0.89, η^2=.004	$F_{(2,44)}$=2.30, p=0.11, η^2=.09
Condition X Sex:	$F_{(2,52)}$=0.54, p=0.59, η^2=.02	$F_{(2,44)}$=0.13, p=0.87, η^2=.006
Condition X Group X Sex:	$F_{(2,52)}$=0.28, p=0.76, η^2=.01	$F_{(2,44)}$=1.81, p=0.18, η^2=.08

Manuscript III. Neonatal amygdala lesions alter normal functioning of the hypothalamic-pituitary-adrenal axis in juvenile rhesus monkeys.

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Abstract

The current study examined the effects of neonatal amygdala (Neo-A) lesions on hypothalamic-pituitary-adrenal (HPA) axis functioning in rhesus monkeys reared with their mothers in large complex social groups. At 12 months of age, stress reactive HPA axis response, diurnal cortisol rhythm, and response to HPA axis challenges were examined. Compared to controls, Neo-A animals exhibited increased reactivity to a stressor as well as increased cortisol secretion across the diurnal rhythm as compared to controls. This hyper-secretion of basal cortisol was associated with reduced HPA response to corticotrophin releasing hormone (CRH) challenge and increased levels of corticotrophin releasing factor (CRF) in cerebrospinal fluid, suggesting that early amygdala damage altered hypothalamic secretions. In addition, unlike controls, Neo-A animals also exhibited insufficient escape from glucocorticoid negative feedback after a dexamethasone suppression test. Taken together these data indicate that early amygdala damage impacts the normal development of the HPA axis, presumably via participation of other neural structures involved in the HPA axis regulation.

Introduction

The ability to recognize and react to potential threats encountered is essential to survival and requires integrated activation of autonomic and endocrine systems to promote rapid changes in behavioral response and redistribution of resources (Herman, et al., 2003, 2005; Myers, et al., 2012). Modulation of both behavioral and physiological responses to potential threats is mediated at least in part by the amygdala, which has an excitatory influence on the hypothalamic-pituitary-adrenal (HPA) axis through both direct projections to the lateral hypothalamus and indirect projections to the paraventricular nucleus (PVN) of the hypothalamus (Price & Amaral, 1981; Amaral, et al., 1992; Freese & Amaral, 2009). The central and basal nuclei of the amygdala also project to the bed nucleus of the stria terminalis, which in turn projects to the PVN providing an indirect pathway by which the amygdala can influence the HPA axis neuroendocrine response to perceived threats (Price & Amaral, 1981; Amaral, et al., 1992; Pitkänen, 2000; Feldman, et al., 1990; 1995; Herman, et al., 2003; 2005). Specifically, in response to a threat, activation of these stressor-specific pathways causes the PVN to release corticotrophin releasing hormone (CRH) into the hypophyseal portal blood system, which is transported to the anterior pituitary to stimulate the release of adrenocorticotrophic hormone (ACTH) into systemic circulation. ACTH then binds to receptors in cells of the adrenal cortex and stimulates the synthesis and release of glucocorticoids, such as cortisol in primates (Herman, et al., 2003, 2005). Previous studies in adult animals have shown that electrical stimulation of the amygdala results in increased cortisol secretion (Mason, 1959; Ehle, et al., 1977), whereas amygdala lesions result in decreased HPA axis response to a stressor (Beaulieu, et al., 1986; Feldman, et al., 1994; Kalin, et al., 2004; Machado & Bachevalier, 2008). Thus, although the role of the amygdala in the reactive stress response has received some support, its potential

contribution to basal cortisol secretion has been less studied.

The HPA axis basal secretory rhythm is essential for the body's homeostasis and normal functioning of other systems in the organism, such as metabolism, growth, and immune function (Munck, Guyre, & Holbrook, 1984). In diurnal animals, this basal HPA axis activity follows a circadian rhythm characterized by a peak of cortisol secretion during the early morning hours (upon awakening), and a decline across the day with a trough at night (during sleep) (Weitzman, et al., 1971; Keller-Wood & Dallman, 1984). An earlier study in rodents (Allen & Allen, 1975) reported that the amygdala was necessary to maintain ACTH secretion after adrenalectomy, suggesting a potential role of the amygdala for tonic (basal) control of the HPA axis in the absence of glucocorticoid regulation. A more recent study showed that CRH knockdown in the CeA of adult mice leads to increased basal corticosterone (Regev, et al., 2012). However, studies in primates with adult-onset amygdala lesions have reported no changes in baseline (pre-stressor) cortisol levels (Sapolsky, et al., 1991; Kalin, et al., 2004; Machado & Bachevalier, 2008), but blood samples in these studies were collected mid-morning or afternoon and could not inform on the role of the amygdala on the basal cortisol secretory rhythm.

Finally, the role of the amygdala on HPA axis functioning has been mostly studied in adult animals in which both the amygdala and hypothalamus are fully mature at the time of experimental manipulations (typically, stimulation or lesion). Thus, little is known about the influence of the amygdala on the HPA axis during development. From the few reports on the effects of amygdala lesions performed either during the juvenile period (Norman & Spies, 1981) or during the neonatal phase (Goursaud, et al., 2006; Raper, et al., 2012a, 2012b), the data are inconsistent. The former study examined cortisol response to dexamethasone suppression and ACTH neuropeptide challenge in infant monkeys with neonatal amygdala lesions (Goursaud, et

al., 2006) and indicated no changes in cortisol secretion between the amygdalectomized animals and controls. However, the lack of effects of amygdala lesions on the HPA axis may have resulted from an absence of a true baseline sample for comparison or from the long delay (16 hours) after which the dexamethasone suppression was examined, which could have missed the peak suppression time. In the most recent study, Raper and colleagues (2012a) showed that neonatal amygdala lesions yielded a flattened diurnal HPA axis rhythm (i.e., lower basal cortisol at "lights on") as well as a blunted cortisol response to a stressor that was associated with altered modulation of emotional reactivity. These more recent results suggest a critical influence of the amygdala not only on the HPA axis reactive stress response but also on basal HPA functioning, although the changes after neonatal amygdalectomy were only examined in adulthood.

Given the paucity of information on the role of the amygdala on the HPA axis during development, we initiated a longitudinal investigation to measure the effects of neonatal amygdala lesions on HPA functioning from 2.5 months to adulthood. In a first report (Raper, et al., 2012b), the effects of neonatal amygdala lesions were examined in infancy and the results demonstrated a flattened diurnal cortisol rhythm as early as five months of age, with neonatally amygdalectomized infants showing higher cortisol at night compared to sham-operated controls. To further examine the effects of neonatal neurotoxic amygdala lesions across development, the present study re-assessed basal and stress reactive HPA axis function of these same animals during the juvenile period. Given our previous findings (Raper, et al., 2012a, 2012b), we predicted that the flattened HPA rhythm observed at 5 months will still be present in pre-adolescent monkeys. In addition, consistent with previous data on amygdala lesions received either in adulthood (Kalin, et al., 2004; Machado & Bachevalier, 2008) or in infancy (Raper, et al., 2012a), we predicted that neonatal amygdalectomized juvenile monkeys will exhibit a

blunted cortisol response to psychological stressors. Lastly, we predicted that neonatal amygdalaectomy would alter the normal functioning of the HPA axis at the feed-forward loop examined by CRH challenge and the escape from glucocorticoid negative feedback after dexamethasone suppression.

Methods

Subjects

All animals were raised by middle-ranking mothers and lived in large social groups (38 X 39 m outdoor compounds) at the Yerkes National Primate Research Center (YNPRC) Field Station (Lawrenceville, GA), Emory University. From the 30 juvenile rhesus monkeys (*Macaca mulatta*) that were included in the original longitudinal developmental study (Raper, et al., 2012b, 2012c), only 26 participated in Experiments 1 and 2 below. At 24.8 ± 1.2 days of age, 13 animals (7 males, 6 females) had received neonatal neurotoxic lesions of the amygdala (Neo-A) and 13 animals (4 males, 5 females) had received sham operations (Neo-C). In addition, three animals (2 males, 1 female) had received the same experimental manipulation given to the other animals except the neuroimaging and surgical procedures and served as behavioral controls (Neo-BC). All neuroimaging and surgical procedures were performed at the YNPRC Main Station (Atlanta, GA). In the current study, their neuroendocrine reactivity to a Human Intruder was assessed at 12 months of age. Procedures for neuroimaging, surgical, estimation of lesion extent as well as reunions with the mother after surgery have been described in detail earlier (Raper, et al., 2012b submitted) and will be briefly summarized below. The Animal Care and Use Committee of Emory University has approved all procedures, which were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory

Animals.

Imaging and surgical procedures:

Magnetic Resonance Imaging (MRI) Procedure: On the day of surgery, the infant was removed from their mother, sedated (Ketamine hydrochloride, 100mg/ml), intubated, and anesthetized with isoflurane (1-2% to effect). Their head was shaved and secured in a nonferromagnetic stereotaxic apparatus, an intravenous drip of 0.45% dextrose and sodium chloride was placed to maintain normal hydration and vital signs (heart rate, respirations, blood pressure, expired CO2) were monitored throughout the procedures. Two MRI sequences were acquired using a Siemens 3.0T/90 cm whole body scanner and a 3" circular surface coil. A high resolution T1-weighted scan (spin-echo sequence, echo time [TE] = 11ms, repetition time [TR] = 450ms, contiguous 1mm section, 12cm field of view [FOV], 256x256 matrix) obtained in the coronal plane was used to determine the coordinates of injection sites in the amygdala. Three additionally fluid attenuated inversion recovery (FLAIR) scans (3D T2-weighted fast spoiled gradient [FSPGR]-echo sequence, TE = 2.6ms, TR = 10.2ms, 25 flip angle, 12 cm FOV, 256x256 matrix) were obtained in the coronal plane at 3.0 mm (each offset of 1 mm posteriorly) throughout the brain. These two MR sequences were then repeated 7-10 days after the surgical procedure for Neo-A animals, whereas Neo-C animals were just separated from their mothers and placed in an isolette incubator for the same amount of time.

Surgical Procedure: After the pre-surgical imaging session, the anesthetized infants were immediately brought to the surgical suite where they were prepared for aseptic surgical procedures. Nolvasan Solution was used to disinfect the scalp and a local anesthetic

(Bupivicaine 0.25% concentration, 1.5ml) was injected subcutaneously along the midline to reduce the pain during skin incision. After the skin and underlying connective tissue were gently displaced laterally, two small craniotomies were made in front of bregma and above the amygdala, and the dura was cut and retracted to expose the brain.

Animals in group Neo-A, injections of ibotenic acid (PH 7.8-7.9, 10 mg/ml concentration) were made in 6-8 sites within the center of the amygdala using 10µl Hamilton syringes. Needles were lowered simultaneously in both hemispheres and a total of 0.6-0.8 µl of ibotenic acid was manually injected at a rate of 0.2µl/minute. After each injection, 3-minute waiting period was allotted to minimize neurotoxin spread during needle retractions.

At the completion of the injections, the dura was closed with silk sutures, the bone opening was covered with Surgicel NU-KNIT (absorbable hemostat), and connective tissues and skin were closed. The animal was removed from anesthesia, and placed in a temperature controlled incubator ventilated with oxygen until full recovery from anesthesia. All animals received banamine (1mg/kg for 3 days), dexamethasone (0.5mg/kg for 3 days) and antibiotic (rocephin, 25mg/kg for 7 days) after surgery to prevent pain, edema, and infection, respectively.

The sham-operated controls (Neo-C) received the same surgical procedures except no needles were lowered and no injections were given. In addition, the behavioral controls (Neo-BC) were separated from their mother and sedated (Ketamine hydrochloride, 100mg/ml). Their head was shaved and scalp was disinfected with Nolvasan Solution, but no surgery was performed. They were also given the same "post-surgery" medication treatments.

Mother-infant Reunions and Cross-fostering: The day after surgery, after ensuring that the infant was alert, it was returned to the mother and monitored constantly via a secured internet

web camera. Most mothers immediately retrieved their infants and the infants were observed nursing on the mother that same day. In a few cases, however, infants demonstrated difficulty nursing during the first reunion and the mother-infant pairs had to be separated overnight for a few days to monitor nutrition until the infant was able to nurse on the mother adequately.

In three cases, the mother-infant reunions were unsuccessful despite repeated attempts. However, one amygdalectomized male, was successfully cross-fostered to a mother that had recently lost her infant. In the remaining two cases (Neo-A female and Neo-C male), the "abandoned" infant was nursed and raised by another adult female who already had an infant that birth season. Close monitoring of these two cases of 'twin' mother-infant pairs revealed that both infants were growing normally and remained healthy.

Lesion Verification: Estimation of the extent of intended and unintended damage for Neo-A animals was made using pre- and post-surgical MR images (Malkova, et al, 2001; Nemanic, et al, 2002). The high-resolution T1 images were used to help identify the borders of each structure and lower resolution FLAIR images were used to identify extent of hypersignals which were then plotted onto corresponding coronal drawings from a normalized infant rhesus monkey brain (J. Bachevalier, unpublished atlas) using Adobe Photoshop software. Drawings were imported into image analysis program Image-J® (version 1.44) to measure the surface area (in pixels squared) containing hypersignals in the amygdala and in all surrounding structures (entorhinal and perirhinal cortex and hippocampus). Surface areas measured by the image thickness (i.e., 1mm) to obtain an estimate of volume, and the volume of damage for each structure was then divided by the normal volume of that structure (obtained from the template brain in the same manner) and multiplied by 100 to estimate a percentage of the total damage volume.

Lesion Extent

Estimated bilateral damage to the amygdala, averaged 80.3 % across both hemispheres (see Table 1), but varied from case to case. Thus, substantial bilateral damage to the amygdala in both hemispheres was found in 9 cases, averaging 87.72% and 92.09% on the right and left, respectively. Three other cases had either moderate bilateral damage (Neo-A-F5: right: 61.6%, left: 58.4%) or more asymmetrical amygdala damage (Neo-A-F3: right 100%, left 32.2%; Neo-A-M4: right 50.5%, left 84.9%). Lastly, in one case (Neo-A-F1), the damage was only unilateral (right: 82.3%, left 0%). Unintended damage to surrounding structures was negligible in 13 cases, and only two cases had moderate damage to the right entorhinal cortex (Neo-A-F1: 18.3%; Neo-A-F3: 21.8%), whereas two others had moderate damage to the tail of the putamen (Neo-A-F5 and Neo-A-F6). Figure 1 illustrates the extent of bilateral amygdala damage in animal Neo-A-F6 as reflected by the location and extent of hypersignals seen in the post-surgical FLAIR images.

Experiment 1

A human intruder was used as a threatening stimulus in a paradigm that has previously shown significant activation of the HPA axis reactive stress response as revealed by rapid increases in ACTH and cortisol in blood following the presence of the intruder (Kalin, et al., 1991b; Jahn, et al., 2010; Raper, et al., 2012a). In an earlier report, we described the impact of neonatal amygdala lesion on the behavioral reactions of the juvenile monkeys toward the human intruder (Raper, et al., 2012c). This study will focus on investigating the neuroendocrine stress response of the same animals after being confronted with the human intruder.

Human Intruder Paradigm and Blood collection

Experimental procedures were performed at sunrise prior to subjects being fed to avoid meal-induced HPA axis activation and to minimize arousal occurring during routine animal husbandry procedures in the early morning. Animals' were trained for quick capture from the social group and to present a leg for awake (unanesthetized) blood drawing procedures to minimize arousal (see Sanchez, et al., 2010; Raper, et al., 2012a). After collecting a "pre-stressor" blood sample immediately after capture and within 10 minutes of initial disturbance (when the experimenters first enter the social group), the animal was transported to a testing room away from their social group, and then transferred to a stainless steel cage (65 cm X 65 cm x 150 cm) with one side made of clear plexiglass for video recording. The human intruder paradigm lasted 30 minutes and consisted of three conditions (Alone, No Eye Contact, Stare) presented in this order for all animals (for details see Raper, et al., 2012b). Immediately following the end of the test, an additional blood sample (post-stressor) was collected to examine HPA axis stress reactive response. All blood samples were collected in pre-chilled 2 ml vacutainer tubes containing EDTA (3.6 mg) and immediately placed on ice. Samples were centrifuged at 3,000 rpm for 15 minutes in a refrigerated centrifuge (4°C) and plasma samples were stored at -80°C until assayed.

Plasma Hormonal Assays:

All assays were performed by the YNPRC Biomarker Core Laboratory. Plasma samples during the Human Intruder task and on baseline days were assayed for both ACTH and cortisol. Plasma concentrations of ACTH were assayed in duplicate by radioimmunoassay (RIA) using commercially available kits (DiaSorin, Inc., Stillwater, MN). The sensitivity of the DiaSorin

assay was 7.10 pg/ml and intra- and inter-assay coefficients of variation in each assay were < 9.2%. Plasma concentrations of cortisol were assayed using liquid chromatography – mass spectroscopy (LC-MS). LC-MS analyses were performed via reverse phase chromatography on an LTQ-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). Quantitation was achieved using a deuterated cortisol internal standard (CDN Isotopes, Cortisol-9,11,-12,12-d4). The assay range was 2.5-60 ug/dl with intra- and inter-assay coefficients of variation < 8%.

Data Analysis

Because the control group was made of sham-operated controls (Neo-C) and behavioral controls (Neo-BC), we first conducted a preliminary statistical analysis to ensure that the two control groups did not differ in any neuroendocrine measures taken in the present study. All group comparisons were non-significant, and all control animals were combined into a signle control group (Neo-C) for all subsequent analyses. Additionally, three females had to be dropped from hormonal analyses due to illness or injury at the time of testing (one Neo-C female, two Neo-A females), reducing the sample size to 23 subjects for the final analysis (Neo-C: males = 6, females = 6; Neo-A: males = 7, females = 4).

A preliminary analysis was performed to ascertain if the time from disturbance until collection of the blood sample had any influence on pre-stress cortisol levels. Two Hierarchical Linear Model (HLM) Regression analyses were performed on pre-stress cortisol and ACTH levels separately. The amount of time it took to collect the blood sample could explain a significant portion of the variance in pre-stress cortisol levels ($R^2 = 0.20$, $F(1,20) = 3.57$, $p = 0.047$), but not in pre-stress ACTH levels ($R^2 = 0.10$, $F(1,20) = 1.08$, $p = 0.36$), thus this variable of time to collect the sample was used as a covariate in subsequent analyses of group differences

for cortisol levels only.

Changes in cortisol and ACTH levels were assessed using repeated measures ANOVA with Group (2) and Sex as between subjects factor and Time (pre- vs post-stressor) as the within subjects repeated measures. Post hoc analyses to further examine interactions effects were performed with One-way ANOVA's. Lastly, the relationship between the extent of amygdala lesion and hormone levels were examined with Partial correlations to correct for the amount of time it took to collect the blood sample.

Results

HPA axis stress reactivity after neonatal amygdala lesion:

At one year of age, neonatal amygdalectomy produced profound changes in neuroendocrine stress response. Thus, as shown in Figure 2a, Neo-A animals exhibited greater cortisol levels during the post-stress as compared to Neo-C animals (Group: $F[1,18] = 7.79$, $p = 0.012$, $\eta^2 = 0.30$). There was also a significant Group X Sex X Time interaction for cortisol levels ($F[1,18] = 4.31$, $p = 0.05$, $\eta^2 = 0.19$), indicating that females with neonatal amygdalectomies had the highest cortisol levels after exposure to the human intruder (see Figure 2a). By contrast, both groups demonstrated significant increase in ACTH level from pre- to post-stressor (Time: $F[1,19] = 13.28$, $p = 0.002$, $\eta^2 = 0.41$), but there was no significant effects of Group or Sex ($F[1,19] = 0.59$, $p = 0.45$, $\eta^2 = 0.03$; $F[1,21] = 0.20$, $p = 0.65$, $\eta^2 = 0.01$, respectively). There were no significant correlations between the extent of amygdala lesion and hormone levels.

Experiment 2

Results of Experiment 1 demonstrate that neonatal amygdalectomy results in an increased cortisol stress response to a threatening stimulus in juvenile monkeys. In addition, although both males and females with neonatal amygdala lesions exhibited higher cortisol levels during the post-stressor as compared to their same sex controls, the Neo-A females exhibited the highest cortisol response to the stressor. To investigate whether the effects of early amygdala lesions on stress reactivity is the result of alterations to the HPA axis, diurnal cortisol rhythm as well as pharmacological challenges were used to examine the basic functioning of different levels of the HPA axis.

Neuropeptide Challenges and CSF collection

From the 26 animals participating in this study, six could not be included in Experiment 2 due to illnesses or injuries reducing the sample size to 20 animals (Neo-C: males = 5, females = 5; Neo-A: males = 6, females = 4). All animals received intravenous neuropeptide challenges consisting of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) or a saline vehicle injections. Animals were accessed at sunrise prior to feeding or other routine care procedures to avoid both meal-induced or arousal HPA axis activation. Since the animals live outdoors under natural lighting conditions, sunrise times were obtained from the US Naval Observatory (http://aa.usno.navy.mil/data/docs/RS_OneYear.php) and used as daylight time instead of clock time. Animals were separated from their social group according to the procedures outlined in Experiment 1, and immediately anesthetized with an intramuscular injection of Telazol (5mg/kg) to minimize the stress associated with repeated handling and blood sampling after the drug injection. The response to neuropeptide challenges was assessed while

the animals were under anesthesia to minimize the stress associated with repeated handling and blood sampling after the drug injection. A basal blood sample was taken (0 minute) by saphenous venipuncture, prior to anesthesia injection, to assess pre-challenge cortisol and ACTH levels. An intravenous bolus of r/h CRH (50ug/kg), r/h ACTH (ug/kg) or a vehicle solution (10mM acetic acid/sterile 0.9% saline) was administered into the saphenous vein of the animal, and blood samples were collected from the femoral vein at 15, 30, and 45 minutes after the injection. These intravenous neuropeptide challenges were done according to published protocols (Sanchez, et al., 2005) and interval between drug treatments were a minimum of one week. Administration of a saline vehicle solution was used to control for the effects of anesthesia and blood drawing procedures on the hormonal variables of interest.

A cerebral spinal fluid (CSF) sample was collected, immediately prior to the administration of neuropeptides or saline, but only one CSF sample was collected per subject before one of the three neuropeptide challenges. The CSF sample was obtained by cisternal tap within 15 minutes of initial disturbance in the anesthetized animal.

Diurnal cortisol rhythm and Dexamethasone Suppression Test

For the diurnal rhythm, blood samples were collected at sunrise, mid-day and sunset according to the sun times obtained from the US Naval Observatory. All blood samples were collected by quickly separating subjects from their social group according to the procedures outlined in Experiment 1. After the sunset blood sample collection and 10 hours before sunrise, subjects were given an intramuscular injection of dexamethasone sodium phosphate (0.25 mg/kg). The following day after the dexamethasone injection, blood samples were collected at sunrise to examine the suppression of the morning cortisol rise and then at mid-day to examine

the escape from suppression. The sunrise and mid-day samples collected after dexamethasone suppression were compared to the diurnal cortisol samples collected at the same time of day.

Hormonal Assays: All plasma assays were performed by the YNPRC Biomarker Core Laboratory. Plasma samples for neuropeptide challenges, diurnal rhythm, and dexamethasone suppression were assayed for cortisol. Plasma concentrations of cortisol were assayed in duplicate by RIA using commercially available kits (DSL kit: Diagnostic Systems Laboratories, Webster, TX). The sensitivity of the DSL assay was 1.25µg/dl and intra- and inter-assay coefficients of variation in each assay were < 10%.

Assay for corticotropin releasing factor (CRF) in CSF samples was conducted by Dr. Patrick Roseboom at the University of Wisconsin. CRH was measured in cerebrospinal fluid using an antibody (rC68 – 5/31/83 bleed) generously provided by Wylie Vale (Salk Institute for Biological Studies, La Jolla, CA). Briefly the CSF was incubated for 72 hours in the presence of the antibody, and this was followed by a 48 hour incubation with [^{125}I-Tyr^{0}]r,hCRH (Perkin-Elmer, Downers Grove, IL). The final dilution of the antibody was 1:1,000,000. Following the addition of normal rabbit serum (Bachem, Torrence, CA) as a carrier, the antigen-antibody complex was pelleted using goat anti-rabbit gamma globin (Bachem) at 1,700 x g for 20 min. The supernatant was removed by aspiration and the radioactivity remaining in the pellet was counted in a gamma counter (Perkin-Elmer). The amount of CRH in the sample was determined using a standard curve ranging from 3.91 pg/ml to 1000 pg/ml of r,hCRH (Sigma-Aldrich, St. Louis, MO). The limit of detection was 0.4 pg, and the majority of samples were assayed in triplicate with the exception that three samples were assayed in duplicate as a result of low sample volume. All samples in the current study were assessed for CRH in a single assay run.

Data Analysis

Due to illness at the start of this experiment six animals were unable to undergo neuropeptide challenge or dexamethasone suppression testing. Additionally, one Neo-C female was excluded from neuropeptide challenge analyses due to illness unrelated to the experiment, but this female was able to be included in the diurnal and dexamethasone suppression tests. Another Neo-C female was excluded from the CRH challenge analyses because she did not receive the full injection of the neuropeptide, but this female was included in ACTH challenge, dexamethasone, and diurnal analyses. Therefore, 20 subjects were included in the final analyses for diurnal and dexamethasone suppression (Neo-C: males = 5, females = 6; Neo-A: males = 6, females = 3), and only 18 subjects were included in the final analyses of neuropeptide challenges (Neo-C: males = 5, females = 4; Neo-A: males = 6, females = 3).

Area under the curve with respect to increase (AUCi) calculates the change in hormones with respect to the baseline value, thereby indexing change beyond baseline over time (0/baseline, 15, 30, 45 minutes post-injection; Pruessner, et al., 2003). Therefore, AUCi was calculated for cortisol and ACTH levels response to neuropeptide challenges. Mix-model design ANOVA with Drug (Saline vehicle vs ACTH or CRH), Group (Neo-C, Neo-A), and Sex as between subjects factors were used to analyze the AUCi for cortisol and ACTH.

To ascertain whether the time from disturbance until collection of the blood sample had any influence on basal cortisol levels, Hierarchical Linear Model (HLM) Regression analyses were performed on basal cortisol levels. Variance in basal cortisol level was not significantly explained by the amount of time it took to collect the sample ($R^2 = 0.15$, $F(1,17) = 1.59$, $p = 0.23$), therefore time to collect the sample was not covaried in subsequent analyses of group differences in diurnal cortisol. However, a significant amount of variance in cortisol was

accounted for in samples collected after dexamethasone suppression ($R^2 = 0.35$, $F(1,17) = 6.33$, $p = 0.009$), in this case time to collect the sample was used as a covariate in subsequent analyses of group differences in dexamethasone suppression.

The diurnal cortisol rhythm was assessed using a repeated measures ANOVA with Group (2) and Sex as between subjects factors and Time (sunrise, mid-day, sunset) as the within subjects factor with repeated measures and the "time from initial disturbance" as covariate. The dexamethasone suppression test was assessed with mixed-model ANOVA with Group (2), Drug (diurnal/non drug, dexamethasone), and Sex as between subjects factors and Time (sunrise, mid-day) and "time from initial disturbance" as covariate. Escape from glucocorticoid negative feedback was examined by taking the difference between the mid-day and sunrise cortisol level after dexamethasone suppression, then using ANOVA with Group (2) and Sex as between subject factors.

Results

During preadolescence, both groups showed significant reduction in cortisol secretion across the day (Time of day: $F[2,32] = 54.21$, $p < 0.001$, $\eta^2 = 0.77$; Figure 2b), and more specifically, the diurnal cortisol rhythm was linear and adult-like at this age (repeated contrasts: sunrise vs mid-day $F[1,16] = 31.7$, $p < 0.001$, $\eta^2 = 0.67$; mid-day vs sunset $F[1,16] = 27.74$, $p < 0.001$, $\eta^2 = 0.63$). However, despite similar patterns of cortisol rhythm, Neo-A animals had significantly higher cortisol secretion compared to Neo-C animals throughout the day (Group: $F[1,16] = 4.78$, $p = 0.044$, $\eta^2 = 0.23$) and this hypersecretion of cortisol was similar in both sex (Sex: $F[1,16] = 0.92$, $p = 0.35$, $\eta^2 = 0.06$).

Thus, the elevated cortisol secretion observed after post-stress HPA axis reactivity

(Experiment 1) and across the diurnal rhythm (Experiment 2) suggests that early damage to the amygdala has altered the normal functioning of the HPA axis. To examine the precise nature of the altered HPA axis functioning neuropeptide challenges were employed. Overall, the results indicate that HPA axis neuropeptide challenges elicited significant changes in neuroendocrine responses in juvenile monkeys with or without neonatal amygdalectomy.

First, as shown in Figure 3a, after the ACTH neuropeptide challenge, both groups responded with a significant increase in cortisol compared to a saline injection (Drug: $F[1,38] = 102.2$, $p < 0.001$, $\eta^2 = 0.77$) and the effects of Group and Sex and their interactions did not reach significance. Second, as shown in Figure 3b, after CRH challenge, again both groups responded with a significant increase in cortisol compared to saline (Drug: $F[1,36] = 28.4$, $p < 0.001$, $\eta^2 = 0.50$). However, the cortisol response to CRH was less in magnitude in Neo-A animals as compared to Neo-C animals (Group: ($F[1,36] = 5.42$, $p = 0.027$, $\eta^2 = 0.16$; see Figure 3b). This lower response to CRH challenge among Neo-A animals was also confirmed when comparing ACTH levels after CRH and saline injections. It is first important to note that ACTH levels were high in the baseline samples because animals exhibited a physiological response to capture, and CRH administration delayed the ACTH decrease compared to saline. The results showed a significant interaction between Drug, Group, and Sex for ACTH response to CRH challenge ($F[1,36] = 4.2$, $p = 0.05$, $\eta^2 = 0.13$; see Figure 3c). Although both groups responded similarly to the CRH challenge, showing less reduction in ACTH levels compared to saline injection, this blunted response to the CRH challenge was present in both Neo-C males and females, but only in Neo-A males. Neo-A females did not show changes in ACTH levels after the CRH challenge (see AUCi Figure 3c). Third, to confirm the effects of neonatal amygdalectomy on cortisol levels after CRH challenge, CRF concentration was measures in CSF samples. There was a

significant Group X Sex interaction for CRF concentration ($F[1,19] = 6.5$, $p = 0.022$, $\eta^2 = 0.30$; see Figure 2c), indicating that Neo-A females exhibited higher CRF in CSF compared to Neo-A males and Neo-C animals of both sex.

Lastly, glucocorticoid negative feedback was examined using the dexamethasone suppression test. As shown in Figure 2b, dexamethasone administration significantly suppressed cortisol levels at sunrise, 10 hours after its administration in both groups and animals escaped the glucocorticoid negative feedback by mid-day (Drug X Time ($F[1,30] = 43.5$, $p < 0.001$, $\eta^2 = 0.59$). Nevertheless, the effect of dexamethasone suppression was altered by neonatal amygdalectomy (Drug X Group: $F[1,30] = 4.91$, $p = 0.035$, $\eta^2 = 0.14$). Thus, Neo-C animals ($M = 7.92 \pm 1.4$) exhibited a significantly greater escape from dexamethasone suppression test than Neo-A animals ($M = 2.52 \pm 1.6$; Group: $F[1,20] = 6.09$, $p = 0.026$, $\eta^2 = 0.29$).

Discussion

The present study provides conclusive evidence that damage to the amygdala in infancy alters the normal development of the HPA axis in monkeys. First, contrary to previous studies demonstrating a blunted HPA axis response to a stressor in adult animals with either adult-onset (Kalin, et al., 2004; Machado & Bachevalier, 2008) or neonatal (Raper, et al., 2012a) amygdala lesions, the current results indicate that neonatal amygdala lesions lead to increased cortisol stress response to a threatening stimulus in juvenile monkeys. Second, neonatal amygdala damage also impacted the normal functioning of the HPA axis as reflected in the increased basal cortisol rhythm, decreased response to CRH challenge and impaired ability to escape from dexamethasone suppression test. Finally, these changes in the normal development of the HPA axis functions were confirmed by the increased CRF found in CSF of animals with neonatal

amygdala lesions.

Amygdala and development of the HPA axis stress reactivity

Juvenile monkeys responded to the psychological stress of the Human Intruder paradigm with increased plasma cortisol and ACTH, and the increase in cortisol was even greater in animals with neonatal amygdala lesions. Interestingly, females with neonatal amygdala lesions expressed significantly greater cortisol secretion in response to the stressor compared to all other animals. The increased stress reactive cortisol response from neonatal amygdalectomized animals was unexpected given that previous lesion studies have reported blunted stress response after amygdalectomy (Kalin, et al., 2004; Machado & Bachevalier, 2008; Raper, et al., 2012a). One potential factor that could explain these unexpected findings is the age at which the stress response was examined. All previous lesion studies have examined the effects of amygdala lesions on stress response in adulthood (after puberty), whereas the current study focused on preadolescence (before puberty). Puberty is a period of continued development of neural pathways involved in coordinating the HPA axis stress response (Lidow, et al., 1991; Andersen, 2003). For example, prior to puberty neurotransmitter receptor density is steadily declining until tapering off at puberty (Lidow, et al., 1991). In contrast, glucocorticoid receptors (GR) density increases from infancy to adolescence at which time GR expression reaches a plateau and then declines in old age (Perlman, et al., 2007; Sinclair, et al., 2011b). In fact, prepubertal rodents exhibit an exaggerated stress response and delayed return to baseline after stressors compared to adults (Romeo, et al., 2004a, 2004b, 2006), suggesting an immaturity of the HPA axis glucocorticoid negative feedback before puberty. Additionally, primate studies have reported that pre-pubertal female monkeys exhibit greater cortisol response to stressors (Clarke, 1993;

Davenport, et al., 2003; Sanchez, et al., 2005). Thus, the cortisol hyper-secretion observed after neonatal amygdala lesions, particularly in females, suggests that the neonatal lesions have impacted the normal development of the HPA axis and, more specifically, the normal changes in receptor density, leading to a hyperactive stress response. Further examining the stress reactive response of these animals after puberty when the animals will reach maturity will demonstrate whether the effects of neonatal amygdala lesions are transitory or in the contrary will lead to blunted cortisol secretion as it has been shown in the earlier studies (Kalin, et al., 2004; Machado & Bachevalier, 2008; Raper, et al., 2012a).

Despite the exaggerated stress reactive response, neonatal amygdala-lesioned animals exhibited blunted fear reactivity during the human intruder as reflected by a reduction of freezing in the presence of the intruder as compared to controls (Raper, et al., 2012c). Thus, the data indicate a dichotomy between the effects of neonatal amygdala lesions on fear responses and on HPA axis functioning. This dichotomy could be explained by the different connections of the amygdala with brain systems critical for the production of fear behaviors as those responsible for HPA axis fear reactivity. Thus, the regulation of freezing behaviors requires activation of the periaqueductal gray matter which receives direct inputs from the amygdala (Kalin, et al., 2001; Walker & Davis, 1997). For the reactive stress response, although the amygdala could directly impact hypothalamic activation, there exist alternative pathways that could differently impact the HPA axis reactivity in the absence of a functional amygdala. Thus, both the prefrontal limbic cortex and the bed nucleus of the stria terminalis have strong connections with the hypothalamus and both receive strong inputs from the amygdala (Rempel-Clower & Barbas, 1998; Myers, et al., 2012; Radley, 2012). Therefore, in the absence of a functional amygdala, it is possible that the HPA axis reactive stress response could still be stimulated by other structures.

Amygdala and development of diurnal cortisol rhythm

To date, only few studies have examined the diurnal cortisol rhythm of juvenile rhesus monkeys and have primarily focused on the impact of early life experience on the HPA axis (Sanchez, et al., 2005; Barrett, et al., 2009). Those studies demonstrate a linear pattern in diurnal cortisol secretion in juvenile monkeys, which was replicated in the current study. Thus, both control and neonatal amygdala-lesioned animals exhibited the highest cortisol levels at sunrise, with progressive decline at mid-day and further decline from mid-day to sunset. Interestingly, despite this overall change in diurnal cortisol secretion, the shape of the decline through the day differed with maturation of the animals. At the youngest age (5 months), infant monkeys exhibited equal levels of cortisol at sunrise and mid-day with a significant decline occurring only at sunset (Raper, et al., 2012b). In contrast, by 12 months of age, the linear pattern of cortisol decline from sunrise to mid-day and mid-day to sunset reached an adult pattern (Sanchez, et al., 2005; Goncharova, et al., 2006; Barrett, et al., 2009; Collura, et al., 2009; Arce, et al., 2010. Similar to controls, animals with neonatal amygdala lesions exhibited a linear decline in diurnal cortisol, but had elevated cortisol levels across all time points. This higher cortisol rhythm suggests that damage to the amygdala early in life impacts the typical basal HPA axis secretion. Therefore, disruption of the amygdalar inputs to the hypothalamus and other neural structures involved in HPA axis functioning may have impacted the basal tone of the HPA axis. Support for this proposition comes from adult rodent studies (Allen & Allen, 1975; Regev, et al., 2012). In adult rats, the amygdala is necessary to maintain ACTH secretion after adrenalectomy, suggesting that in the absence of glucocorticoid regulation, the amygdala plays a critical role in maintaining the tonic (basal) control of the HPA axis (Allen & Allen, 1975). Further, CRH

knockdown in the central nucleus of the amygdala in adult mice leads to increased basal corticosterone secretion (Regev, et al., 2012). Although the exact mechanism by which the amygdala exerts its influence on the basal tone of the HPA axis is unknown, the results obtained with the use of pharmacological challenges that targeted different levels of the HPA axis offer some potential explanations.

Putative mechanisms

Increased stress reactivity and diurnal cortisol secretion after neonatal amygdalectomy suggest a significant impact on the neural changes in neurotransmitters and glucocorticoid receptors that normally occur during development. To inform on the potential changes in receptors involved in HPA axis functioning after early amygdala damage, specific neuropeptide pharmacological challenges were used to assess functioning of different levels of the HPA axis. Functioning of the adrenal cortex was examined using an ACTH neuropeptide challenge. Because the ACTH challenge was performed under anesthesia to ensure that animals were not undergoing psychological stress at the time of the challenge, the results reflect only the activation of adrenal receptors by the exogenous ACTH leading to cortisol synthesis and secretion. Thus, as expected, after the ACTH challenge controls exhibited an increase in cortisol section and a similar increase was found in animals with neonatal amygdala lesions. The lack of a group difference after exogenous administration of ACTH rules out the possibility that the increased stress reactivity after neonatal amygdalectomy was due to changes in responsiveness of the adrenal cortex. In addition, animals of both sex reacted similarly to the ACTH challenge. These findings confirm earlier results indicating that sex differences in response to ACTH challenges normally emerge after puberty when gonadal hormones are present in systemic

circulation (Meyer & Bowman, 1972; Wilson, et al., 2005).

The normal functioning of the pituitary was examined using exogenous CRH. The results demonstrated that both groups exhibited an increase plasma cortisol and ACTH secretion to administration of CRH, yet this increase was greater in controls than in animals with neonatal amygdala lesion, regardless of sex. The baseline sample did exhibit elevated plasma ACTH in all subjects, presumably due to the physical activation of the HPA axis during the capture procedure, and the CRH administration delayed the ACTH decrease while subjects were under anesthesia. The results of this CRH challenge suggest that damage to the amygdala early in life has altered the normal function of the HPA axis, potentially through alterations in CRH and glucocorticoid receptors in the hypothalamus and pituitary. Taken together, the elevated diurnal cortisol rhythm and reduced response to CRH challenge among neonatal amygdala-lesioned animals suggest elevated secretion of CRF in these animals. This conclusion is further supported by examination of CRF within the CSF. The results revealed higher CRF concentration in CSF of females with neonatal amygdala lesions as compared to controls and amygdala-lesioned males. Elevated CRF secretion from the hypothalamus results in a down-regulation of CRH receptors in the anterior pituitary (Wynn, et al., 1985; Hauger & Aguilera, 1993), resulting in fewer CRH receptors available for binding the exogenous CRH, thus leading to the lower ACTH and cortisol response seen among neonatal amygdalecomized animals as compared to controls. Thus, these data provide indirect evidence that early amygdala damage alters the normal developmental changes in receptors and neural pathways involved in coordinating the stress response.

Lastly, assessment of glucocorticoid negative feedback using the dexamethasone suppression test revealed that both groups exhibited cortisol suppression 10 hours after

dexamethasone administration and this effect was observed for both sex. Studies in adult animals have demonstrated the direct influence of estrogen, such that estrogen administered to females resulted in non-suppression and a faster escapes from maximal suppression compared to control females (Wilson, et al., 2005). The lack of sex differences in the current study is likely due to the fact that the juvenile animals were at a developmental stage of gonadal quiescence. The ability of amygdalectomized animals to suppress the morning rise in cortisol secretion after exposure to dexamethasone suggests that these animals have a sufficient density of GR's to impose negative feedback, such that the elevated diurnal cortisol secretion is not due to a lack of glucocorticoid negative feedback. Rather, the elevated basal cortisol secretion could be due to increased hypothalamic output, a conclusion supported by the increased CRF concentrations found in animals with neonatal amygdala lesions.

Despite their normal glucocorticoid negative feedback, the release from dexamethasone suppression in animals with neonatal amygdala lesions was blunted as compared to controls. This super-suppression after early amygdala damage suggests that other neural structures (e.g. prefrontal cortex, hippocampus) involved in negative feedback on the HPA axis may be compensating for the elevated CRF concentration and diurnal cortisol rhythm. Studies examining the anti-inflammatory actions of glucocorticoids demonstrate that GR can be up-regulated in peripheral mononuclear cells after exposure to endotoxins or glucocorticoids (Bartholome, et al., 2004; review Stahn, et al., 2007), but whether this same type of GR up-regulation occurs in neurons has yet to be demonstrated. However, if such an up-regulation of GR's existed in the neural pathways coordinating negative feedback control of the HPA axis, it is likely that dexamethasone will have a much stronger effect on these neural GR's. Given that GR's have a higher affinity for dexamethasone compared to cortisol (Dallman, 2010),

dexamethasone would more readily bind to GR's in neonatal amygdalectomized animals, resulting in an increase in glucocortiocoid negative feedback response.

As mentioned above, up-regulation of GR's have been demonstrated in peripheral cells (Bartholome, et al., 2004; review Stahn, et al., 2007), but whether this occurs in neurons is unknown. Most studies have focused on the deleterious effects of glucocorticoids on GR down-regulation and neuronal atrophy in the hippocampus, an area implicated in negative feedback control of the HPA axis (Sapolsky, et al., 1986; Patil, et al., 2007; Oitzl, et al. 2009). Thus, an up-regulation of GR's is still questionable. Alternatively, in contrast to the hippocampus, exposure to high levels of glucocorticoids results in an increased arborization of dendrites in neurons of the bed nucleus of the stria terminalis (BnST; Vyas, et al., 2003). If this scenario will prove to be true, elevated HPA activity in animals with neonatal amygdala lesions may yield to an enlarged BnST. The BnST has not only been implicated in anxiety disorders and sustained fear (review Walker & Davis, 2008), but also in the modulation of the HPA axis (Myers, et al., 2012; Radley, 2012). It is interesting to note that the amygdalectomized animals participating in this study exhibited an inability to modulate fear and anxious behaviors (Raper, et al., 2012c), suggesting a potential overactivity of the BnST. Therefore, an alternative hypothesis to explain the dexamethasone super-suppression among neonatal amygdalectomized animals may be the disconnections between the amygdala and the BnST and its influence on the HPA axis. The major outputs of the amygdala are GABA-ergic projections to the BnST, which in turn sends direct GABA-ergic inputs to the PVN (Freese & Amaral, 2009; Myers, et al., 2012; Radley, 2012). Therefore, the amygdala's excitatory influence on the HPA axis is likely mediated through trans-synaptic disinhibition of the PVN (Myers, et al., 2012; Radley, 2012). Taken together, these data suggest that animals with neonatal amygdalectomies lack the ability to

disinhibit the BnST, resulting in an inability to overcome the suppression from dexamethasone. The current data cannot determine whether the super-suppression after dexamethasone administration in neonatal amygdala lesion animals is the result of up-regulated GR's as a compensatory mechanism for elevated HPA output, or due to the lack of amygdala inputs to the BnST to stimulate the escape from glucocorticoid negative feedback. Future PET imaging or *in situ* hybridization studies in these animals may help determine which of these alternative hypotheses is correct.

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Figure captions

Figure 1. Two coronal MR images through the amygdala: T1-weighted images in one sham-operated control (Neo-C-1) and Fluid Attenuated Inversion Reversal (FLAIR) images in a representative case with neonatal amygdala lesions (Neo-A-F6). The numerals to the left of each coronal section indicate the distance in millimeters from the interaural plane. Black arrows point to the hypersignal resulting from the cell death from neurotoxic injections.

Figure 2. Mean ± SEM of cortisol during Human Intruder stressor (A), Diurnal rhythm and dexamethasone suppression (B). Also the mean ± SEM of corticotropin releasing factor (CRF) measured in cerebrospinal fluid (C). Sham operated controls (Neo-C) are represented by squares (males = open squares, females = grey squares) or open bars, neonatal amygdala lesion animals (Neo-A) are represented by circles (males = black circles, females = grey circles) or black bars. Dashed lines indicate cortisol levels after dexamethasone administration. * indicates a significant difference ($p < 0.05$).

Figure 3. Mean ± SEM of cortisol levels after an ACTH, CRH (C), or saline vehicle (Veh or V) challenge, as well as mean ± SEM of ACTH level after CRH or saline vehicle challenge. Sham operated controls (Neo-C) are represented by squares or open bars, neonatal amygdala lesion animals (Neo-A) are represented by circles or black bars. Response to a saline vehicle injection is indicated by solid lines and shapes, ACTH injections are indicated by dashed lines and striped shapes, and CRH injections are indicated by double lines and checked shapes. * indicates a significant difference ($p < 0.05$).

Figure 1. Cortisol and corticotropin releasing factor levels

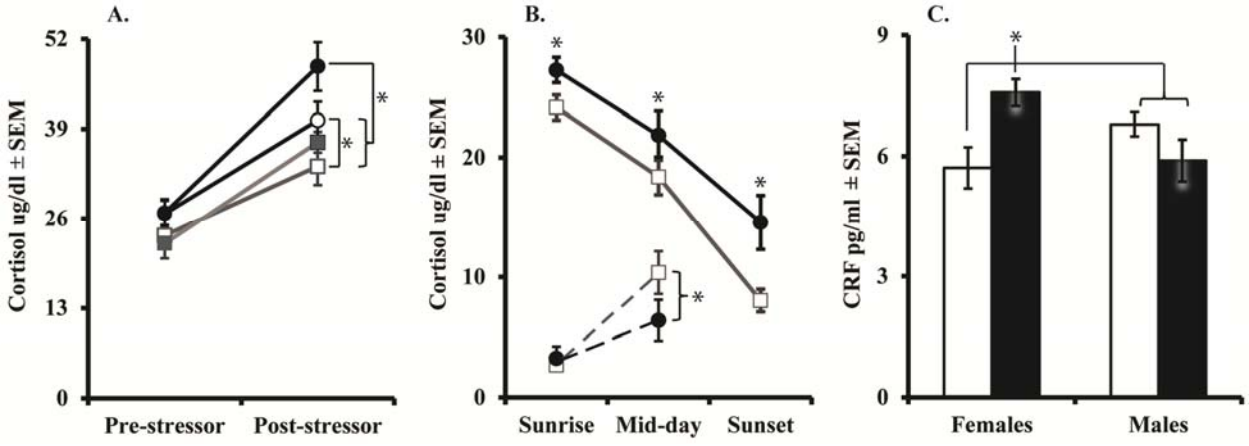

Figure 2. Response to neuropeptide pharmacological challenges

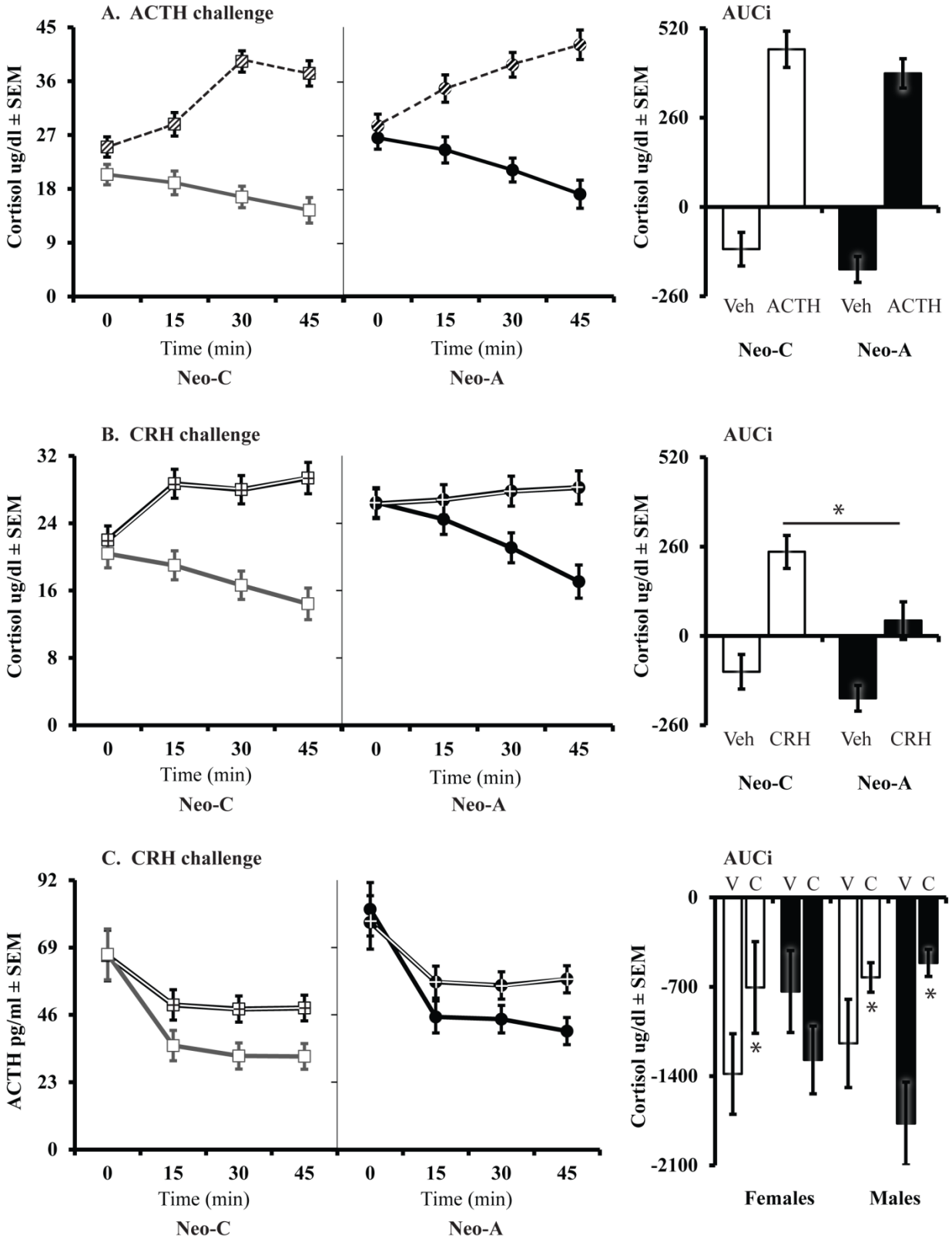

Table 1. Intended and unintended damage after neurotoxic lesions of the amygdala

	Intended Damage				Unintended Damage							
	Amygdala				Hippocampus				Entorhinal			
Subjects	Rt%	Lf%	X%	W%	Rt%	Lf%	X%	W%	Rt%	Lf%	X%	W%
Neo-A-F1	82.3	0.0	41.2	0.0	0.0	0.0	0.0	0.0	18.3	0.0	9.2	0.0
Neo-A-F3	100	32.2	66.1	32.2	2.5	0.0	1.2	0.0	21.8	2.9	12.4	0.6
Neo-A-F4	90.9	89.3	90.1	81.1	1.9	0.0	1.0	0.0	12.3	0.0	6.2	0.0
Neo-A-F5	61.6	58.4	60.0	36.0	0.0	0.0	0.0	0.0	1.6	0.0	0.8	0.0
Neo-A-F6	100	97.7	98.8	97.7	2.4	7.9	5.1	0.2	1.3	2.0	1.6	0.03
Neo-A-F7	98.3	99.0	98.6	97.3	4.3	2.1	3.2	0.1	7.3	2.5	4.9	0.2
Mean	**88.9**	**62.8**	**75.8**	**57.4**	**1.9**	**1.7**	**1.8**	**0.05**	**10.4**	**1.2**	**5.9**	**0.18**
Neo-A-M1	100	80.6	90.3	80.6	8.9	9.0	9.0	0.8	8.3	15.8	12.0	1.3
Neo-A-M3	70.3	90.8	80.6	63.9	3.1	5.3	4.2	0.2	2.1	31.4	16.8	0.7
Neo-A-M4	50.5	84.9	67.7	42.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Neo-A-M6	77.3	92.3	84.8	71.3	4.4	0.0	2.2	0.0	0.0	0.3	0.2	0.0
Neo-A-M7	90.9	98.9	94.9	90.0	5.1	0.6	2.9	0.03	1.4	0.0	0.7	0.0
Neo-A-M8	100	87.0	93.5	87.0	6.4	0.4	3.4	0.03	7.1	0.0	3.6	0.0
Neo-A-M9	61.8	93.2	77.5	57.6	0.0	0.0	0.0	0.0	0.0	0.7	0.4	0.0
Mean	**78.7**	**89.7**	**84.2**	**70.5**	**4.0**	**2.2**	**3.1**	**0.15**	**2.7**	**6.9**	**4.8**	**0.3**

L%: percent damage in the left hemisphere; R%: percent damage in the right hemisphere; X%: average damage to both hemispheres; W%: weighted average damage to both hemispheres (W%=(L%×R%)/100). Neo-A-F: female amygdala lesion subject, Neo-A-M: male amygdala lesion subject.

General Discussion

The primary goal of this project was to investigate the role of the amygdala in the development of emotional behavior and hypothalamic-pituitary-adrenal (HPA) axis function. Understanding how the amygdala influences the development of these processes will provide valuable insights into developmental neuropsychiatric disorders. Aberrant amygdala development as well as abnormal fear reactivity and emotional dysregulation are present in developmental neuropsychiatric disorders such as autism, schizophrenia, and mood disorders (see review Schumann, et al., 2011). Neuropsychiatric disorders are also characterized by HPA-axis dysfunction (Kaufman, et al., 2001; Walker, Mittal, & Tessner, 2008; Corbett, et al., 2009). Children with autism lack consistency in day-to-day diurnal cortisol production, generally exhibiting higher cortisol levels at night (Corbett, et al., 2006, 2008, 2009). Children with depression or schizophrenia exhibit an elevated diurnal cortisol rhythm (Kaufman, et al., 2001; Walker, Mittal, & Tessner, 2008). There is also a sex difference in prevalence and behavioral profile of neuropsychiatric disorders. Men exhibit a great preponderance of autism, schizophrenia, and conduct disorder, whereas women display more mood disorders; e.g. anxiety, depression, post-traumatic stress disorder, (Häfner, 2003; Zahn-Waxler, Shirtcliff, & Marceau, 2008; Rinehart, Cornish, & Tonge, 2011). The clinical profile of neuropsychiatric disorders differs in a way that is consistent with sex differences in the normal population, such that children with disorders exhibit an exaggerated pattern of typical sex differences (Häfner, 2003; Rinehart, Cornish, & Tonge, 2011). Furthermore, the amygdala's sexual dimorphism in size and androgen receptors (Pomerantz & Sholl, 1987; Micheal, Rees, Bonsall, 1989; Abdelgardir, et al., 1999) position it as an important area in the expression of sexually dimorphic behaviors (McClure, et al., 2004; Zosuls, et al., 2009; Saint-Maurice, et al., 2011). Taken together these

data suggest that the amygdala plays a critical role in the behavioral and neuroendocrine features of neuropsychiatric disorders, such that early perturbation results in emotional and HPA-axis dysfunction. In order to examine the precise role of the amygdala in normal behavior and HPA-axis functioning throughout development, we utilized a non-human primate neonatal lesion model.

There are several reasons that a non-human primate serves as a good animal model to study the amygdala's involvement in developmental neuropsychiatric disorders in humans. Studies utilizing rodents to model neuropsychiatric disorders have provided important findings, although they suffer from a lack of homology with higher order brain structures in humans (e.g., prefrontal cortex). The non-human primate brain has a complex prefrontal cortex, which is more similar to humans, perhaps making it a better animal model for translational research. It is important for an animal model to take into consideration the early ontogeny of many neuropsychiatric disorders, including autism, schizophrenia, and mood disorders and to follow how progression of symptoms manifests throughout the lifespan. To this end, the present study explored the amygdala's involvement in behavioral and neuroendocrine aspects of neuropsychiatric disorders using a nonhuman primate lesion model followed from birth to adulthood. This paper primarily focuses on the infancy and preadolescent stages of development.

We predicted that neonatal amygdala lesions would a) alter emotional reactivity toward a novel human intruder, such that neonatal amygdala lesion animals would still express emotional and defensive behaviors but would lack the ability to modulate their behaviors based on the presence and gaze direction of the intruder; b) impact the basal functioning of the HPA-axis, such that the diurnal cortisol rhythm would be flattened and lower compared to controls; c) result

in a blunted cortisol response to a stressor; and d) alter the normal functioning of the HPA-axis at the feed-forward loop examined by CRH challenge and the escape from negative feedback loop after dexamethasone suppression, but leave cortisol production unaffected as assessed via ACTH challenge. As summarized in Tables 1 and 2, the current project revealed some intriguing group differences, though not all were consistent with our predictions.

Is the amygdala important for the development of emotional behavior expression?

Previous studies have shown that the ability to modulate defensive and emotional behaviors based on contextual information is mature by approximately three months of age in rhesus monkeys (Kalin, et al., 1989, 1991; Raper, et al., 2012a submitted). Therefore, we predicted that neonatal amygdala lesions would impair animals' ability to modulate emotional behaviors based on the contextual information in the environment (e.g., presence and gaze direction of the intruder). In addition to our prediction of dysregulation of emotional modulation, we noted novel findings of behavioral sex differences during the task, and the impact of neonatal amygdala lesions differed depending on the sex of the animal and age at examination (see Table 1).

It is important to point out that damage to the amygdala early in life did not disrupt the emergence of species-typical defensive and emotional behaviors. When tested in infancy, both sham-operated controls and neonatal amygdala-lesioned animals exhibited the appropriate pattern of defensive behaviors; i.e. more freezing during the Profile condition and hostility during the Stare condition. Despite their ability to express species-typical behaviors, infants with neonatal amygdala lesions were unable to modulate their coo vocalizations, fearful defensive behaviors, or tooth grinding based on the salience of the threat in the condition. Considering the

role that the amygdala plays in the expression of fear and anxiety, it is logical that early amygdala damage would impair the ability to modulate the above behaviors. The impact of early amygdala damage on defensive behaviors became more apparent later in development, when subjects were tested again as juveniles. During preadolescence, neonatal amygdala lesion animals exhibited less freezing and hostility, but continued to exhibit more coo vocalizations as compared to controls. At both ages, neonatal amygdala-lesioned animals expressed less anxiety, as indicated by decreased tooth grinding. These results suggest that the impact of neonatal amygdala lesions on the expression of defensive and emotional behaviors becomes more evident later in development. Therefore, despite the increased potential for brain plasticity and compensation after early brain insult, neonatal amygdala damage resulted in deficits in defensive and emotional expressions, which worsened with age.

Another major finding of the current study pertained to the presence of behavioral sex differences during the Human Intruder task. To date, only one study has examined potential sex differences in this task, reporting no effects of sex on the duration of freezing (Kalin, et al., 1998). The current study also demonstrated no sex differences in freezing, but an overall sex difference in yawning and self-soothing behaviors, as well as an interaction between lesion and sex for fearful defensive and anxious behaviors, such as tooth grinding (see Table 1). In the cases of yawning, fearful defensive, and tooth grinding, the sex differences were evident during infancy, but were no longer present when animals were re-tested as juveniles. This contrasts with the findings of previous studies showing that sex differences do not emerge until after puberty when gonadal hormones are in high circulation (Mason, et al., 1960; Hadidian, 1980; Troisi, et al., 1990). At the time of testing during infancy, males were experiencing the postnatal testosterone (T) surge, which typically occurs from birth through four months of age in male

rhesus monkeys (Robinson & Bridson, 1978; Mann, et al., 1989). Therefore, the sex difference and Group X Sex interactions found during infancy may be influenced by the activational effects of circulating testosterone at this age. For example, in adult monkeys, serum T levels have been shown to directly influence yawning behavior with an increased T levels resulting in increased yawning (Phoenix, et al., 1973; Graves & Wallen, 2005). The current study also reported that this positive relationship between T levels and yawning during infancy was present at a time when the males were under activational influence of high levels of T in circulation. Interestingly, some other sexually dimorphic behavioral responses are impacted by neonatal amygdala damage (e.g., fearful defensive and tooth grinding), whereas other behaviors are not (e.g., yawning and self-soothing). Taken together, these findings suggest that the amygdala is essential for some but not all sexually dimorphic behaviors, and despite the loss of androgen receptors due to amygdala lesion, testosterone can still exert an activational influence on sexually dimorphic behaviors like yawning. More sexually dimorphic behaviors will likely emerge with the onset of puberty, when gonadal hormones turn on again after the period of quiescence during late infancy and preadolescence. It will be interesting to see which sex differences are spared after puberty, and thus not amygdala-centric, versus which sexually dimorphic behaviors are negatively impacted by this early perturbation. Continued observation of these same animals throughout puberty and into adulthood will shed valuable light on the amygdala's role in the expression of behavioral sex differences across development.

Overall, the data from the current study appears to indicate that the amygdala is important early in life for the normal development of emotional reactivity. Although animals with neonatal amygdala lesion can produce emotional behaviors, they cannot modulate their emotional

behaviors based on the contextual information in the environment. Additionally, the amygdala is essential for the normal expression of some, but not all, sexually dimorphic behaviors.

How does the present results inform on the role of the amygdala on emotional reactivity in neuropsychiatric disorders?

First, there are many similarities between characteristics of neuropsychiatric disorders and the progression of symptoms observed in animals with neonatal amygdala lesions. First, the clinical characteristics of neuropsychiatric disorders, such as schizophrenia and mood disorders, become more apparent later in development (Häfner, 2003; Zahn-Waxler, et al., 2008; Rinehart, Cornish, & Tonge, 2011). The impact of early amygdala damage on emotional behavior in rhesus monkeys also becomes more apparent with age. During infancy, neonatal amygdala-lesioned animals exhibited normal freezing and hostile expressions, whereas later, during preadolescence, they exhibited impairments in those defensive behaviors. The later emergence of deficits in emotional behavior suggests that early damage to the amygdala impacts developmental changes in other neural structures involved in threat appraisal and coordination of the reactive stress response. In fact, there are a myriad of developmental changes occurring between infancy and adolescence in both human and non-human primates: the amygdala increases in volume (Giedd, et al., 1996, 1997; Schumann, et al., 2004; Ostby, et al., 2009; Payne, et al., 2010), glucocorticoid receptors density increases (Perlman, et al., 2007; Sinclair, et al., 2011b), other neurotransmitter receptor density steadily decline (Lidow, et al., 1991), and the prefrontal cortex continues to experience growth and refinement (Lambe, et al., 2000, 2011; Raznahan, et al., 2011). Therefore, early damage to the amygdala may negatively impact the

normal development of other systems, leading to the increased alterations of emotional behavior later in development versus infancy.

Second, although the core symptoms of neuropsychiatric disorders are similar between males and females, the behavioral profile differs in a way that is consistent with sex differences seen in the normal population (Häfner, 2003; Rinehart, Cornish, & Tonge, 2011). Normally developing children exhibit a sex difference in externalizing behaviors and toy preference. Boys exhibit more hyperactivity and prefer wheeled toys, whereas girls are more inhibited, shy, and prefer to play with dolls. These normal sex differences are exaggerated among individuals with neuropsychiatric disorders; autistic boys exhibit a fixation on vehicles (Rinehart, et al., 2011), and pre-schizophrenic males exhibit more externalizing behaviors compared with normally developing siblings (Walker, et al., 1995; Häfner, 2003). Similar exaggerations of normal behavioral sex differences in rhesus monkeys are seen in animals with neonatal amygdala lesions. Female rhesus monkeys typically exhibit more fear grimaces and hostility toward social stimuli compared to males (Mason, et al., 1960). This pattern is exaggerated in infant females with neonatal amygdala lesions, such that they display more fearful defensive and screaming compared to control females. The findings of exaggerated sex differences among neonatal amygdalectomized animals lend validity to comparisons between this lesion model and neuropsychiatric disorders. These findings also suggest that the amygdala is important for the normal expression of some sexually dimorphic behaviors.

Lastly, along with aberrant amygdala development, neuropsychiatric disorders share common features of abnormal fear reactivity and altered threat appraisal. Individuals with autism, schizophrenia, and anxiety disorders exhibit high fear reactivity, suggesting a hyperactive threat appraisal level (review Schumann, et al., 2011). For example, in a test of

temperament, autistic children show decreased approach of novel foods or objects compared to typically developing or developmentally delayed children (Brock, et al., 2012). A similar result was found in the current study. Neonatal amygdala lesion males spent significantly less time exploring a novel fruit compared to controls. Additionally, unlike controls, neonatal amygdala-lesioned animals express more coo vocalizations across all conditions and threat levels of the Human Intruder paradigm, suggesting that amygdala damage has altered their ability to discern the differences in threat level. These data support previous work outlining the amygdala's role in the detecting danger in the environment and one's ability to select the appropriate emotional response according to the threat level (Davis & Whalen, 2001). Overall, the current study demonstrates the critical role of the amygdala for the normal development of emotions, expression of sexually dimorphic behaviors, threat detection, and potentially the normal development of other neural systems.

Is the amygdala important for normal functioning of the HPA-axis?

The HPA-axis has two main functions: 1) a reactive response is important for promoting activation of neural systems to rapidly respond to threats and redistribute resources (Herman, et al., 2003, 2005); and 2) a basal secretory rhythm that is essential for the body's homeostasis and normal functioning of other systems in the organism, such as metabolism, growth, and immune function (Munck, Guyre, & Holbrook, 1984). Studies in adult animals have demonstrated the excitatory influence of the amygdala on the HPA-axis, such that electrical stimulation results in increased cortisol secretion (Mason, 1959; Ehle, Mason, & Pennington, 1977). In contrast, lesions of the amygdala result in lower HPA-axis response to stressors (Knigge, 1961; Knigge & Hays, 1963; Beaulieu, DiPaolo, & Barden, 1986; Feldman, et al., 1994; Kalin, et al, 2004;

Machado & Bachevalier, 2008; Raper, et al., 2012a). Additional evidence from rodent studies (Allen, & Allen, 1975; Beaulieu, et al., 1989; Regev, et al., 2011) and neonatal lesions in primates (Raper, et al., 2012a) indicate that the amygdala plays an important role in the basal HPA-axis function. Therefore, we predicted that early damage to the amygdala would alter the normal functioning of the HPA-axis. Specifically, amygdala damage would impact the feed-forward loop of the HPA-axis, resulting in lower basal cortisol rhythm, blunted stress reactive cortisol response, and increased HPA-activity to an exogenous CRH challenge. In contrast, neonatal amygdalectomy would not impact the responsiveness of the adrenal cortex to ACTH, nor would it impair glucocorticoid negative feedback, as examined by an exogenous ACTH challenge and dexamethasone suppression test, respectively. The majority of our predictions were not supported, with a few exceptions (see Table 2).

First, the basal cortisol rhythm was examined at three different ages during development: 2.5 months, 5 months, and 12 months of age. At 2.5 months of age, neonatal amygdala lesions produced significant decreases in basal cortisol secretion in males, thus eliminating the sex difference in cortisol during the postnatal testosterone (T) surge without affecting the T surge itself. After the postnatal T surge was over, neonatal amygdala lesions resulted in a flattened diurnal rhythm at 5 months of age compared to controls, driven by higher cortisol levels secreted at night. However, later in development, during preadolescence (12 months of age), animals with neonatal amygdala lesions exhibited the typical linear pattern in cortisol, yet they secreted higher levels of cortisol across the day compared to controls. These results are partially in line with our predictions. As predicted, at 2.5 months neonatal amygdalectomized males had lower cortisol compared to control males; however, females with amygdala lesions did not differ from control females or amygdala lesion males. Again, as predicted, neonatal amygdalectomy resulted

in a flattened diurnal cortisol rhythm at 5 months of age; however, the flattened rhythm is a result of higher cortisol at sunset, thus the rhythm was not flattened in the direction predicted. Increased cortisol secretion at 12 months of age was also unexpected given that previous studies in adult animals indicated that the amygdala has an excitatory influence on the HPA-axis, such that lesion result in decreased cortisol secretion (Allen, & Allen, 1975; Beaulieu, et al., 1989; Regev, et al., 2011; Raper, et al., 2012a).

Examination of the stress reactive HPA-axis response also revealed unexpected results. At 12 months of age, animals with neonatal amygdala lesions secreted significantly more cortisol after the stressor compared to same sex controls. Additionally, a significant interaction revealed that females with neonatal amygdala lesions exhibited greater increase in cortisol secretion compared to males with neonatal amygdalectomies as well as controls. These findings were unexpected given that previous studies have shown that lesions of the amygdala in adulthood (Kalin, et al., 2004; Machado & Bachevalier, 2008) or neonatally (Raper, et al., 2012a) resulted in a blunted reactive HPA-axis response to an acute stressor. However, the latter study only examined the effect of neonatal amygdala lesions in adulthood, after puberty (Raper, et al., 2012a). Considering that puberty is a period of continued development of the neural pathways involved in coordinating the stress response (Lidow, et al, 1991; Anderson, 2003, Perlman, et al., 2007; Sinclair, et al., 2011b), it is possible that the neonatal amygdalectomized animals in the previous study had underwent such developmental changes during puberty, which lead to their blunted HPA-axis reactivity similar to adult-onset lesions. Although the results of the basal cortisol rhythm and reactive HPA-axis response were unexpected, the current data support the prediction that amygdala damage alters the feed-forward loop of the HPA-axis, but the alteration is not in the direction predicted.

Direct examination of the feed-forward loop of the HPA-axis was conducted at 12 months of age with exogenous CRH and ACTH challenges. As predicted, adrenal responsiveness to exogenous ACTH was not affected by neonatal amygdala damage, whereas response to exogenous CRH was impaired. Compared to controls, neonatal amygdala-lesioned animals exhibited a reduced ACTH and cortisol response to the CRH challenge. The combination of neonatal amgydalectomized animals exhibiting elevated basal cortisol rhythm and reduced response to exogenous CRH challenge suggests that elevated CRF secretion. In fact, neonatal amygdalectomized females exhibited increased CRF concentration in cerebrospinal fluid. Evidence from rodent studies demonstrate that above normal secretion of CRF from the hypothalamus causes a down-regulation of CRH receptors in the anterior pituitary (Wynn, et al., 1985; Hauger & Aguilera, 1993). A down-regulation of CRH receptors in the pituitary of amygdalectomized animals would result in reduced response to exogenous CRH due to the reduction in receptors to which CRH could bind. These data give direct evidence that early amygdala damage alters the normal developmental maturation in receptors in other neural structures.

Predictions about glucocorticoid negative feedback were partially supported. Neonatal amygdala lesions did not impair the ability to suppress the morning rise in cortisol after administration of dexamethasone, an artificial glucocorticoid. However, neonatal amygdala-lesioned animals were unable to escape from maximal suppression by mid-day, as shown by their lower cortisol levels at mid-day, after dexamethasone administration, compared to controls. The dexamethasone suppression test data suggest that early amygdala damage may alter normal developmental changes in glucocorticoid receptors (GR) and neural pathways involved in coordinating negative feedback control on the HPA-axis. Taken together, all of the results from

the current study of both the basal rhythm, reactive HPA-axis response, CRH challenge, and dexamethasone suppression suggest that the amygdala may have a very different role during early development compared to adulthood, thus leading to this different HPA-axis profile compared to previous work in adult animals (Allen, & Allen, 1975; Beaulieu, et al., 1989; Kalin, et al., 2004; Machado & Bachevalier, 2008; Regev, et al., 2011; Raper, et al., 2012a).

Studies in adult animals and humans have shown that the amygdala facilitates the HPA-axis stress response, whereas the prefrontal cortex (PFC) has an inhibitory feedback on the HPA-axis (Herman, et al, 2003, 2005). Additionally, the medial PFC has been shown to be involved in top-down inhibitory control over the amygdala (Phillips, et al., 2003; Quirk & Beer, 2006), thereby modulating amygdala activity during emotional tasks and regulating affective responses (Etkin, et al., 2006; Gianaros, et al., 2008; Wager, et al., 2009). Magnetic resonance imaging (MRI) studies utilizing resting-state functional connectivity have reported strong negative connectivity between the mPFC and amygdala; higher activity in the mPFC corresponds with lower activity in the amygdala (Urry, et al., 2006; Roy, et al., 2009; Veer, et al., 2011). Additionally, basal cortisol levels are associated with the connection between the amygdala and mPFC (Urry, et al., 2006; Veer, et al., 2011). Urry and colleagues (2006) found that, among healthy adults, a steeper diurnal cortisol curve was related to higher mPFC activity, lower amygdala activity, and better performance during an affective regulation task. This suggests that, in adulthood, the mPFC and amygdala exhibit negative connectivity, and that cortisol levels either influence the crosstalk between these structures, or that the cross-talk between these structures influences basal cortisol.

Yet, this adult-like pattern of negative connectivity between mPFC and amygdala is not the same as the pattern exhibited during development. Healthy children ages 4 to 9 years of age

exhibit positive connectivity between the mPFC and amygdala compared to adolescents and adults when viewing fearful faces (Tottenham, personal communication). Dr. Tottenham hypothesizes that, early in life, the amygdala and mPFC have an excitatory connection, with inhibitory inputs from the mPFC to the amygdala developing during childhood, such that the adult-like pattern of negative connectivity is seen later in development. This hypothesis of a developmental change in PFC and amygdala connectivity is supported by data from Gee and colleagues (2012), which show that ventral lateral PFC activation exhibits an age-related increase, whereas the amygdala exhibits a decrease in activity from adolescence to early adulthood. Additional evidence comes from a resting-state fMRI study in rhesus monkeys, which showed a positive connectivity between the mPFC and amygdala at 3 months of age (Sanchez, et al., 2011; Howell, et al., 2011, 2012). Taken together, these data suggest that the connectivity between the PFC and amygdala is different early in development compared to adulthood; therefore, the impact of basal cortisol on this connectivity may also be quite different early in development.

If the connectivity between the amygdala and PFC is different during development compared with adulthood, and the cross-talk between these structures influences basal cortisol levels, then this may explain the divergent results in cortisol found in the current study. Presumably, early damage to the amygdala could alter activity of the PFC, which in turn, will result in differential activation of the HPA-axis (e.g., increased CRH and cortisol secretion). Damage to the amygdala in adulthood, after negative connectivity between the amygdala and PFC had fully developed, would result in increased negative feedback on the HPA-axis (e.g., decreased CRH and cortisol secretion). Given the protracted development of the PFC and developmental changes in the HPA-axis during puberty, it is also possible that the inhibitory

influence of the PFC on the HPA-axis would still emerge later in development (e.g., after puberty), despite neonatal amygdala damage. This may explain why an earlier study of neonatal amygdala lesions found a blunted cortisol response to a stressor and flattened (lower cortisol at awakening) diurnal cortisol rhythm (Raper, et al., 2012a) when these measures were taken in adulthood. Another study of adult monkeys with neonatal amygdala lesions reported altered resting brain metabolism in multiple brain areas, including the frontal lobe, hippocampus, caudate, and putamen (Machado, et al., 2008). These studies demonstrate that amygdala damage early in development results in long-term alterations of brain metabolism and HPA-axis function. Unfortunately, these two studies did not investigate basal HPA-axis function or brain activity during development, thus limiting the conclusions that can be drawn from them about the role of the amygdala during development. To further investigate this hypothesis of the amygdala having a different role on the HPA-axis during development, future studies should utilize either resting-state MRI or resting-state PET technology to examine the impact of amygdala damage during infancy.

As suggested above, early amygdala damage could alter activity of the PFC, which in turn results in differential activation of the HPA-axis (e.g., increased CRH and cortisol secretion). Elevated glucocorticoids can have a deleterious effect on the brain and body (Dallman, 2010); therefore, compensatory mechanisms may emerge in an attempt to restore homeostasis. One such compensatory mechanism was seen in our study. The reduced response to exogenous CRH suggests that neonatal amygdala-lesioned animals have down-regulated CRH receptors in the anterior pituitary to compensate for high levels of CRF being secreted from the hypothalamus. Other compensatory mechanisms may also be present in these animals in an attempt to restore homeostasis. Evidence in anti-inflammatory research demonstrates the up-

regulation of GR in peripheral mononuclear cells (Bartholome, et al., 2004; review Stahn, et al., 2007). Whether this type of GR up-regulation may occur in neurons is unknown. If GR up-regulation occurred in neural pathways that coordinate the negative feedback control of the HPA-axis (e.g., PFC, hippocampus), this may explain why the neonatal amygdalectomized animals showed an inability to escape dexamethasone suppression by mid-day. GRs have a higher affinity for dexamethasone over cortisol compared to mineralocorticoid receptors (MR), such that under basal conditions, MRs more readily bind cortisol (Dallman, 2010). Since GRs preferentially bind dexamethasone, and if neonatal amygdalectomized animals' exhibit increased GRs due to up-regulation, then one would expect the greater glucocorticoid negative feedback response exhibited by neonatal amygdala-lesioned animals. Quantifying the number of GRs in neonatal amygdala-lesioned animals compared to control animals would be needed to assess the validity of this hypothesis. However, these animals are still involved in additional aims of this longitudinal study, therefore histological assessment is not possible at this time.

Alternatively, the amygdala may influence the basal tone of the HPA-axis via GABA-ergic projections to the bed nucleus of the stria terminalis (BnST), which in turn send GABA-ergic projections to the paraventricular nucleus (PVN) of the hypothalamus (Radley, 2012; see Figure 1). Disruption of the GABAergic tone of the BnST has been shown to impact anxiety-like behaviors and autonomic responses (e.g., increased heart rate) to a social interaction (Sajdyk, et al., 2008). Therefore, the loss of GABAergic input into the BnST after damage to the amygdala may impact the neuroendocrine as well as autonomic output. One way to potentially assess this hypothesis is to utilize [F-18] fluorodoxyglucose positron emission tomography (FDG-PET) combined with behavioral tests to examine the activity of the BnST in animals with neonatal amygdala lesions compared to control animals. This technique has been successfully

used to assess alterations in brain metabolism of the amygdala and BnST after orbital frontal cortex lesions in adult rhesus monkeys (Fox, et al., 2010). Future examination of the animals in the current study utilizing FDG-PET may clarify the potential for increased BnST activity among neonatal amygdala lesion animals.

The BnST not only plays an important role in the modulation of the HPA-axis (Myers, et al., 2012; Radley, 2012), it is also involved in anxiety and sustained fear responses (review Walker & Davis, 2008). Interestingly, the BnST exhibits increased arborization of dendrites after exposure to high levels of glucocorticoids (Vyas, et al., 2003). Therefore, the elevated cortisol levels secreted by neonatal amygdalectomized animals could result in an enlarged BnST and consequently lead to increased anxiety in these animals. Certainly, data presented above demonstrate that neonatal amygdalectomized animals exhibit increased fearful defensive behaviors and an inability to modulate other emotional behaviors, suggesting BnST activity influences behavioral responses toward threatening stimuli.

Overall, the data from the current study indicates that the amygdala is important early in life for the normal development and functioning of the HPA-axis. Though previous research in adult animals has shown that amygdala lesions result in lower HPA-axis responses, the elevated pattern of HPA-axis secretion in the current study suggests that the amygdala and its connection may be very different early in life compared to adulthood.

How do these data inform on the role of the amygdala on HPA-axis activity in neuropsychiatric disorders?

Dysregulation of basal HPA-axis secretion is commonly seen in individuals with neuropsychiatric disorders, which is similar to those exhibited by the neonatal amygdala-lesioned

animals in the current study. Elevated basal cortisol secretion has been demonstrated in neuropsychiatric disorders, such as autism, schizophrenia, and depression. Specifically, children with autism exhibit increased cortisol at night (Corbett, et al., 2006, 2008, 2009), which is similar to the findings of increased cortisol at sunset in 5-month-old infant monkeys with neonatal amygdala lesions. An elevated diurnal cortisol rhythm is exhibited by children with either depression or schizophrenia (Kaufman, et al., 2001; for review see Walker, et al., 2008). Examination of the diurnal cortisol rhythm during preadolescence revealed that neonatal amygdala-lesioned animals exhibited an overall higher cortisol rhythm compared to controls. The elevated basal cortisol secretion exhibited by individuals with depression and schizophrenia has been found to be linked to increased concentrations of CRF found in cerebrospinal fluid (Nemeroff, et al., 1984; Banki, et al., 1987; 1992). The current study also demonstrated elevated CRF in females with neonatal amygdala lesions. Therefore, the impact of early amygdala damage results in an elevated basal HPA-axis profile similar to neuropsychiatric disorders. These data demonstrate that the amygdala is essential during development for the normal functioning of the HPA-axis during infancy and later in preadolescence.

Along with elevated basal cortisol secretion, neuropsychiatric patients often exhibit non-suppression to dexamethasone, which is most likely the result of an impaired negative feedback caused by a reduction in glucocorticoid receptors (Knable, et al., 2001, 2004; Sinclair, et al., 2011a). Despite the elevated diurnal cortisol rhythm and CRF secretion, neonatal amygdala-lesioned animals were able to suppress the morning rise in cortisol, demonstrating that neonatal amygdalectomy does not impair glucocorticoid negative feedback. Interestingly, amygdalectomized animals exhibit super-suppression from dexamethasone, as evidenced by their inability to escape from dexamethasone at mid-day. Super-suppression to low doses of

dexamethasone is commonly found in individuals with post-traumatic stress disorder (PTSD; review de Kloet, et al., 2006; Savic, et al., 2012), suggesting increased glucocorticoid negative feedback. A recent study found that soldiers who developed PTSD exhibited an increase GR in peripheral mononuclear cells both prior to, and up to 6 months after, deployment (van Zuiden, et al., 2011). If GR number in peripheral cells is a proxy for the amount of GR in neural systems controlling negative feedback of the HPA-axis, then this could explain the super-suppression from dexamethasone exhibited by individuals with PTSD. Lastly, since GRs can be up-regulated in peripheral cells, it is possible that neonatal amygdala-lesioned animals are up-regulating GR in neural systems involved in negative feedback to compensate and attempting to restore homeostatic balance. The glucocorticoid binding capacity of peripheral cells in neonatal amygdala lesion animals may further enlighten the hypothesis of increased GR's and compensatory mechanisms.

The neuroendocrine profile of neonatal amygdala-lesioned monkeys is similar to that observed in several neuropsychiatric disorders. The characteristics displayed by animals with neonatal amygdala lesions do not fully capture the complexity of any single neuropsychiatric disorder. This model is useful in elucidating the precise role that the amygdala plays during development on the normal functioning of the HPA-axis.

Summary

The current study has demonstrated that damage to the amygdala early in life results in a cascade of dysfunction in emotional and neuroendocrine regulation. Although previous studies have merely focused on the role of the amygdala in the reactive stress response (Herman, et al., 2003, 2005), the current study demonstrates the critical role that the amygdala plays in basal

cortisol rhythm, suggesting that the amygdala plays a larger role beyond that of threat detection and rapid HPA-axis response. The current study also demonstrates the importance of normal amygdala functioning in the normal development of other systems, such as emotional regulation and neuroendocrine function. Lastly, the impact of early perturbation to the amygdala results in behavioral and neuroendocrine features similar to those found in neuropsychiatric disorders, suggesting that the amygdala is a key structure that influences the clinical profile of these disorders.

Table 1. Summary of emotional reactivity results

Behavioral measure	Age	
	2.5 months	12 months
Cage Exploration	Neo-A < Neo-C M < F	Neo-A = Neo-C M = F
Affiliative	Neo-A = Neo-C	Neo-A M > Neo-C M in Stare Neo-A F > Neo-C F in Alone
Coo vocalizations	Neo-A > Neo-C	Neo-A > Neo-C
Fearful defensive	Neo-A M > Neo-C M in Profile, Stare Neo-A F > Neo-C F in Alone, Stare	Neo-C: Profile > Alone, Stare Neo-A: Alone = Profile > Stare
Freezing	Neo-A = Neo-C	Neo-A < Neo-C
Hostile defensive	Neo-A = Neo-C	Neo-A < Neo-C
Scream vocalizations	Neo-A F > Neo-C, Neo-A M	Neo-A = Neo-C
Anxious behaviors	Neo-A, Neo-C F < Neo-C M	Neo-A = Neo-C
Tooth grinding	Neo-A, Neo-C F < Neo-C M	Neo-A < Neo-C
Yawning	Neo-A = Neo-C M > F	Neo-A = Neo-C M = F
Stereotypies	Neo-A = Neo-C	Neo-A = Neo-C
Self-soothing	Neo-A = Neo-C M < F	Neo-A = Neo-C M < F

Results of behavioral responses to the Human Intruder paradigm across development in males (M) and females (F) with neonatal amygdala lesions (Neo-A) or sham operated controls (Neo-C).

Table 2. Summary of hormone results

Age (months)	Endocrine measure	Sex Differences		Group Differences
		Neo-C	Neo-A	
2.5	Basal cortisol	M > F	M = F	Neo-A M < Neo-C M Neo-A M = Neo-A F, Neo-C F
	Testosterone	M > F	M > F	Neo-A M = Neo-C M Neo-A F = Neo-C F
5	Diunral rhythm cortisol	M = F	M = F	Neo-A = Neo-C at sunrise, mid-day Neo-A > Neo-C at sunset
	Testosterone	M = F	M = F	Neo-A = Neo-C
12	Diurnal rhythm cortisol	M = F	M = F	Neo-A > Neo-C at sunrise, mid-day, sunset
	Testosterone	M = F	M = F	Neo-A = Neo-C
	Stress response: cortisol	M = F	M < F	Neo-A > Neo-C Neo-A F > Neo-C, Neo-A M at post-stress
	Stress response: ACTH	M = F	M = F	Neo-A = Neo-C
	ACTH challenge: cortisol	M = F	M = F	Neo-A = Neo-C
	CRH challenge: cortisol	M = F	M = F	Neo-A < Neo-C
	CRH challenge: ACTH	M = F	M > F	Neo-A < Neo-C
	Dexamethasone: cortisol	M=F	M = F	Neo-A = Neo-C at sunrise Neo-A < Neo-C at mid-day
	Cerebrospinal fluid: CRF	M = F	M < F	Neo-A F > Neo-A M, Neo-C M, Neo-C F

Results of hormone analyses across development in males (M) and females (F) with neonatal amygdala lesions (Neo-A) or sham operated controls (Neo-C).

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