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Emily Johnson April 23, 2013

THE ROLE OF THE PERIRHINAL CORTEX IN EMOTIONAL REGULATION IN NON HUMAN PRIMATES

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By

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B.S., University of Maryland - College Park, 2011

Advisor: Jocelyne Bachevalier, Ph.D

An abstract of a thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Arts in Psychology, 2013

Abstract

THE ROLE OF THE PERIRHINAL CORTEX IN EMOTIONAL REGULATION IN NON HUMAN PRIMATES

By Emily Johnson

Recent efforts to define the functions of the primate perirhinal (PRh) cortex have focused on its interaction with the hippocampus in the mediation of normal memory. Less is known on the functions of its strong connections to the amygdala (Pitkanen, 2006; Amaral, 2002), a key substrate for emotion regulation. Previous studies (Meunier & Bachevalier, 2002; Meunier et al., 2006) showed that PRh lesions in adult monkeys enhanced defensive responses to threatening stimuli, an effect exactly opposite to the effects of amygdala lesions (e.g. reduced defensive responses). Here, we investigated the role of PRh lesions on the development of emotion regulation in monkeys from infancy through adulthood. Infant monkeys with either bilateral neurotoxic PRh lesions (Neo-PRh; males = 3, females = 3), or sham operations (Neo-C; male = 2, females = 2), performed between 7-14 days of age were used. Emotional reactivity towards a Human Intruder was evaluated at 2 months, 4.5 months, and 5 years of age using three conditions (Alone, Profile/no eye contact, and Stare). Additionally, in adulthood, blood cortisol and ACTH levels were assessed pre- and post- Human Intruder stressor and across the day. As compared to controls, group Neo-PRh showed profound changes in the modulation of freezing, fearful/defensive behaviors, hostility, self-directed behaviors, stereotypies, cage exploration, locomotion, and vocalizations to the presence and gaze direction of a human intruder that were present in infancy and persisted into adulthood. However, the neonatal lesions of the PRh cortex did not alter neuroendocrine functions. These behavioral changes were reminiscent to the

heightened defensive responses reported after adult-onset PRh lesions (Meunier et al., 2006). Therefore, neonatal lesions to the PRh cortex disrupt the normal development of emotional regulation, sparing hormonal regulation, in ways distinctively different from those reported after neonatal amygdala lesions (Raper et al., 2012). The data suggest that early dysfunction of the PRh results in abnormal fear reactivity and provide insights onto the neural underpinning of several developmental neuropsychiatric disorders associated with anxiety.

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Emotional Regulation and the Medial Temporal Lobe in Primates

Introduction

Extensive work in nonhuman primates has led to important discoveries on the key neural pathways involved in emotional regulation and social behavior. Neural structures of the medial temporal lobe (MTL), such as the amygdala, and hippocampus have proven to be important in modulating emotion and memory, and impairments in these functions together with MTL alterations are often seen in many human psychopathologies, such as autism, social anxiety, schizophrenia, and phobias (Machado & Bachevalier, 2003; Cohen 1996). Thus, defining the specific roles played by MTL structures in emotional regulation has become a critical endeavor to help in determining the specific neural substrate of these disorders and to explore potential therapies.

In order to understand current views on the functions of the MTL structures, a description of the anatomical organization of the MTL will be provided. We will then address the role of these structures in emotional and neuroendocrine regulation, and summarize a brief history of the research that led to these discoveries. Nonhuman primate research, and more specifically lesion studies, has played a strong role in our current understanding of MTL functions. Although the amygdala is a key structure contributing to emotional and neuroendocrine regulation, recent research has also pointed to a critical involvement of the rhinal cortices in this regulation. Cortical areas, such as the perirhinal (PRh) cortex, are now known to contribute to affect and emotional memory, although its role in hormonal regulation is still unknown. Further support for the role of the PRh cortex in emotional and endocrine regulation is provided by studies of neuropathologies and psychopathologies in humans, such as in patients with phobias or social

anxiety. Given that these pathologies develop over time, beginning in childhood or adolescence, one of the main goals of my research was to explore the role of the PRh cortex on emotional reactivity and on stress hormone (Hypothalamic-Pituitary-Adrenal, or HPA axis) regulation at different points in development.

Functional organization of the medial temporal lobe:

The MTL is made up of subcortical nuclei and several cortical areas, including the hippocampus, amygdala, and surrounding entorhinal (ERh), PRh, and parahippocampal cortices. As shown in Figure 1, the connectional organization of the MTL is well conserved across mammalian species and primates. The parahippocampal cortex mainly receives projections from the parietal and lateral prefrontal cortex and is involved in spatial and context memory. The PRh cortex surrounds the rhinal sulcus in the medial portion of the anterior MTL and is made up of two cortical areas, Broca's areas 35 and 36, differing from their cytoarchitectural organization (Sukuzi & Amaral, 1994). The PRh cortex receives projections from all sensory areas (visual, auditory, and somatosensory), such as the inferotemporal cortex, and is involved in object identification and memory (Suzuki, 1996). Both the parahippocampal and PRh cortices project to the ERh cortex, which in turn sends information to the hippocampus, a structure responsible for many aspects of memory, including integrating objects and context, spatial memory, and episodic memory (Zeamer et al., 2010). Nevertheless, the heavy projections from the PRh cortex to the amygdala, a complex of several nuclei known to share direct reciprocal projections with the hippocampus and rhinal cortices and to process emotional memories and modulate the HPA-axis (Pitkanen, 2006) make the PRh cortex a critical candidate for the regulation of emotional reactivity and HPA-axis regulation, either via its direct connections to the amygdala or via

connections to other brain structures critical for the regulation of behavioral and hormonal stress reactivity. It has already been shown that the PRh cortex mediates and maintains associations between objects and their behavioral meaning or their emotional content (Meunier & Bachevalier, 2002).

The MTL and emotion regulation

The role of the MTL in modulating social and emotional behaviors was first discovered from lesion studies. In nonhuman primates, damage to the MTL yields severe impairment in social and emotional regulation. The first description of this impairment was reported by Heinrich Klüver and Paul Bucy in 1939. Animals with temporal lobectomy exhibited abnormal emotional and exploratory behaviors post-surgery. These changes were labeled as the "Klüver-Bucy syndrome", which includes symptoms such as hyperorality, hypermetamorphosis, visual agnosia, and decreased expression of fear and aggression, or hypoemotionality. Following Klüver and Bucy's study, research was conducted to parse out the roles of different MTL structures in the symptoms characterizing the Klüver-Bucy syndrome, and to determine if the symptoms were due to specific or more generalized damage to the brain.

The first dissociation attempted was between the amygdala and the temporal cortical areas. Emotional responses of monkeys with bilateral aspiration lesions of the temporal neocortex were compared to those of monkeys with bilateral aspiration lesions of the amygdala (Horel et al., 1975). An approach-avoidance task was used to assess the emotional reactivity of the operated animals and normal controls when presented with three different stimuli, i.e. a human intruder staring at them, a stuffed toy bear, and a metal bar thrust into the cage. Both operated groups showed comparable changes in emotional behavior as compared to control

animals, characterized by hypoemotionality (decreased aggression and defensive behaviors), and increased approach and explorative behaviors towards the stimuli similar to the emotional changes seen after large MTL lesions (Klüver & Bucy, 1939). Thus, either medial temporal cortical damage or amygdalar damage separately was sufficient to induce these emotional deficits. Horel and colleagues (1975) concluded that, because these two areas are anatomically interconnected, damage to either one of them may result in emotional deficits characterized by indiscriminate emotional reactions to stimuli. However, in an earlier study comparing monkeys with bilateral amygdalar ablations to monkeys with bilateral inferotemporal cortex lesions, Weiskrantz (1956) argued that the lack of control on the behavioral task used and of specificity of the location and size of lesions across studies made it impossible to determine whether the emotional changes were due to the disruption of an emotional focal center, or due to generally large amounts of damage to the MTL.

One critical study that attempted to dissociate the emotional changes of the Klüver-Bucy syndrome with the visual agnosia also seen after MTL cortical damage in monkeys was conducted by Downer and colleagues (1961). In this study, monkeys received a mid-sagittal surgical division of the two hemispheres that abolished connections traveling from both hemispheres through the corpus callosum, optic chiasm, and anterior commissure. A second surgical procedure involved a unilateral aspiration amygdalectomy, resulting in a hemisphere with an intact amygdala and the other with a damaged amygdala. When the eye contralateral to the hemisphere with the amygdala lesion was sutured closed and a picture of a threatening monkey was presented to the eye ipsilateral to the amygdala lesion, the typical emotional reactivity changes (decreased fear and aggression and increased approach) were observed in the subject, without visual agnosia symptoms being present. By contrast, when the eye ipsilateral to

the hemisphere with the amygdala damage was shut and the picture of the threatening monkey was presented to the eye contralateral to the amygdala lesion, emotional reactivity was normal. These data clearly established for the first time that the amygdala was a key structure within the MTL for emotional regulation. This conclusion was supported by the lesion studies that followed Downer's study.

Amygdala and emotional regulation

Aggleton and Passingham (1981) compared complete amygdala ablations with partial amygdalar nuclei lesions to further determine the role of the amygdala in emotional regulation. Although small amygdalar lesions resulted in increased approach behavior in monkeys towards novel inanimate objects, the hypoemotionality associated with the Klüver-Bucy Syndrome was only seen in the larger amygdala ablations (Aggleton & Passingham, 1981). Furthermore, human lesion studies confirm the results in nonhuman primates by showing that damage to the amygdala disrupts emotional regulation to stimuli but not to the visual information itself. In one rare case, a patient (SM) with Urbach-Wiethe disease with selective calcification of the amygdala shows deficits in recognizing multiple emotional and fearful facial expressions, but not in identifying faces (Adolphs, 1994). Further research with SM and similar patients has shown that amygdala damage disrupts the connections necessary for emotional memory modulation. For example, the amygdala is activated by emotional arousal, which is thought to modulate the emotional memory storage processes through connections with neocortex and the hippocampus (Fine & Blair, 2000). Recent evidence, however, has demonstrated that patients such as SM can respond to selective emotional stimuli, such as a CO_2 challenge that induces panic attacks in some patients (Feinstein et al., 2013). In this study, patients with amygdala damage experienced

fear for the first time, having a heightened panic response compared to controls, although they did not exhibit any physiological anticipatory responses. This lack of an anticipatory response provides evidence to support the role of the amygdala in threat detection, and the experience of fear by these patients suggests that the amygdala is not required for the expression of fear or anxiety. On the contrary, the heightened panic response seen in patients with amygdala damage suggests that the amygdala may be responsible for modulating panic responses to emotional stimuli. In conclusion, although these studies indicate a role of the amygdala in emotional regulation, the data do not provide conclusive evidence that other brain regions are not involved, considering that both aspiration lesions in animals and calcification in humans do not limit damage to the amygdala. Therefore further investigation on how the surrounding areas may contribute to emotional regulation processes in still warranted.

Aspiration lesions of the amygdala in monkeys and calcification of the amygdala region in humans produced further damage to the surrounding ERh and PRh cortices as well as damage of cortical fibers passing through and around it. These cortical fibers originate from the medial temporal cortex and rhinal cortical areas and project to the thalamus and the orbital frontal cortex (Goulet et al., 1998; Meunier et al., 1999). Due to the spread of damage associated with aspiration amygdala lesions, it remains possible that the emotional changes observed after aspiration lesions of the amygdala may instead be due to damage to the rhinal cortex or its fibers. The use of neurotoxins, such as ibotenic acid, provides a useful tool to destroy cell bodies in a particular brain region more selectively, sparing fibers of passage and avoiding damage to the adjacent cortex. Ibotenic acid is an excitotoxin that functions as an agonist of N-methyl-D-aspartate (NMDA) receptors found on cell bodies. Ibotenic acid therefore over-activates NMDA receptors, causing an influx of calcium into the cell and increasing cell firing rate, leading to

delayed cell death. Excitotoxins allow for selective cell death, but spare axons traveling through and around the injected region. Thus, the use of neurotoxin combined with neuro-imaging techniques to selectively choose the injection target areas for each animal allows researchers to target more specific brain areas, and therefore to more clearly dissociate structure and function between the MTL structures.

When aspiration lesions and neurotoxic lesions of the amygdala were compared in nonhuman primates, similar effects were seen in both lesion groups (Meunier et al., 1999). In this study, changes in emotional responses were evaluated using four different emotionally arousing stimuli displayed in a Wisconsin General Testing Apparatus (WGTA). Two of the stimuli were social stimuli – an unfamiliar human wearing a mask, and a taxidermic conspecific monkey head. The other two stimuli were a negative and a positive inanimate object (a toy snake and an object concealing a treat, respectively). Behavioral responses were videotaped and later coded for aggression, submission, defense, and approach behaviors (Meunier et al., 1999). The results of this study showed that both amygdala lesion groups exhibited decreased levels of aggression, decreased fear towards objects, and hyperorality. However, there was a difference in the severity of these symptoms based on the type of lesions received. The magnitude of the emotional changes was greater after aspiration lesions than after neurotoxic lesions, suggesting that damage to the cortices surrounding the amygdala and/or destruction of fibers of passage may exacerbate the Klüver-Bucy symptoms (Meunier et al., 1999).

The Rhinal cortices and emotional regulation

Despite the strong anatomical connections between the PRh cortex and the amygdala, there is very limited research on the role of the PRh cortex on emotion regulation. Most research

so far has focused on the role of the PRh cortex on object perception and object memory, and the few studies that have investigated its role on emotion regulation have provided conflicting results.

An earlier study by Zola-Morgan et al. (1991) provided support to the view that the PRh cortex plays a significant role in memory but not in emotion regulation. Monkeys received lesions to various parts of the MTL (either the amygdala (2 groups), the hippocampus and surrounding cortices (2 groups), both the amygdala and the hippocampus with the surrounding cortices, or the PRh and parahippocampal cortices). A task was used to observe emotional reactivity by displaying inanimate object and social stimuli to the monkeys, and emotional reactivity was scored based on approach and exploratory behaviors, and avoidance. Monkeys with amygdalar lesions sparing the hippocampus displayed changes in emotional reactivity similar to the hypoemotionality described by Klüver and Bucy, whereas those with hippocampal lesions including the rhinal cortices but sparing the amygdala produced only memory deficits. Lesions of the rhinal cortices alone produced memory deficits and no change in emotional reactivity. When both hippocampal and amygdala structures and the rhinal cortical areas were damaged, the emotional and memory changes observed did not significantly increase as compared to those seen after separate hippocampal or amygdalar lesions alone (Zola-Morgan et al., 1991). However, there was a caveat in this study in that emotional reactivity seen in any of the lesion groups was observed when viewing inanimate object stimuli but not when viewing social stimuli (human lunging at cage, staring at monkey, lip-smacking at monkey, blowing air at monkey, and a conspecific). These findings could have resulted because only approach behaviors, avoidant behaviors, and eye contacts were recorded, without sufficient measure of aggressive, anxious, or defensive behaviors. In addition, as noted by the authors all monkeys had

extensive exposure to humans, which may have impacted the animals' reactivity to the social stimuli used. Therefore, this study had several methodological weaknesses that may have prevented them from fully addressing how rhinal cortex lesions affect emotional processing.

Meunier and Bachevalier (2002) compared emotional reactivity of adult monkeys with selective lesions of the rhinal cortex (including both the ERh and PRh cortices) with that of animals that had received neurotoxic amygdala lesions. Although emotional reactivity of both groups differed from that of controls animals, the behavioral changes of monkeys with rhinal cortex ablations differed from those reported after selective lesions of the amygdala in an approach-avoidance task (described above, see Meunier et al., 1999). The amygdala lesions resulted in a decrease in aggression and defensive behaviors, and an increase in submissive and approach behaviors, as typically seen in Klüver-Bucy syndrome. In contrast, the rhinal lesions resulted in a general decrease in emotional reactivity and withdrawal-like behaviors from the environment, including increased defensiveness, decreased submissive and approach behaviors, and an increase in stereotypic behaviors. None of the symptoms seen in monkeys with rhinal cortex lesions resemble the Klüver-Bucy symptoms. Therefore, the exacerbation of Klüver-Bucy symptoms in monkeys with aspiration lesions of the amygdala seen previously does not seem to be due to additive damage to the surrounding rhinal cortices. The rhinal cortices may play a role in emotional processing, regulation, and memory, but this role differs from that of the amygdala (Meunier & Bachevalier, 2002).

In a recent follow up study, Meunier et al. (2006) investigated which of the cortical components of the rhinal cortex (i.e. ERh and PRh) was responsible for the emotional deficits seen by Meunier and Bachevalier (2002). Specifically, the authors examined the behavioral effects of selective lesions to either the ERh or PRh cortices in monkeys. Using the same

approach-avoidance task described above (i.e. presentation of a toy snake, a rewarded object, a conspecific monkey head, and an unfamiliar masked human), the authors reported that both lesions to the ERh cortex and the PRh cortex yielded emotional changes similar to those seen after complete rhinal ablations, i.e. increased defensiveness and decreased affiliation and submission behaviors. Thus, both PRh and ERh cortical lesions affected the monkeys' behavioral responses to emotional and social stimuli. It is possible that the rhinal cortices are both involved in modulation of anxiety in social and emotional environments (Meunier et al., 2006).

In another study, (Chudasama et al., 2008), emotional behavioral responses were assessed in PRh-operated monkeys viewing emotionally charged stimuli and neutral stimuli. The behavioral response measured in this study was the speed at which the animal will retrieve a food reward placed on the far side of a fearful stimulus (spider and snake). As compared to control animals, monkeys with PRh damage were faster to approach and retrieve the food despite the presence of the snake and spider stimuli. The PRh-operated animals also showed less approach behavior but intact defensive behaviors, which is paradoxical to the faster latency to retrieve the reward. The authors claimed that the faster latencies are similar to those seen in monkeys with amygdala damage, and therefore the PRh cortex could be responsible for mediating this behavior through connections to the amygdala. By contrast, the incongruent intact defensive behaviors may be mediated by another brain region, such as the inferior temporal area (TE), sending direct visual input to the amygdala despite the PRh damage.

Thus, all together the evidence gathered up to now suggests that the PRh cortex is critical for emotional regulation but in a way that differs from that of the amygdala. Whereas amygdala lesions yielded disinhibited behavioral responses to fearful stimuli, the PRh lesions resulted in

more anxious behavior. One hypothesis posited by Meunier and Bachevalier (2002) is that the PRh cortex is responsible for emotional memories and for forming associations between visual stimuli through its interactions with the amygdala. Meunier and Bachevalier (2002) suggested that the amygdala is involved in monitoring the environment, specifically for ambiguous and therefore potentially dangerous situations. When presented with an ambiguous situation, previous experiences as well as creating new associations and memories of the current experience are necessary to understand a situation and therefore behave appropriately. The amygdala plays a more prominent role in vigilance, and lesions of the amygdala result in hypoemotionality. The PRh cortex, however, could be primarily involved in sending information to the amygdala about previous experience and known associations with encountered stimuli. Therefore, lesions to the PRh cortex would result in experiences being both novel and ambiguous, potentially increasing anxiety and defensiveness more generally, regardless of the stimulus encountered or previous experiences. Furthermore, previous studies looking at aspiration lesions to the amygdala that damage the surrounding rhinal cortices would result in an exacerbation of symptoms, since these animals would exhibit symptoms of decreased vigilance or reactivity, as well as lack the knowledge of previous experiences that could help the animals assess ambiguous situations.

MTL structures and HPA-axis regulation

In association with their role in the regulation of emotional reactivity, MTL structures are also known to play a significant role in the modulation of the HPA axis functioning during stressful or fearful situations. The HPA axis is known to regulate the release of stress hormones, such as cortisol, which allows the organism to increase metabolic functions and blood sugar and

suppresses the immune system. Both the hippocampus and the amygdala play a role in regulating stress via the HPA axis. The hippocampus is involved in inhibition of the HPA axis responses to stress. Damage to the hippocampus can cause prolonged corticosterone or ACTH release, and increase basal glucocorticoid levels. Stimulation of the hippocampus alternately reduces HPA axis activity (Herman et al., 2003). In contrast, the amygdala activates the HPA axis. Stimulation of the amygdala promotes corticosteroid or ACTH secretion, and amygdala lesions reduce the levels of glucocorticoids after stressful situations or stimuli. Monkeys with amygdala lesions have a decreased cortisol response after presentation of a stressor, such as isolating them from their peers (Machado & Bachevalier, 2008). Different nuclei in the amygdala are thought to control ACTH secretion under different types of sensory stimuli inducing stress (Herman et al., 2003). Nevertheless, despite the role of the PRh cortex in controlling emotional reactivity, and the evidence that animals with PRh lesions display more anxious behaviors, no studies have investigated whether the PRh cortex also modulates HPA-axis functioning. Thus, future research is needed to fill this gap in our knowledge.

Translational significance

A better understanding of the contribution of the PRh cortex in the regulation of emotional responses and HPA-axis functioning has important clinical applications. For example, phobias are defined by extreme anxiety and emotional reactivity provoked by environmental stimuli. This emotional reaction is sometimes caused by a previous traumatic experience or emotional memory Recent brain-imaging studies measuring cerebral blood flow in the brain have had conflicting results, specifically regarding the temporal cortex, and therefore the brain regions responsible for this behavior remain unknown. Some studies have shown decreases in temporal

cortical blood flow, whereas others have shown increases (Fredrikson et al., 2007; Reiman, 1997). Thus, changes in blood flow in the MTL are common in phobic patients, but the directionality (increase or decrease) of these changes has been difficult to correlate with behavior. Positron Emission Tomography (PET) scans conducted on spider phobic patients to investigate cerebral blood flow during habituation to prolonged exposure to the phobic stimuli showed decreased bilateral blood flow in the MTL. The decreased cerebral blood flow in the PRh cortex was correlated with reduced state anxiety, suggesting that the PRh cortex may be involved in emotional modulation of sensory input and memory. The role of the PRh cortex in anxiety might also suggest that this cortex plays a role in the sympathetic nervous system symptoms related to heightened anxiety, such as increasing cortisol levels and hormonal regulation (Veltman et al., 2004).

In another PET study of patients with social phobias compared to controls, participants were asked to speak in private and in front of an audience while self-reported anxiety levels, heart rate, and regional cerebral blood flow (rCBF) were measured. Results showed increased rCBF in the right amygdala of socially phobic patients as compared to controls. This increase in blood flow was seen in both the private and public speaking conditions, but was of greater magnitude during public speaking, presumably because of higher anxiety levels. Increased blood flow was seen bilaterally in the PRh cortex in controls during the public speaking condition but not during the private speaking condition. This pattern of results may have resulted from a greater general anxiety response in controls to public speaking compared to the private condition (Tillfors, 2001).

Thus, from a clinical standpoint, additional experimental studies on the role of the PRh cortex in the regulation of emotions and HPA axis stress reactivity are clearly warranted. In

addition, many of the human psychopathologies associated with emotional dysregulation and HPA axis dysfunction have their origin in early development. Emotional modulation and social conduct develop through childhood, and many emotional disorders or symptoms of abnormal affect and social behavior become apparent during these years. Autism is detectable in children at a very young age (for review see Machado & Bachevalier, 2003). Some children have high levels of anxiety, which later develop into more serious emotional disorders (Feder, 2004). However, very little is known on the role of the MTL structures in the development of emotional and neuroendocrine functions. Thus, our laboratory has recently designed developmental studies in monkeys to fill this gap.

Neonatal MTL lesions and emotional regulation

Recent studies in our laboratory have investigated how neonatal lesions of the amygdala alter the development of emotional reactivity and are associated with changes in the HPA axis. To be able to make comparisons between the effects of neonatal lesions with those of the adult-onset lesions, emotional reactivity was assessed using the human intruder paradigm (Kalin & Shelton, 1989). In this task, animals are placed in a novel cage and left alone for ten minutes. A human intruder wearing a mask enters the room and presents his profile to the animal, in a no eye contact condition, for ten minutes. The intruder leaves the room to give the animal a three minute break, and then returns and stares at the animal for ten minutes, maintaining eye contact. Finally, the intruder leaves the room, and the animal is left alone in the cage for a final ten minutes. In this task, the animal is videotaped so that animals' behavioral responses can later be coded using a detailed ethogram. This paradigm presents two conditions of different stress levels. In the no eye contact condition, the animal is subjected to a lower stress level because the

intruder in passive and not attending to the animal. The normal predicted reaction for the monkey is to freeze and not attract attention to them. In the stare condition, the animal is subjected to a higher stress level because eye contact signals threat. The normal response to threat for monkeys is aggression. This paradigm can be used to compare the emotional reactivity of monkeys with different variables, such as sex differences, lesion differences, and rearing environment differences (Corcoran et al., 2011).

In a recent study conducted in our lab, the effects of neonatal neurotoxic lesions of the amygdala were compared to controls (neonatal sham surgeries) using the Human Intruder task during development (i.e. 2 months, 4.5 months and adults; Raper et al., 2013). The results showed that monkeys with neonatal amygdala lesions exhibited more freezing then controls at 2 months of age in the no eye contact condition. At 4.5 months of age, however, controls modulate the magnitude of freezing behavior across the Profile and Stare conditions, and the amygdala-operated animals did not. Thus, the emergence of the emotional modulation was altered by early amygdala damage. Upon reaching adulthood, the monkeys with neonatal amygdala lesions were again unable to modulate their behavior as controls did (i.e. freezing during the no eye contact condition, as seen at 4.5 months of age). The monkeys with neonatal amygdala damage exhibited more fearful-defensive behaviors while alone, but they displayed anxious and hostile behaviors across all conditions. This stands in contrast to controls that displayed the most anxious and hostile behaviors during the stare condition. When the effects of early-onset lesions were compared to those of adult-onset lesions (Machado & Bachevalier, 2008), the neonatal amygdala lesions resulted in similar emotional changes as those described with the adult-onset lesions. Thus, the data suggest that neonatal amygdala lesions have long-lasting effects on behavior, and very little functional compensation. The changes in emotional

modulation after neonatal amygdala lesions were correlated with lower basal cortisol levels in adulthood, as well as a blunted cortisol response to stressors in the human intruder paradigm, giving support that the amygdala is important for activating the HPA axis in reaction to stressful situations. This study has led to more detailed information about the role of the amygdala in the development of emotional regulation, and contributes to the activation of the HPA axis and endocrine activity during stressful events.

Current study

The purpose of the current study was an attempt to parse out the selective role of the PRh cortex in behavioral and neuroendocrine stress responses. More specifically, the study tracked the development of emotional reactivity in monkeys that have received neonatal PRh damage as compared to sham-operated controls and normal controls. Emotional reactivity was measured with the Human Intruder task at 2 months, 4 months, and 5 years. In addition, at 5 years of age, basal HPA-axis functioning (cortisol and ACTH levels) was measured at three time points during the day and HPA-axis responses to a social stressor was assessed by measuring stress hormone levels before and after the Human Intruder task. This longitudinal study attempted to further our understanding of the PRh cortex function in emotional development and reactivity to stress. Preliminary results from this experiment were presented in an abstract form (Johnson, Raper, & Bachevalier, 2012).

Hypotheses

We hypothesized that neonatal PRh lesions would cause emotional dysregulation to occur early in development, affecting the monkey's ability to modulate anxious and defensive

behaviors. Given that in normally developing monkeys the modulation of defensive behaviors occurs after 2 months of age (Kalin et al., 1991a), we expected to see very little modulation of behavior at 2 months in both the controls and the PRh operated monkeys, since this is before the critical period in development where there is intentional, planned, and modulated behavior in response to complex environmental situations. In contrast, by 4 months of age, normal control monkeys should demonstrate increased freezing and defensive behavior in the profile condition, and increased aggressive behaviors and threats in the stare condition. Given the previous data obtained in adult monkeys with PRh lesions (Meunier et al., 2006), we hypothesized that monkeys with neonatal PRh cortex lesions will show enhanced defensive behaviors during the Profile condition and decreased affiliative behaviors towards the intruder during the Stare condition. We hypothesized that they may also display more stereotypies or oral exploration as well. These emotional reactivity changes may remain present when the animals are re-tested at 5 years. If the neonatal PRh cortex lesions resulted in long-term heightened freezing and defensive behavior, we also hypothesized a greater increase in blood cortisol and ACTH levels as compared to control animals from pre- to post-stress testing at 5 years of age. Alternatively, our null hypothesis was that the neonatal PRh lesions will result in no, or less, emotional and neuroendocrine changes due to greater functional plasticity after neonatal brain lesions.

Methods

Subjects

The subjects were 10 infant rhesus monkeys (*Macaca mulatta*), 5 male and 5 female, born at the Yerkes National Primate Research Center (YNPRC) field station (Lawrenceville, Georgia). The monkeys were brought to the primate nursery of the YNPRC main station

(Atlanta, Georgia) at 1-2 days of age, and received either bilateral stereotaxic guided ibotenic acid lesions to the PRh cortex (Neo-PRh; males = 3, females = 3), or sham operations or no lesions (Neo-C; male = 2, females = 2) between 10-12 days of age. Surgery and behavioral testing were conducted at the YNPRC main station, and all procedures were approved by the Institutional Animal Care and Use Committee for Emory University.

Animals were surrogate-peer reared with human caregivers and age-matched peers according to similar procedures described previously (Zeamer, 2009; Goursaud & Bachevalier, 2007). Infants were housed individually in wire cages under an incubator until 3 months of age, with 2 wire cages adjacent to each other to allow for visual, auditory, and olfactory contact between individuals. Infants were handled, played with, and fed several times a day by a principle caregiver, and from 3 to 9 months of age, infants were socialized daily with age- and sex-matched peers in a central cage containing toys for 3-4 hours. Infants were hand-fed Similac formula until 3-4 weeks of age, when they were able to self-feed, and their diet was supplemented with banana pellets (190 mg, P.J. Noyes, Cleveland, OH). By 3 months of age, animals were pair-housed, and diets were supplemented with Purina monkey chow and fresh fruit daily, given ad libitum after completion of testing once a day, with access to water at all times. Rooms had automatically regulated lighting (12 hour light, 12 hour dark cycle).

Evaluation of emotional reactivity using the Human Intruder Paradigm took place at about 2 months of age, 4.5 months of age, and at 5 years (post-puberty) to assess short-term and long-term developmental effects on behavior. Imaging and surgical procedures were described in details previously by Zeamer (2009), and are summarized here.

Neuro-imaging Procedures

Subjects underwent a series of magnetic resonance imaging (MRI) scans immediately before surgery, and one week and one year after surgery. The animals were anesthetized with Ketamine Hydrochloride and Xylazine (10 mg/kgg of 7:3 Ketamine Hydrochloride, 100 mg/ml, and Xylazine, 20 mg/ml, i.m.). Animals were then intubated, given an intravenous drip (dextrose and 0.45% sodium chloride) for hydration, placed on a heating pad to keep the animal at normal body temperatures, and maintained under general anesthesia (isoflurane, 1-2% to effect) during scanning and surgical procedures. Vital signs, such as heart rate, respiration rate, blood pressure, and CO_2 exhalation were monitored during the procedures by veterinary staff. Preparation for the scan included shaving the animal's head, applying EMLA cream (lidocaine 2.5% and prilocaine 2.5%) to the ear canals and skin below the eye orbits, and securing its head in a nonferromagnetic stereotaxic apparatus. Opthalmic ointment was also applied to the eyes to avoid dryness or irritation to the eyes during surgery. Each scanning session included a coronal 3D T1-weighted structural scan (spin-echo sequence, echo time [TE] = 11 ms, repetition time [TR] = 450 ms, contiguous 1 mm sections, 12 cm FOV, 256 x 256 matrix) and additionally for Neo-PRh animals, three series of coronal 3D T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) scans (fast spoiled gradient (FSPGR)-echo sequence, TE = 2.6 ms, TR = 10.2 ms, 25° flip angle, contiguous 3 mm sections, 12 cm FOV, 256 x 256 matrix), offset by 1 mm posteriorly, using a 3 T Siemens Magnetom Trio system (Siemens Medical Solutions, Malvern, PA at YNPRC). Pre-surgical T1 images were used to determine stereotaxic coordinates for neurotoxin injections for group Neo-PRh. Post-surgical FLAIR images were compared to pre-surgical scans to quantify the extent of the lesions. At completion of the pre-surgical scanning sessions, animals were maintained anesthetized in the stereotaxic apparatus and immediately transported to the surgical suite.

Surgery

Surgical procedures were conducted under general anesthesia using aseptic techniques. An IV drip containing 5.0% dextrose and 0.45% sodium chloride maintained normal hydration, and a blanket placed around the animals and attached to a Bair Hugger® Therapy warming unit provided warm air to maintain body temperature. The shaved skin on the scalp was disinfected with Nolvasan, and a local anesthetic (Marcain 25%, 1.5 ml) was injected subcutaneously along the incision line (along the midline). The skin was opened and the galea tissue was moved laterally to expose the skull. Craniotomies (1 cm wide x 2.5 cm long) were created using an electric drill directly above the injection sites (bilaterally), and the dura mater was cut and retracted to expose the brain. Neo-PRh animals received simultaneous bilateral injections of 0.4 µl ibotenic acid (Biosearch Technologies, Novato, CA, 10 mg/ml in PBS, pH 7.4) to Brodmann's areas 35 and 36 in 3 injection sites, 2 mm apart along the antero-posterior axis of the PRh cortex, using a Hamilton syringe (see Figure 2). Each injection was delivered 0.2 µl per minute and when the injection was completed the needle was maintained in place for an additional 3 minutes before being retracted. After injections were completed, the dura was sewn closed with interrupted sutures (2.0 Vicryl; Ethicon, Somerville, NJ), and the bone opening was covered with Surgicel. Then the galea was sutured closed with interrupted sutures (3.0 Dexon with a T5 needle; Ethicon, Somerville, NJ) and skin were sutured closed with a continuous subcuticular suture (3.0 Ethilon with a cutting needle; Ethicon, Somerville, NJ). The animal was fully recovered from the anesthesia.

The same surgical procedures were used for the sham operations, with the exception of the needles that were not descended in the brain and the injections were not made. Instead, after opening of the dura, the tissue layers were sutured and the opening covered with Surgicel.

Animals were treated 12 hours pre-surgically and 7 days post-surgically with dexamethasone sodium phosphate (0.4 mg/kg, i.m.) and Cephazolin (25 mg/kg, i.m.) to reduce edema and prevent infection, respectively. Acetaminophen (10 mg/kg, p.o.) was administered 4 times a day for 3 days for pain reduction.

MRI-based Lesion Reconstruction

Histological evaluations of the lesion extents were unavailable, since the animals are currently participating in additional cognitive testing. Instead, the one week post-surgical Fluid attenuated inversion recovery (FLAIR) and T1 MRI images were used to estimate the lesion extent using the hypersignals, indicative of edema induced by cell death, seen on the FLAIR images. The extent of hypersignals seen on each coronal section at 1-mm intervals was drawn onto corresponding coronal sections of a normal one week old rhesus monkey brain (J. Bachevalier, unpublished atlas) using Adobe Photoshop software (for reviews of procedures, see Malkova et al., 2001; Nemanic et al., 2002). These images were imported into Image J® to measure the surface area in square pixels of hypersignals seen in the PRh cortex as well as adjacent brain regions (ERh cortex, parahippocampal cortex, amygdala and hippocampus). Surface area of each section were added and divided by image thickness (1mm) to obtain the volume of hypersignals in each hemisphere for each structure. The volumes were then divided by the volume of each structure in the control brain and multiply by 100 to obtain the percentage of total damage for each structure individually.

Behavioral Assessment

Emotional reactivity towards an unfamiliar Human Intruder was evaluated at 2 months (range: 59 to 64 days), 4.5 months (range: 136 to 140 days), and 5 years of age using the four 10-minute conditions of the Human Intruder Paradigm (Alone-pre, Profile/no eye contact (NEC), Stare, and Alone-post) in both Neo-C (males = 2, females = 2) and Neo-PRh (males = 3, females = 3) animals.

Testing at 2 and 4.5 months: On the day of testing, the subject was removed from its home cage at 7:00 am and transported to a testing cage in a novel room. The testing cage had been modified to have a plexiglass side to allow video recording of animal's behaviors without obstruction. A video camera was set up facing the plexiglass side of the cage. In the first condition (Alone-pre), the subject was given 10 minutes alone in the cage as an acclimation period, and a baseline control condition for behavior. In the second condition (Profile), the experimenter entered the room wearing a rubber human mask, and, while presenting his/her profile and avoiding eye contact, sat 2 meters away from the plexiglass front of the cage, and remained motionless for ten minutes. The intruder then left the room for three minutes to give the animal a brief break. For the third condition (Stare), the Intruder re-entered the room and sat facing the subject, making direct eye contact for ten minutes. The Intruder then stood and left the room, leaving the animal alone for another 10 minutes for the alone-post condition. The experimenter then removed the mask and re-entered the room to return the subject to its home cage. The same procedure was repeated the next day with the presentation of the Profile and Stare conditions reversed. Order of presentation of the Profile and Stare conditions were counterbalanced across day and across subjects.

Testing at 5 years: The Human Intruder task was slightly modified to accommodate the collection of blood samples immediately before, after, and 45 minutes after the social stressor to assess HPA axis activation and regulation after a stressor through hormone analyses (see Figure 3). The task was given in only one session instead of two days of testing, the Alone-post condition was removed, and the duration of each remaining condition was reduced such that the post-test blood sample occurred at the peak of cortisol secretion (approximately 30 minutes). Thus, adult testing included an Alone-pre condition of 9 minutes, a Profile condition of 9 minutes followed by a break of 3 minutes, and finally a Stare condition of 9 minutes.

Sample Collection

For collection of the blood samples in unanesthetized animals, subjects were trained to voluntarily present their leg through a central hole in the side of the testing cage using positive reinforcement to allow access to the saphenous vein for a blood draw. Animals trained under these conditions show reliable basal hormone levels (Blank, Gordon, &Wilson, 1983; Raper et al., 2013). Elevations in cortisol can be detected in the blood within 10 minutes of initial disturbance or stress, so all baseline blood samples were collected within 10 minutes of entrance to the housing room.

To assess HPA axis stress response activity during an acute social stressor (the human intruder), three blood draws were taken on the day of testing to later be analyzed for levels of cortisol and ACTH. The first sample was taken at lights-on (7:00 am), after the subject had been transported to the novel cage. The first sample serves as a baseline control immediately before the human intruder task. A second sample was taken immediately after the stare condition of the Human Intruder task, 30 minutes after the baseline sample. The animal was then returned to the

home cage. Forty five minutes after being returned to their normal housing conditions, the experimenter then transported the animal to a familiar cage and testing room for a third sample, to determine any dysfunction in the HPA axis, or the ability to regulate cortisol levels back to basal levels after an acute stressor.

As a control, baseline blood samples were taken on a different day (two days before human intruder testing) without presentation of the stressor (human intruder task) to ensure that changes in cortisol or ACTH levels in the presence of the social stressor did not result from handling of the animals and blood draws. For sample collection, the animal was transported to a familiar room and placed in a familiar cage. The first sample was collected at lights-on (7:00 am). Instead of the human intruder task, the subject was returned to their home cage after sample collection. This procedure was repeated after 30 minutes, and then after 45 minutes, to obtain baseline blood hormone levels at the same time points as were obtained during human intruder testing.

Finally, basal functioning of the HPA axis was measured by collecting diurnal blood hormone samples at lights-on (7:00 am), mid-day (1:00 pm), and evening (lights-off, or 7:00 pm) on three different days, several months after the Human Intruder and Baseline testing days, to track the circadian rhythm of cortisol. These samples were collected under the same conditions and with the same procedure as the baseline samples. One diurnal sample was collected per week on each monkey, taking a total of three weeks to collect all three samples. Animals were counterbalanced for the order in which samples were collected.

Hormonal Assays

All blood samples were collected in chilled vacutainer tubes containing EDTA (3.6 mg) and kept 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. All assays were performed by the YNPRC Biomarker Core Laboratory. Plasma samples from both the Human Intruder task and baseline days were assayed first for ACTH, and then for cortisol. ACTH samples were assayed in duplicate by radioimmunoassay (RIA) using commercially prepared kits (DiaSorin, Inc., Stillwater, MN). The DiaSorin assay sensitivity was 12.50 pg/ml, and intra-assay and inter-assay coefficients of variation were less than 5.4%. All plasma samples (including diurnal samples) were assayed for cortisol using liquid chromatography – mass spectroscopy (LC-MS). LC-MS analyses were performed using reverse phase chromatography on an LTQ-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). Quantitation was achieved by treating samples with an internal standard d4-Cortisol (CDN Isotopes, Pointe-Claire Quebec, Canada). The assay range was 2.5-60.0 ug/dl, with intra-assay and inter-assay coefficients of variation being less than 8.6%.

Data Analysis

Video data was coded with Observer XT 10 (Noldus Inc.) using the behavioral ethogram previously used to study other types of MTL lesions in this same task, documenting defensive, anxious, hostile, affiliative, and exploratory behaviors (See Table 1). One experimenter coded all videos, but had an inter-rater reliability of Cohen's Kappa = .80 with experimenters who coded videos of the same task using the same ethogram. Statistical analysis was calculated on frequency and duration of behaviors across treatment (lesion and control), condition (alone-pre, profile, stare, and alone-post), and age (2 months, 4.5 months, and 5 years) using ANOVAs.

Preliminary analyses were first conducted on behavioral data at 2 months and 4.5 months of age to compare the emotional reactivity between day 1 and day 2 of testing at each time point. Repeated measures ANOVA (Condition X Testing Day) revealed no significant interactions indicating no differences in results of Day 1 and Day 2, and therefore the behavioral data from these two days were averaged for each animal to create one composite score for each behavior at each age. All behavioral measures that do not satisfy normality were transformed using an LnX +1 constant. One Neo-C animal exhibited freezing behaviors 2 standard deviations from the group mean across all conditions at 2 months and 4.5 months of age, and was therefore excluded from analysis at these time points.

First, Neo-C behavior was assessed at 2 and 4.5 months of age to evaluate the normal development of emotional reactivity through repeated measure ANOVAs (Condition (4) X Age (2)) with age as the repeated measure. Differences in the development of behavior between Neo-C and Neo-PRh groups were established using repeated measure MANOVAs (Group (2) X Condition (4) X Age (2)) with Group and Condition as main factors and age as the within subject repeated measure.

Next, adult behaviors were analyzed separately, and the normal expression of emotionally reactive behaviors in adulthood was evaluated through one-way repeated measures ANOVAs (with Condition (3) as the repeated factor) on group Neo-C. To assess the differences in behavioral expression between Neo-PRh and Neo-C animals as adults, repeated measure ANOVAs (Group (2) X Condition (3)) were used, with Condition as the repeated measure.

Finally, adult behaviors were analyzed in comparison to behaviors at 2 months and 4.5 months of age to assess changes that occurred across development. First, a ratio of each behavior during the 2 month and 4.5 month time points was created to correct for the time differences in

the task during infancy compared to adulthood (at 2 and 4.5 months, each condition was 10 minutes long while during adulthood, each condition was 9 minutes long). The second alone condition at 2 and 4.5 months was dropped from the analysis, since this condition was not run on the adults. Finally, the Neo-C that was excluded from analysis in the infant behavioral data as an outlier was also excluded here in the comparison to adult behaviors. The normal development of emotional reactivity was assessed through repeated measure ANOVAs on the Neo-C group (Condition (3) X Age (3)) with age as the repeated measure. To evaluate the differences between Neo-PRh and Neo-C groups on the development of these behaviors, repeated measures MANOVAs were used (Group (2) X Condition (3) X Age (3)) with group and condition as the main factors and age as the within group repeated measure. Finally, correlations were run between behaviors with statistically significant differences and the extent of total damage and total weighted damage of PRh and ERh of each subject.

To assess differences between animals with neonatal PRh damage and neonatal Amygdalar damage, one-way repeated measures ANOVAs (with condition (3) as the repeated factor) were conducted on the adult behavioral data from group Neo-PRh in the current study, and group Neo-A from a previous study conducted in our lab (Raper et al., 2012).

Discriminant function analyses were conducted for each age (2 months, 4.5 months, and adulthood) to test if specific behaviors during the human intruder task could accurately classify individual animals by group (Neo-C, Neo-PRh). Behavioral factors included in the discriminant function analyses included coo vocalizations, fearful defensive behaviors, freezing, hostility, stereotypies/pacing, and affiliative behaviors. These behavioral factors were selected based on previous studies that demonstrated changes in these behaviors due to PRh cortex damage (Meunier & Bachevalier, 2002; Meunier et al., 2006). To test if the discriminant function

analysis categorized animals above chance levels, the Press's Q statistic was calculated (Hair et al., 2009).

Hormonal levels of cortisol and ACTH during adulthood were compared using a repeated measures ANOVA between subjects across Group, with Time (pre-stressor, post-stressor, and +45 minutes for Human Intruder test, or lights-on, +30 minutes, and +45 minutes for baseline day) as the within subjects repeated measure. Diurnal cortisol rhythm was analyzed using a repeated measures ANOVA between subjects across groups, with Time (lights-on, mid-day, and lights-off) as the within subjects measure.

Results

Lesion assessment

All Neo-PRh subjects received extensive bilateral PRh damage, ranging from 67.1% to 83.3%, with an average total damage of 73.6% (see Table 2). Unintended damage to the ERh was found in all subjects, from as little as 5.4%, to 34.5% (averaging at 20.6%). All subjects also had varying levels of unintended damage to area TE, from 0.1% to 7.11% (averaging at 2.5%). Four of the six subjects had negligible damage to the anterior hippocampus (average 0.8%), and three of the six subjects had minor damage to the amygdala (average 2.5%). Pre-surgical and post-surgical MR FLAIR images of two representative cases are presented in Figure 2, demonstrating the injection sites on the pre-surgical images and the resulting extent of hypersignals post-surgery.

Development of normal regulatory behaviors

Table 3 provides the results of the statistical analyses for all behaviors and the 3 ages for the control animals. Only significant results will be discussed below.

Previous studies quantifying the ability of rhesus monkey infants to modulate their behavior based on the presence and gaze direction of an intruder have shown that normally developing monkeys begin freezing most during the profile condition, and being hostile mostly during the stare condition when they reach 9-12 weeks of age (Kalin et al., 1991a). The current study showed that Neo-C animals displayed an increase in freezing behaviors during the profile condition at both 2 and 4.5 months of age, as shown by a main effect of condition ($F [3, 8] = 6.46$, $p = 0.02$, $\eta^2 = 0.71$; Fig. 4a). Control animals also expressed an increase in hostile behaviors during the stare condition ($F [3, 8] = 14.93$, $p = 0.001$, $\eta^2 = 0.85$; Fig. 4b), and although these behaviors were produced at 2 months, there was a significant increase in the frequency of these behaviors at 4.5 months of age, as shown by a main effect of age ($F [1, 8] = 6.64$, $p = 0.03$, $\eta^2 = 0.45$). There was an Age by Condition interaction in affiliative behaviors ($F [3, 8] = 5.20$, $p = 0.03$, $\eta^2 = 0.66$; Fig. 4d), with controls displaying less affiliative behaviors during the stare condition at 4.5 months of age. Control animals also displayed more anxious behaviors at both 2 and 4.5 months during the stare condition ($F [3, 8] = 14.87$, $p = 0.001$, $\eta^2 = 0.85$; Fig. 5c). Finally, when examining vocalizations in group Neo-C, there were main effects of age for both the frequency of coo vocalizations and the frequency of screams, where control animals screamed more at 2 months of age ($F [1, 8] = 15.64$, $p = 0.004$, $\eta^2 = 0.66$; Fig. 6b), and made more coo vocalizations at 4.5 months of age ($F [1, 8] = 9.86$, $p = 0.01$, $\eta^2 = 0.55$; Fig. 6a).

Effect of neonatal PRh cortex lesions on the development of regulatory behaviors

Table 4 provides the results of statistical analyses comparing Neo-C and Neo-PRh for all behaviors and the 3 ages. Only significant results will be discussed below.

As shown in Figure 4, Neo-PRh animals display very different patterns of freezing and hostile behaviors across conditions compared to controls. Neo-PRh animals exhibit overall higher levels of freezing behaviors across all conditions ($F [1, 28] = 5.38$, $p = 0.03$, $\eta^2 = 0.16$; Fig. 4a), instead of modulating the amount of freezing in response to the direction of gaze of the intruder. For hostile behaviors, there was a significant Group by Condition interaction ($F [3, 28] = 3.21$, $p = 0.04$, $\eta^2 = 0.26$; Fig. 4b), indicating that the Neo-PRh group displayed less hostile behaviors compared to Neo-C animals. Planned comparisons revealed that Neo-PRh animals displayed a lower frequency of hostile behaviors during both the profile and stare conditions compared to group Neo-C, regardless of age ($F [1, 8] = 106.90$, $p < 0.001$; $F [1, 8] = 16.79$, $p = 0.005$, respectively). Similar to the pattern seen in freezing duration, Neo-PRh animals also displayed a higher frequency of fearful/defensive behaviors overall, across all conditions compared to controls (Group: $F [1, 28] = 4.32$, $p = 0.05$, $\eta^2 = 0.13$; Fig. 4c). Furthermore, there were significant main effects of group on both self-soothing ($F [1, 28] = 7.81$, $p = 0.009$, $\eta^2 = 0.22$; Fig. 5d) and self-directed behaviors ($F [1, 28] = 6.37$, $p = 0.02$, $\eta^2 = 0.19$; Fig. 5b), with Neo-PRh animals displaying longer durations of each behavior compared to controls across conditions. Despite these findings of increased self-soothing and self-directed behaviors, there were no effects of species-typical anxious behaviors between groups (Group: $F [1, 28] = 0.88$, $p = 0.34$, $\eta^2 = 0.03$; Fig. 5c) or stereotypy (Group: $F [1, 28] = 0.003$, $p = 0.74$, $\eta^2 = 0.001$; Fig. 5a). Comparison of Neo-PRh and Neo-C groups on overall activity levels revealed that Neo-PRh animals locomoted less ($F [1, 28] = 9.32$, $p = 0.005$, $\eta^2 = 0.25$; Fig. 6a), and explored the cage less ($F [1, 28] = 9.77$, $p = 0.004$, $\eta^2 = 0.26$; Fig. 6b) compared to controls, irrespective of age or

condition. When comparing vocalizations across groups, there was a Group by Age interaction for frequency of coo vocalizations (F [1, 28] = 4.89, p = 0.04, η^2 = 0.15; Fig. 7a), although no differences in the frequency of screams. Planned comparisons indicated that Neo-PRh animals were producing more coo vocalizations at 2 months of age compared to controls. Finally, when looking at affiliative behaviors, there was a main effect of condition with more affiliative behaviors during the stare condition, and a main effect of age with more affiliative behaviors at 2 months of age (F [3, 28] = 8.19, p = 0.001, η^2 =0.47; F [1, 28] = 7.86, p = 0.009, η^2 =0.22 respectively; Fig. 4d), with no effects of group.

Emotional reactivity in controls during adulthood

Control animals demonstrated an ability to modulate their freezing, anxious, and affiliative behaviors as adults based on the gaze direction of the intruder. Main effects of condition revealed that controls displayed higher levels of freezing during the profile condition (F [2, 6] = 8.29, p = 0.02, η^2 = 0.73; Fig. 8a), and higher levels of anxious behaviors (F [2, 6] = 14.16, p = 0.005, η^2 = 0.83; Fig. 9c) and affiliative behaviors (F [2, 6] = 7.24, p = 0.03, η^2 = 0.71; Fig. 8d) during the stare condition.

Long-term effects of neonatal PRh cortex lesions on emotional reactivity

Comparisons of the Neo-PRh and Neo-C in adulthood revealed that neonatal PRh cortex lesions had long term effects on emotional reactivity to the presence and gaze direction of the human intruder. For the duration of freezing behavior, the Group by Condition interaction was just short of significance with a large effect size (F [2, 16] = 3.54, p = 0.05, η^2 = 0.31; Fig. 8a). Planned contrasts indicated that Neo-PRh animals did not increase freezing behaviors between

the alone and profile conditions compared to controls (F [1, 8] = 3.90, p = 0.08, η^2 = 0.33), suggesting that as adults, Neo-PRh animals are still unable to modulate their freezing behaviors in relation to the presence and gaze direction of the intruder. Neo-PRh animals trended towards less freezing behaviors compared to controls in the stare condition as well (F [1, 9] = 4.37, p = 0.07). Both groups expressed higher frequencies of hostile behaviors during the stare condition, with a main effect of condition (F [2, 16] = 9.42, p = 0.002, η^2 = 0.54; Fig. 8b), and no significant main effect of group or interaction. Although as adults control animals produced very few, if any vocalizations during the task, Neo-PRh animals made coo vocalizations across all conditions of the task, resulting in a main effect of Group on the frequency of coo vocalizations made (F [1, 8] = 5.59, p = 0.05, η^2 = 0.41; Fig. 10). No significant main effects or interactions were found for any other behaviors (See Table 4).

Comparison of behavior from infancy to adulthood in controls

Normal emotional reactivity to the human intruder was compared between infancy and adulthood to establish long term developmental changes in behavior. There was a significant Condition by Age interaction in freezing duration (F [4, 12] = 4.10, p = 0.03, η^2 = 0.58; Fig. 8a), and planned contrasts revealed that control animals expressed more freezing behavior as adults during the stare condition (F [2, 4] = 39.38, p = 0.002, η^2 = 0.95). The control group also displayed a higher frequency of fearful/defensive behaviors during adulthood, with a main effect of age (F [2, 12] = 4.70, p = 0.03, η^2 = 0.44; Fig. 8c). For hostile behaviors, there was a main effect of age (F [2, 12] = 14.81, p = 0.006, η^2 = 0.71; Fig. 8b), with adults expressing less hostility compared to 4.5 months of age. There was a main effect of Condition for affiliative behaviors, with controls displaying more affiliative behaviors towards the intruder during the

stare condition at 2 months and adults but not at 4.5 months (F [2, 6] = 13.30, p = 0.006, η^2 = 0.82; Fig. 8d). Controls also produced less frequent vocalizations, specifically producing less coos as adults, with a main effect of age (F [2, 12] = 28.96, p < 0.001, η^2 = 0.83; Fig. 10). Finally, in terms of overall activity, controls spend slightly less time locomoting (Age: F [2, 12] = 11.27, p = 0.002, η^2 = 0.65; Fig. 11a), and cage exploring (Age: F [2, 12] = 6.84, p = 0.01, η^2 = 0.53; Fig. 11b) across all conditions as adults as compared to infancy.

Comparison of behavior from infancy to adulthood in Neo-PRh animals

To quantifiably asses the developmental changes due to effects of the PRh cortex lesion, behavioral reactivity to the human intruder during infancy and adulthood is compared. As seen in Figure 8, there is a Group by Age interaction (F [2, 42] =3 .70, p = 0.03, η^2 = 0.15; Fig. 8a) in the duration of freezing indicating that Neo-PRh animals displayed comparable durations of freezing behaviors across conditions at the 3 ages, whereas control animals modulated the amount of this behavior at the 3 ages. Planned comparisons indicate Neo-PRh animals showed an increase in freezing from 2 to 4.5 months (F [1, 17] = 3.42, p = 0.08, η^2 = 0.17; Fig. 8a), but displayed equal durations of freezing between 4.5 months and adulthood, suggesting that effects of the lesion on freezing behavior persisted into adulthood. By contrast, Neo-C animals increase the duration of time spent freezing between 4.5 months and adulthood (F [1, 8] = 5.70, p = 0.04, η^2 = 0.42). A similar pattern is seen for the frequency of fearful/defensive behaviors, with a Group by Age interaction (F [2, 42] = 4.14, p = 0.02, η^2 = 0.17; Fig. 8c). Thus, the Neo-PRh animals showed equal fearful/defensive behaviors across all three ages with no modulation across conditions, whereas the Neo-C group expressed more fearful/defensive behaviors as adults compared to 4.5 months (F [1, 8] = 5.85, p = 0.04, η^2 = 0.42),. There was also a Group by Age interaction for self-

directed behaviors (F [2, 42] = 11.56, p < 0.001, η^2 = 0.36; Fig. 9b) indicating that, as compared to controls, Neo-PRh animals showed longer durations of self-directed behaviors in infancy (F [1, 21] =4.50, p = 0.05, η^2 = 0.18), but then significantly less self-directed behaviors during adulthood (F [1, 21] = 16.01, p = 0.001, η^2 = 0.43). Interestingly, a different pattern emerged in the duration of motor stereotypies and pacing combined (Group by Age interaction: F [2, 42] = 8.06, p = 0.005, η^2 = 0.28; Fig. 9a). Planned contrasts indicated that there is a sharp increase in these behaviors in Neo-PRh animals with maturation as compared to controls (F [1, 21] = 5.24, p = 0.03, η^2 = 0.20). When comparing hostility across ages, all monkeys decreased the amount of hostility with Age (F [2, 42] = 6.36, p = 0.008, η^2 = 0.23; Fig. 8b), Neo-PRh expressed less hostility as compared to Neo-C (F [1, 21] = 8.19, p = 0.009, η^2 = 0.28), and finally all monkeys displayed the most hostility during the stare condition (F [2, 21] = 29.00, p < 0.001, η^2 = 0.73). There were no group differences in affiliative behaviors, but there were main effects of both Condition and Age (F [, 21] = 11.16, p = 0.001, η^2 = 0.52; F [2, 42] = 3.85, p = 0.03, η^2 = 0.16, respectively), with all animals showing an increase in affiliative behaviors during the stare condition, and the highest levels of affiliative behaviors at 4.5 months of age. No significant main effects or interactions were found for frequency of coo vocalizations (Group: F [1, 21] = 3.70, p = 0.07, η^2 = 0.15; Fig. 10), or frequency of species-typical anxious behaviors (Group: F [1, 21] = 1.83, p = 0.19, η^2 = 0.08; Fig. 9c).

Effects of neonatal PRh cortex lesions on HPA axis functioning

No main effects of Group and no interactions were found on HPA axis functioning for either measures of ACTH or cortisol. As seen in Figure 13, the diurnal rhythm of cortisol was present in both groups, with cortisol levels peaking at lights-on and decreasing by both the mid-day and

lights-off time points (F [2, 16] = 82.84, p < 0.001, η^2 = 0.91). When measured on a baseline day to control for stress due to handling 2 days prior to the human intruder testing day, cortisol levels significantly decreased for both groups between 30 minutes after lights-on and 75 minutes after lights-on (F [2, 16] = 7.26, p = 0.006, η^2 = 0.48; Fig. 12a). By contrast, on the human intruder test day, there was a significant increase in cortisol levels for both groups between pre- versus post-stressor (F [2, 16] = 8.43, p = 0.02, η^2 = 0.51; Fig. 12b). Similar changes were seen in levels of ACTH on both the baseline day and human intruder testing day. For baseline measurements, ACTH levels significantly decreased between lights-on and 30 minutes later for both groups (F [2, 16] = 4.12, p = 0.04, η^2 = 0.34; Fig. 12c). On the day of the human intruder task, in both groups, ACTH levels increased significantly from pre- to post-stressor (F [2, 16] = 9.84, p = 0.002, η^2 = 0.55; Fig. 12d).

Behavioral Correlations with Lesion

Correlations were run on behaviors that had significant group differences and total weighted damage to the PRh cortex. Only one positive correlation was found between extent of damage to the PRh and fearful/defensive behaviors at 4.5 months of age (r = 0.43, p = 0.04).

Comparisons between effects of neonatal PRh and neonatal Amygdala lesions

Behavior of group Neo-PRh in the current study was compared with behavior of group Neo-A on the same task from a previous study (Raper et al., 2012) to determine whether neonatal PRh lesions resulted in different behavioral symptoms that those caused by neonatal amygdala lesions (Neo-A). For freezing behaviors, the difference between the two groups did not reach significance but the effect size was large (Group: F [1, 10] = 3.77, p = 0.08, η^2 = 0.27) indicating

that Neo-PRh animals had higher levels of freezing compared to Neo-A animals. Similarly, for self-directed behaviors, Neo-PRh animals displayed more of these behaviors across all conditions as compared to group Neo-A ($F [1, 10] = 4.84$, $p = 0.053$, $\eta^2 = 0.33$). Finally, there was a Group by Condition interaction in the duration of stereotypies/pacing behavior ($F [2, 20] = 4.54$, $p = 0.02$, $\eta^2 = 0.31$). Neo-PRh animals displayed higher numbers of stereotypies from the profile to the stare conditions, whereas Neo-A animals showed a decrease in the number of stereotypies between these two conditions. In the previous study, Neo-A animals were determined to have lower levels of cortisol post-stressor, resulting in a significantly lower percent change in cortisol after the human intruder task compared to controls (Raper et al., 2012). In the current study, Neo-PRh animals were not significantly different from controls in any hormonal response. When hormonal levels were compared directly between Neo-PRh and Neo-A animals, group Neo-A had a significantly smaller percent change in cortisol during the human intruder task ($F [1, 11] = 4.98$, $p = 0.05$). Comparatively, levels of ACTH in Neo-A and Neo-PRh animals resembled controls pre- and post- stressor. When directly compared to each other, Neo-PRh and Neo-A animals were not statistically different in levels of ACTH on either the human intruder task day or the baseline day (Group: $F [1, 10] = 1.06$, $p = 0.33$, $\eta^2 = 0.10$, $F [1, 10] = 1.34$, $p = 0.28$, $\eta^2 = 0.12$, respectively).

Discriminant function analysis

A discriminant function analysis was used to test whether coo vocalizations, fearful defensive behaviors, freezing, hostility, stereotypies/pacing, or affiliative behaviors during human intruder testing at 2 months, 4.5 months, and adulthood could classify individuals by group (Neo-C, Neo-PRh) accurately. At two months of age, the Wilks's Lambda was not

significant, indicating that the Neo-PRh and Neo-C groups could not be classified based on these behaviors at this age. By 4.5 months of age, however, the Wilks's Lambda reached significance ($\Lambda = 0.13$, χ^2 [4, N=9] = 10.14, p = 0.04), which differed significantly from chance (Press's Q = 36.0, df = 4, p < 0.01). The two groups could be discriminated with 100% total variance when using hostility (r = -0.57) and anxious behaviors (r = -0.26) during the stare condition, and freezing (r = 0.22) and self-directed behaviors (r = 0.26) during the profile condition. None of the other behaviors could predict group classification with confidence. When discriminant analyses were run at 5 years of age, the Wilks's Lambda was again significant ($\Lambda = 0.23$, χ^2 [3, N=9] = 8.01, p = 0.046) with discrimination of the two groups with 100% total variance when using stereotypies (r = 0.68) across all conditions of the task, and affiliative behaviors (r = -0.22), and coo vocalizations (r = 0.58) during the alone condition, which differed significantly from chance (Press's Q = 27.0, df = 3, p < 0.01). Other behaviors were not predictive of group classification. This analysis indicates that the expression of hostility, anxiety, freezing, stereotypies, self-directed behaviors, affiliative behaviors, and coo vocalizations during certain conditions of the human intruder task can correctly predict and classify animals with neonatal PRh cortex damage from control animals, but only at 4.5 months and in adulthood.

Discussion

The present study replicates previous findings of the developmental trajectories of normal emotional reactivity in monkeys as well as helps to characterize the role of the PRh cortex in the modulation of emotional behaviors. More specifically, the results showed that neonatal lesions of the PRh cortex disrupted normal modulation of freezing, fearful/defensive behaviors, hostility, self-directed behaviors, stereotypies, cage exploration, locomotion, and vocalizations to the

presence and gaze direction of a human intruder in infancy, and that these profound changes in emotional behaviors after Neo-PRh lesions lasted into adulthood. Nevertheless, the neonatal PRh lesions did not disrupt normal neuroendocrine function of the diurnal rhythm of cortisol, as well as the modulation of cortisol and ACTH levels before and after a stressor, since both the Neo-PRh animals and the controls displayed an increase in cortisol and ACTH after the stressor. Finally, neonatal PRh lesions disrupt the normal development of emotional regulation with little neural compensation with further maturation, and resulted in behavioral changes distinctive from those reported after neonatal amygdala lesions. These results will be discussed in turn below.

Emotional responses to a stressor in sham-operated control monkeys

The results replicate those of previous research (Raper et al., 2012) documenting the normal development of emotional reactivity to a Human Intruder tin monkeys reared in a primate nursery with surrogates and peers. Both Kalin and colleagues (1991a) studying emotional reactivity in mother-reared rhesus monkeys as well as Raper and colleagues (2013) measuring emotional reactivity in mother-reared infants living in large social groups indicated that the ability to modulate defensive freezing and hostile behaviors in relation to the gaze direction of an intruder is present by 9 to 12 weeks of age. In the current study, an increase in freezing during the profile condition in sham-operated controls was similarly present from 2 months of age (8-9 weeks) until adulthood, suggesting that the developmental changes of brain structures necessary to produce contextually relevant freezing had already occurred. In addition, the ability to modulate hostility when the intruder stared at the subjects was expressed slightly later, at 4.5 months of age, suggesting that the brain structures necessary to coordinate aggressive behaviors towards a threat had not fully developed at the earliest age tested. During adulthood, sham-

operated controls continued to express an increase in fearful/defensive behaviors during the profile condition, although the amount of overall hostility directed towards the intruder in the stare condition slightly decreased as compared to those measured in infancy. Sham-operated control animals also modulated their anxiety and affiliative behaviors at all three ages of testing, displaying more species-typical anxious and affiliative behaviors during the stare condition than the other two conditions, suggesting an ability to modulate these behaviors as seen in previous studies (Kalin et al., 1991a). These results suggest that surrogate-peer rearing did not alter the normal development of defensive or aggressive behaviors in the presence of a threat.

This conclusion is further supported by a previous study systematically investigating the effects of rearing condition on stress responses in infant monkeys between 12 and 16 weeks of age (Rommeck et al., 2011). Rommeck and colleagues exposed animals to different nursery and peer rearing conditions (continuous pairing, intermittent pairing, continuous rotational pairing, and intermittent rotational pairing) and compared them to animals reared with their mother in an adapted version of the human intruder task. Contrary to infants reared in either intermittent rotational pairing or intermittent pairing groups, animals reared with continuous pairing and continuous rotational pairing both resembled mother reared controls in levels of activity, self-stroking, and coo vocalizations, as well as levels of cortisol,. Monkeys raised in the intermittent rotational pairing group showed abnormally low levels of activity and more vocalizations compared to the other groups. Monkeys raised in the intermittent pairing group showed abnormally high levels of self-stroking behaviors in addition to lower levels of activity during the human intruder task. Both of these intermittent nursery rearing conditions resulted in lower levels of cortisol as well. The animals nursery-reared in the continuous rotational pairing group (the group most similar to the rearing conditions of the current study) reacted similarly to

mother-reared animals for all behavioral measures. Therefore, the rearing conditions of the animals in the present study did not significantly impact the development of normal emotional reactivity and confirmed our previous results with a different group of animals (Raper et al., 2012).

Additionally, the current study documented developmental changes in behaviors other than defensive or aggressive behaviors, such as vocalization production. For example, the frequency of coos was inversely related to the frequency of screams over time in that Neo-C animals produced more screams at 2 months of age than at 4.5 months and vice-versa for coos. Coo vocalizations are considered to function as a social call to reconnect with the social group after separation, and are thought to communicate distress and fear (Kalin, et al., 1991b). Coo vocalizations develop and change in acoustic structure in infant rhesus macaques through at least 5 months of age, possibly due to development of the vocal tracts, increases in body weight, or practice in producing coo vocalizations over time (Hammerschmidt, 2000). The changes in vocalization production in the current study may suggest an ability to better modulate vocal responses at 4.5 months of age compared to 2 months of age. When tested as adults, Neo-C animals rarely produced coos during any condition of the task, and never produced any scream vocalizations. Similarly, Kalin and Shelton (1998) reported dramatic decreases in the number of coo vocalizations produced between 4 months and 8 months of age, as well as between 8 months and 12 months of age in normal monkeys during the human intruder task in all conditions.

Neonatal PRh cortex lesions on the development of emotional reactivity

Neonatal lesions to the PRh cortex had both immediate and long term effects on the development of emotional responses to a human intruder. As infants, group Neo-PRh showed

heightened freezing and fearful/defensive behaviors across all conditions, including conditions in the absence of a human intruder. This behavior is indicative of an inability to modulate their behavior in response to varying levels of threat saliency. Inversely, although Neo-PRh animals modulated aggressive responses to the stare condition at 4.5 months, the magnitude of these hostile behaviors was less than those displayed by sham-operated controls, most likely because of their heightened defensiveness in the stare condition as compared to controls. At the same age, the PRh-operated animals also spent more time self-soothing, and producing self-directed behaviors, suggesting that as compared to controls, they may have increased their use of coping strategies as a result of increased stress and anxiety. This heightened level of anxiety in animals with PRh lesions when separated from their group was also reflected in the increased amount of coo vocalizations at 2 months of age. Interestingly, this increased defensive behavior did not express into heightened production of species-typical anxious behaviors, such as yawning, scratching, or tooth grinding. Finally, at both 2 and 4.5 months of age, Neo-PRh animals were less active overall, spending less time locomoting and exploring the cage compared to controls. This was likely due to their high levels of freezing in all conditions of the task.

The present findings are in line with those of Rommeck and colleagues (2011) who found that animals reared in intermittent rotational pairs were behaviorally inhibited in that they spent less time being active, and they produced more coo vocalizations across all conditions of the human intruder task compared to controls. The current study is unable to parse out whether the behavioral effects seen in Neo-PRh animals are due exclusively to the cortical damage, or whether there may be an interaction between early damage and the environmental rearing condition of rotational peer rearing. Future studies could observe the effects of these early lesions on behavior in mother reared infants to determine if this interaction exists. Nevertheless,

the discriminant analyses indicated that none of the behavioral responses measured at 2 months could significantly dissociate the animals with Neo-PRh lesions from controls. However, by 4.5 months of age, both Hostility and Freezing differentiated animals with Neo-PRh lesions from controls. The results are interesting, at least for Freezing, given that this behavioral responses is the hallmark characteristic of adult animals that had received adult-onset PRh lesions

The behavioral inhibition we observed after neonatal PRh lesions is reminiscent with that observed by Kalin and colleagues (1991b) when treating 6-8 month old monkeys with an anxiolytic (Alprazolam). The effects of this drug, which was intended to reduce anxiety on the intruder task, resulted in a decreased production of coos in alone and stare conditions (although this was only seen at certain doses of the drug). In a follow-up experiment, the same authors (1992) studied the effects of an anxiogenic drug (β-Carboline-3-carboxylate) on infant (8-9 months old) monkeys during the human intruder task. Following treatment, animals spent more time freezing during the task across all conditions (including during the alone condition in the absence of an intruder), and the emotional changes varied in a dose-dependent manner and were associated with a decreased amount of time locomoting and cage exploring during the task. These results confirm that the symptoms displayed by animals with Neo-PRh lesions, including increased freezing (regardless of conditions), increased vocalizations, and decreased cage exploration and locomotion during the human intruder task in infancy, are all indicative of a more anxious behavioral profile compared to controls . These results are further strengthened by the increases in self-soothing and self-directed behaviors during all conditions of the task observed in the Neo-PRh animals. Additionally, previous research looking at the effects of neonatal TE lesions in monkeys reported an increase in coo vocalizations when animals were separated from their group (but not in neonatal amygdala lesions animals) compared to controls

at one year of age (Newman & Bachevalier, 1997), similar to results of increased coo vocalizations seen in group Neo-PRh. These results suggest that damage to both area TE and PRh cortex affects the ability to modulate vocalizations in stressful circumstances.

Neonatal PRh cortex lesions and long-term effects on emotional reactivity

The emotional changes following neonatal PRh lesions were also long-lasting and present even in adulthood. Unlike control animals in which fearful/defensive behaviors increased but hostile behaviors and coo vocalizations decreased from infancy to adulthood, the Neo-PRh animals continued to display heightened freezing responses to the human intruder in all conditions. The same was true for fearful/defensive behaviors, although they emitted these responses slightly less frequently in adulthood then in infancy, as well as for the increase in coo vocalizations. These same behavioral responses were those that discriminated best the animals with Neo-PRh lesions from the controls, suggesting that they represent the most important emotional changes associated with neonatal PRhl lesions. Thus, continued vocalizations in adulthood from almost all of the Neo-PRh animals together with their inability to modulate freezing and fearful/defensive behaviors across conditions suggest an abnormal and stunted development of emotional reactivity to social stressors.

Contrary to the persistent non-modulated production of fearful/defensive behaviors, abnormal behavioral responses did change overtime in the Neo-PRh animals. Thus, although the production of self-directed behaviors in the control animals remained unchanged across development, the heightened levels of self-directed behaviors expressed by the Neo-PRh animals during infancy were almost completely eliminated in adulthood. Conversely, as compared to the almost complete absence of motor stereotypies in control animals, the number of motor

stereotypies in the Neo-PRh animals increased significantly with age. Thus, although the production of self-soothing activities diminished over time in the Neo-PRh animals, they were replaced by an increase in motor stereotypies. The emission of these abnormal behaviors may be due in part by the increase in anxiety displayed by the Neo-PRh animals.

It is interesting to note that the changes in emotional reactivity found in the animals with neonatal PRh lesions parallel remarkably well those already described in monkeys that had received either PRh or complete rhinal lesions in adulthood. As mentioned previously, Meunier and Bachevalier (2002) demonstrated that adult animals with rhinal cortex ablations displayed lower levels of hostility, heightened levels of defensiveness, and spent more time producing locomotor stereotypies. In addition, heightened defensive responses were also reported in monkeys with adult-onset PRh cortex lesions (Meunier et al., 2006). The present results demonstrate that the abnormal regulation of emotional behaviors during a social stressor after neonatal PRh lesions is expressed in early infancy and persists through adulthood, suggesting that this abnormal development of emotional reactivity cannot be compensated by other brain regions with age.

Neonatal PRh cortex lesions and HPA axis functioning

Interestingly, despite the emotional reactivity changes seen in monkeys with neonatal PRh lesions, these animals expressed normal HPA axis functioning in adulthood. Group Neo-PRh had similar levels of cortisol and ACTH to controls at both baseline and after HPA axis activation by a stressor. They also had a normal diurnal cortisol rhythm. These results suggest that not only is the PRh not involved in activating or inhibiting the HPA axis, but that inputs from the PRh to either the hippocampus or the amygdala, both of which play an important role in

modulating the HPA axis, are not necessary for normal HPA axis function. Therefore, the abnormal emotional reactivity seen in Neo-PRh animals cannot be attributed to the loss of input from the PRh to the amygdala, or else there would be further disruption to the HPA axis as seen in animals that had received Neo-A lesions in previous studies (Raper et al., 2012). These results suggest that the PRh plays a unique role in the regulation of emotions.

Comparisons of the effects of neonatal PRh and amygdala lesions on emotional reactivity and HPA-axis functioning.

The emotional changes displayed by animals with neonatal PRh lesions in the human intruder task are also in sharp contrast with those expressed by animals with damage restricted to the amygdala. In a recent study, Raper and colleagues (2012) evaluated emotional reactivity in animals that had received neonatal amygdala lesions or sham-operations and tested the animals in the human intruder task at the same ages as the present study (i.e. 2, 4.5 months and adulthood). At 2 months of age, Neo-A animals modulated freezing behavior, whereas Neo-PRh animals did not, showing heightened levels of freezing and fearful/defensive behaviors across all conditions. However, both Neo-A and Neo-PRh animals showed blunted levels of hostility at 4.5 months of age compared to controls. Furthermore, unlike Neo-A animals, group Neo-PRh showed increases in coo vocalizations, self-directed behaviors, and self-soothing behaviors, along with decreases in cage exploration, and locomotion when compared to controls.

In adulthood, Neo-A animals displayed less freezing compared to controls, whereas Neo-PRh animals continued to show heightened levels of freezing and fearful/defensive behaviors as compared to controls. Group Neo-A displayed hostile and anxious behaviors across all conditions, unable to modulate their aggression or species-typical anxious behaviors based on the

saliency of threat (Raper et al., 2012). Comparatively, Neo-PRh animals showed persistent lower levels of hostility compared to controls in adulthood in the current study, and there were no differences in species-typical anxious behaviors. However, unlike Neo-A animals which had self-directed and stereotypic behaviors in the normal range, Neo-PRh animals produced more self-directed behaviors and showed a different pattern of stereotypies. Finally, unlike group Neo-A, Neo-PRh showed differences in vocalizations compared to controls during adulthood.

Interestingly, although the emotional changes observed in animals with Neo-A lesions in the intruder task were associated with significant changes in HPA-axis functioning and reactivity, the emotional changes observed in animals with Neo-PRh lesions did not. Thus, Neo-A animals had lower diurnal levels of cortisol compared to controls, as well as lower levels of cortisol at baseline and after the human intruder task, suggesting a blunted hormonal activation of the HPA axis after a stressor, and Neo-PRh animals were similar to controls on all hormonal measures. Therefore, Neo-PRh lesions impacted emotional regulation in significantly different ways from Neo-A lesions, suggesting that the abnormal emotional behaviors seen in group Neo-PRh are not simply due to a lack of sensory inputs from the PRh cortex to the amygdala.

The role of the PRh cortex in emotional regulation

Both the present developmental findings together with similar changes in emotional reactivity reported after adult-onset PRh lesions (Meunier et al., 2006) strongly suggest that the PRh plays a critical role in emotional regulation. Furthermore, the present results demonstrate that this role in entirely independent of an impact of the PRh cortex on the regulation of the HPA-axis functioning. Before discussing how the PRh cortex may participate in the regulation of emotional reactivity, it is important to review the known functions of the PRh cortex and how

disruption of these functions could have altered emotional reactivity to stressor. Recent research has provided supporting evidence that the PRh cortex is necessary for object identification, especially when these stimuli may be ambiguous (Bussey et al., 2002). Thus, it is possible that animals with neonatal PRh lesions may not be able to perceive and/or interpret the ambiguous social signals expressed by the human intruder, thus reacting in an overly fearful/defensive way. This proposal is in fact supported by a recent rodent study. In a study examining the effects of kindling of the PRh cortex in rats, Hannesson and colleagues (2005) reported that the kindled animals showed heightened levels of anxiety as reflected by spending less time in the open arms of an elevated plus maze or in the open center ring of an open field arena. But these elevated levels of anxiety were also associated by an inability to discriminate between novel and familiar objects. However, we believe that poor discrimination ability may not be the source of the emotional changes observed in animals with Neo-PRh for at least two reasons. First, the animals with neonatal PRh lesions expressed heightened fearful/defensive reactivity not only in the presence of the human intruder but also in the alone condition when nothing needed to be discriminated. Second, they showed modulation of Hostile, Affiliative and Anxious behaviors in the Profile and Stare conditions indicating that they appeared to have discriminated the different threat signals presented by the intruder (see Figs. 8 and 9).

Another well-accepted function of the PRh is its critical role in memory processes (for reviews see Zeamer et al., 2010; Brown et al., 2010; Winters et al., 2008). Meunier and colleagues (2002, 2006) had already suggested that the PRh is involved in emotional memory, i.e. forming and maintaining associations between stimuli and their valence through its strong anatomical connections with the amygdala. The role of the amygdala is to monitor the environment and signal responses (such as fight or flight, or to activate the HPA axis) to

ambiguous or threatening situations, and the proposed role of the PRh is to provide the amygdala and hippocampus with information about previous emotional experiences with encountered stimuli to enable the subject to apprehend ambiguous situations and select the most appropriate responses. With this scenario, lesions of the amygdala will result in hypoemotionality or a lack of reactivity to threatening or ambiguous stimuli, whereas lesions of the PRh cortex will result in heightened anxiety and defensiveness, because a lack of access to previous emotional memories will render all experiences to be novel and ambiguous, and therefore potentially threatening. This hypothesis could explain how aspiration lesions to the amygdala, which include extensive unintended damage to the PRh, result in an exacerbation of symptoms, since these animals would be less reactive as well as unable to assess the environment using previous emotional experiences.

This hypothesis could also explain why the heightened levels of fearful defensive behaviors and the blunted levels of hostility expressed by the Neo-PRh group during infancy resemble similar patterns of behavior seen in group Neo-C during adulthood. If damage to the PRh cortex prevents animals from recognizing or learning the emotional salience of experience, and all situations are potentially ambiguous, reacting with hostility would be dangerous without an understanding of the consequences. Therefore, Neo-PRh animals would react more fearfully in infancy. Control animals, however, would become more hostile and aggressive during development as they learn about their environment and test their boundaries, and this hostility would lessen with age and previous knowledge. Therefore in adulthood, Neo-C animals express higher levels of defensiveness compared to hostility. This is further examined using discriminant function analyses, which demonstrated that freezing and hostile behaviors could be used to accurately predict and classify Neo-PRh and Neo-C groups at 4.5 months of age, and that

fearful/defensive behaviors and coo vocalizations are able to predict animal groupings during adulthood. There neonatal damage to the PRh cortex did not have a strong enough impact on behavior at 2 months of age to be able to classify groups, but the effects of the damage become more apparent with age. These results should be interpreted cautiously due to the small sample size included in the discriminant analysis.

The data suggest that early dysfunction of the PRh cortex results in abnormal fear reactivity and heightened overall anxiety. These results therefore provide insights onto the neural underpinning of several developmental neuropsychiatric disorders associated with anxiety, pointing to a key role of the PRh cortex in modulating anxiety through emotional memory.

Figure Legends

Figure 1: Organization and location of MTL structures

Anatomical organization of the MTL structures (top) and location of MTL structures in the rodent, monkey and humans brains (bottom). Not all structures are visible from each view. From Murray *et al.* (2007).

Figure 2: MR images with injection sites and post-surgical damage for case Neo-PRh-3 and Neo-PRh-6

MRI pre-surgical T1 images and MRI post-surgical FLAIR images for two Neo-PRh cases, with white arrows pointing to the PRh cortex, and yellow * indicating injection site on the T1 image. The PRh cortex is outlined in black dashed lines on the FLAIR image. Images are of Anterior, Mid, and Posterior PRh cortex.

Figure 3: Human intruder task procedures timeline

Human Intruder task procedures timeline with hormone sample collection

Figure 4: Freezing, Hostile, Fearful/Defensive, and Affiliative behaviors in infancy

Bars represent the mean ± SEM for both Neo-C (white) and Neo-PRh (purple) animals at 2 months and 4.5 months of age for Freezing (Group: $p=0.03$; A), Hostile (Group by Condition: $p=0.04$; B), Fearful/defensive (Group: $p=0.05$; C), and Affiliative (D) behaviors in the Human Intruder Paradigm.

Figure 5: Stereotypy, Self-Directed, Anxious, and Self-Soothing behaviors in infancy

Bars represent the mean ± SEM for both Neo-C and Neo-PRh animals at 2 months and 4.5 months of age for Stereotypy (A), Self-Directed (Group: p=0.02; B), Anxious (C), and Self-Soothing (Group: p=0.01; D) behaviors in the Human Intruder Paradigm.

Figure 6: Locomotion and Cage Exploration in infancy

Bars represent the mean ± SEM for both Neo-C and Neo-PRh animals at 2 months and 4.5 months of age for Locomotion (Group: p=0.005; A) and Cage Exploration (Group: p=0.004; B) in the Human Intruder Paradigm.

Figure 7: Coo and Scream Vocalizations in infancy

Bars represent the mean ± SEM for both Neo-C and Neo-PRh animals at 2 months and 4.5 months of age for Coo vocalizations (Group by Age: p=0.04; A) and Scream Vocalizations (B) in the Human Intruder Paradigm.

Figure 8: Freezing, Hostile, Fearful/Defensive, and Affiliative behaviors across all ages

Bars represent the ± SEM for Freezing (Group by Age: p=0.03; A), Hostile (Group: p=0.01; B), Fearful/defensive (Group by Age: p=0.02; C), and Affiliative (D) behaviors during the three conditions of the Human Intruder Paradigm at 2 months, 4.5 months, and 5 years of age in Neo-C and Neo-PRh animals.

Figure 9: Stereotypy, Self-Directed, Anxious, and Self-Soothing behaviors across all ages

Bars represent the ± SEM for Stereotypy (Group by Age: p=0.005; A), Self-Directed (Group by Age: p=0.001; B), Anxious (C), and Self-Soothing (Group: 0.02; D) behaviors during the three conditions of the Human Intruder Paradigm at 2 months, 4.5 months, and 5 years of age in Neo-C and Neo-PRh animals.

Figure 10: Coo Vocalizations across all ages

Bars represent the ± SEM for Coo Vocalizations (Adults only, Group: p=0.05) during the three conditions of the Human Intruder Paradigm at 2 months, 4.5 months, and 5 years of age in Neo-C and Neo-PRh animals.

Figure 11: Cortisol and ACTH levels pre- and post- stressor

Scores are mean ± SEM of cortisol in adulthood during baseline (A), cortisol during Human Intruder stressor (B), ACTH in adulthood during baseline (C), and ACTH during Human Intruder stressor (D). Neo-C is represented with open squares with dashed lines, and Neo-PRh is represented with black circles and solid lines.

Figure 12: Diurnal Cortisol levels

Scores are mean ± SEM of cortisol in adulthood diurnal rhythm. All other abbreviations are as in Figure 11.

Figure 1

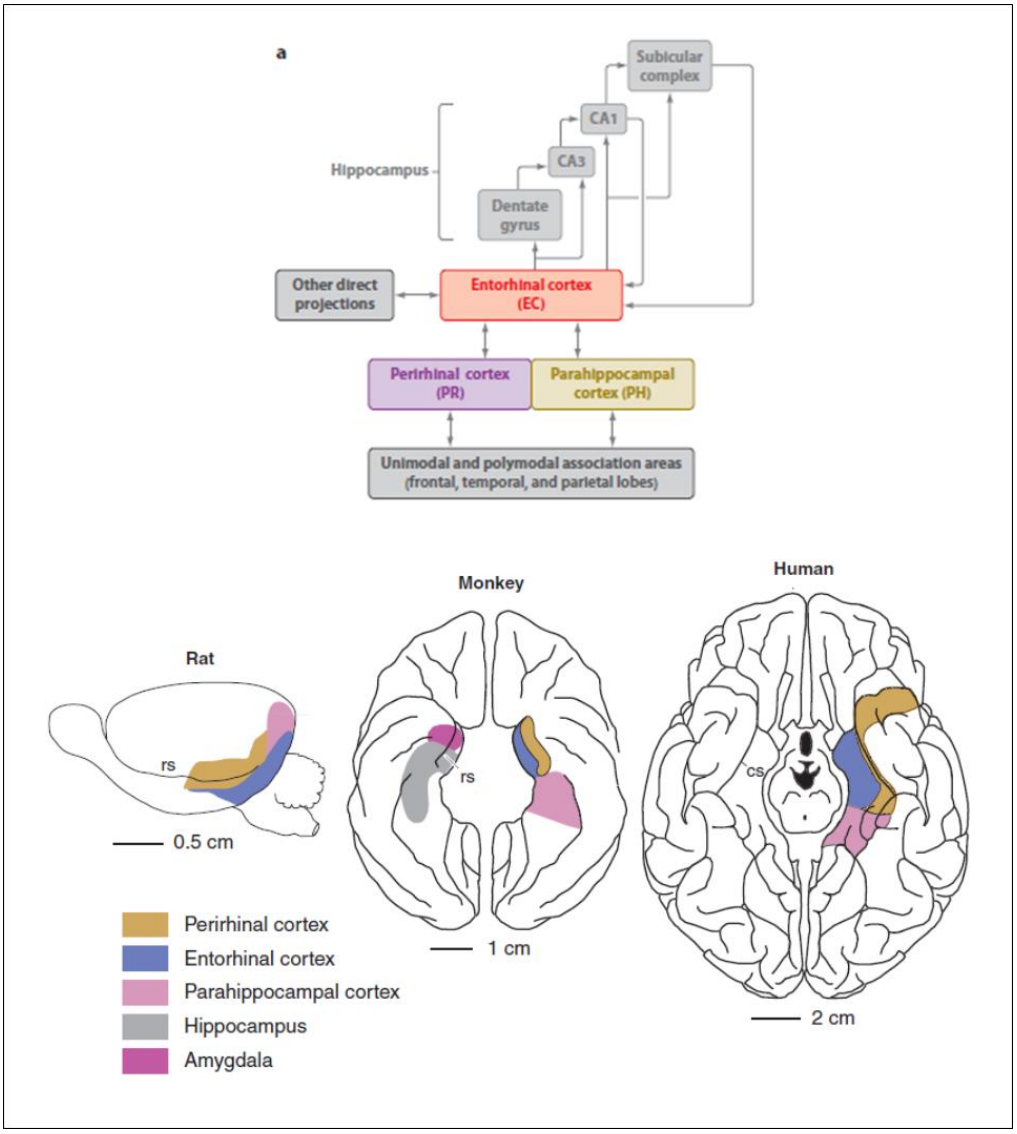

a
Subicular complex
CA1
CA3
Hippocampus
Dentate gyrus
Other direct projections
Entorhinal cortex (EC)
Perirhinal cortex (PR)
Parahippocampal cortex (PH)
Unimodal and polymodal association areas (frontal, temporal, and parietal lobes)
Rat
rs
0.5 cm
Monkey
rs
1 cm
Human
cs
2 cm
Perirhinal cortex
Entorhinal cortex
Parahippocampal cortex
Hippocampus
Amygdala

Figure 2

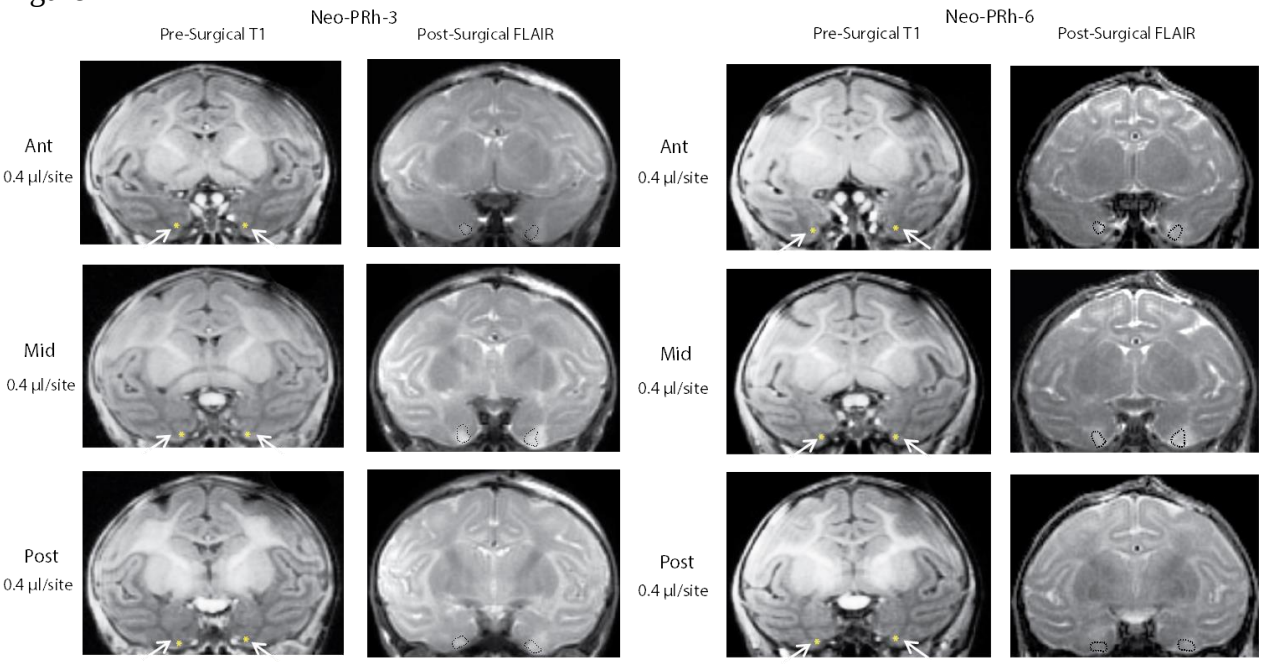

Neo-PRh-3
Pre-Surgical T1
Post-Surgical FLAIR
Ant
0.4 µl/site
Mid
0.4 µl/site
Post
0.4 µl/site
Neo-PRh-6
Pre-Surgical T1
Post-Surgical FLAIR
Ant
0.4 µl/site
Mid
0.4 µl/site
Post
0.4 µl/site

Figure 3

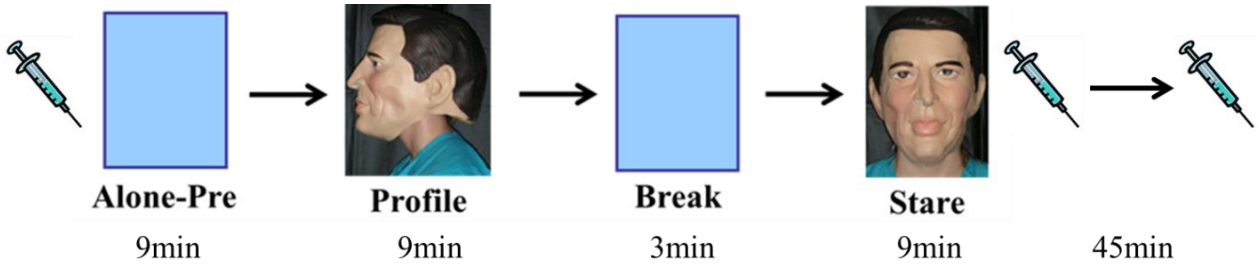


Figure 4

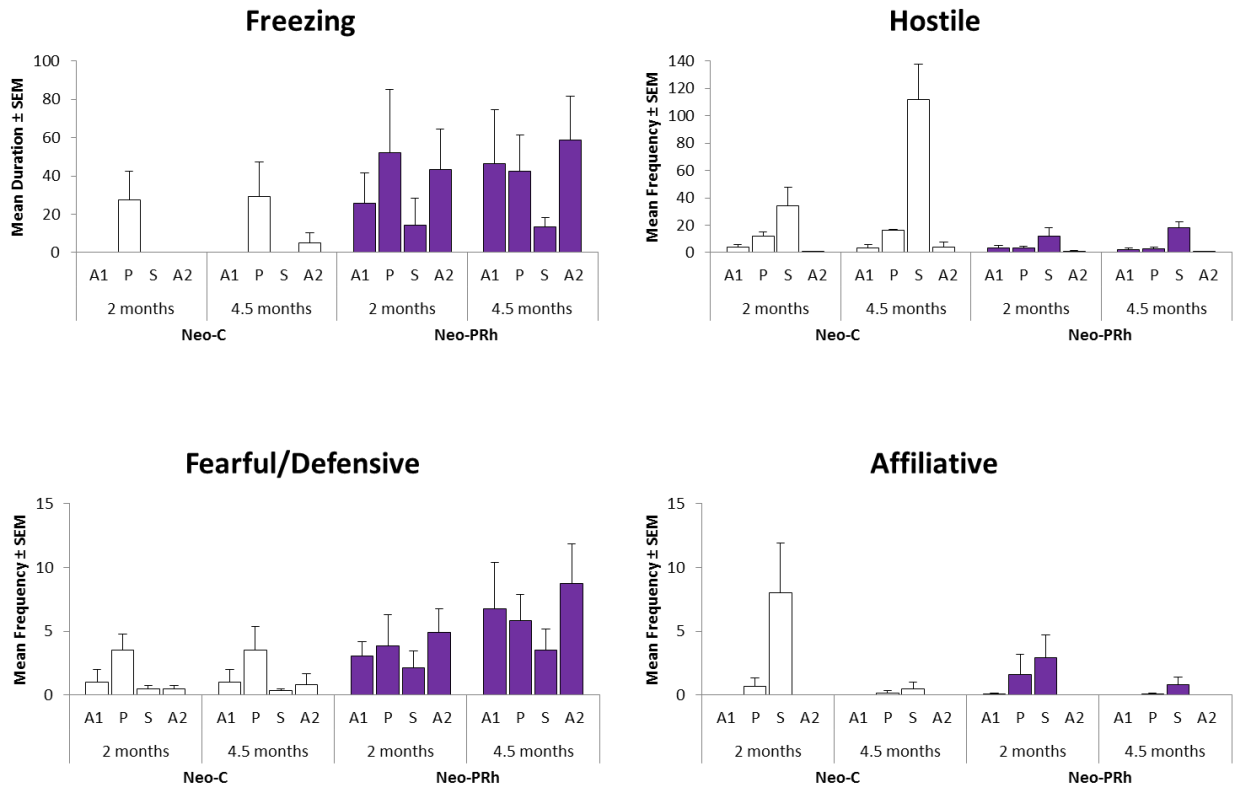

Freezing
Mean Duration ± SEM
100
80
60
40
20
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months 4.5 months 2 months 4.5 months
Neo-C Neo-PRh
Hostile
Mean Frequency ± SEM
140
120
100
80
60
40
20
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months 4.5 months 2 months 4.5 months
Neo-C Neo-PRh
Fearful/Defensive
Mean Frequency ± SEM
15
10
5
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months 4.5 months 2 months 4.5 months
Neo-C Neo-PRh
Affiliative
Mean Frequency ± SEM
15
10
5
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months 4.5 months 2 months 4.5 months
Neo-C Neo-PRh

Figure 5

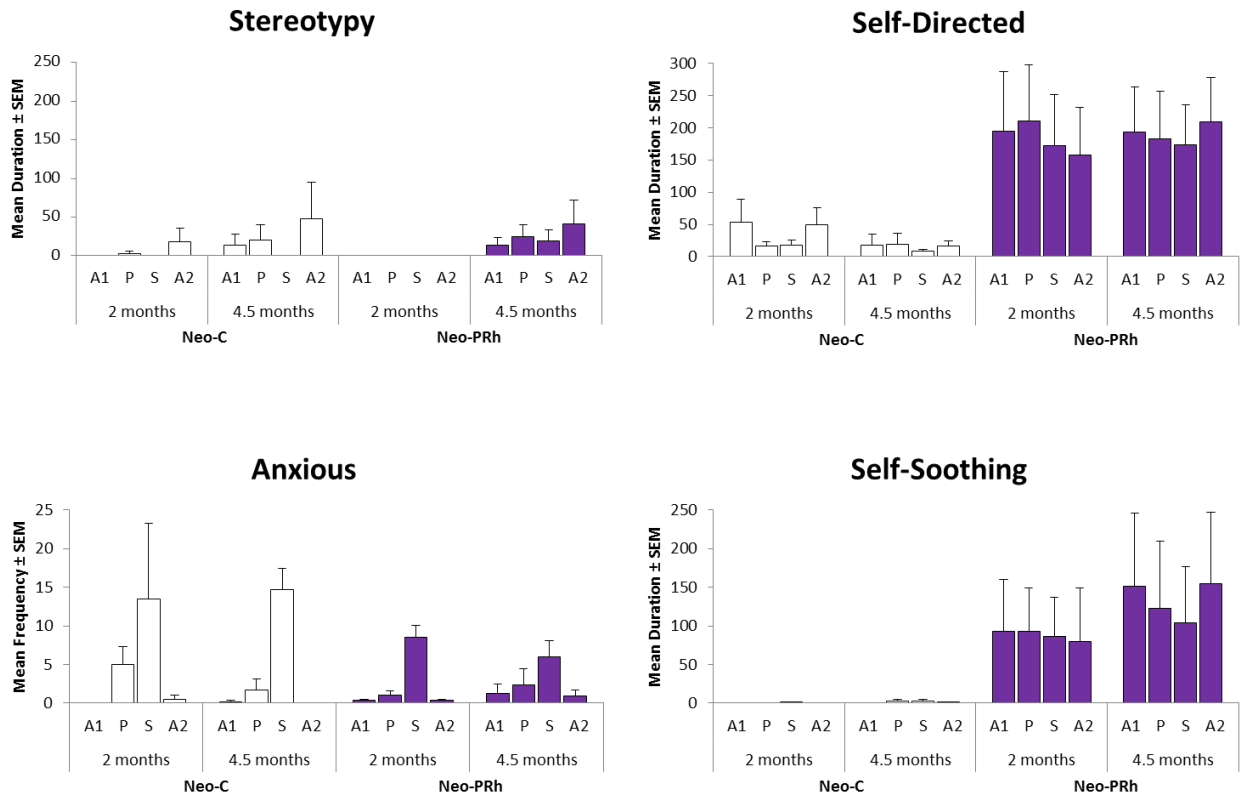

Stereotypy
Mean Duration ± SEM
250
200
150
100
50
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh
Self-Directed
Mean Duration ± SEM
300
250
200
150
100
50
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh
Anxious
Mean Frequency ± SEM
25
20
15
10
5
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh
Self-Soothing
Mean Duration ± SEM
250
200
150
100
50
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh

Figure 6

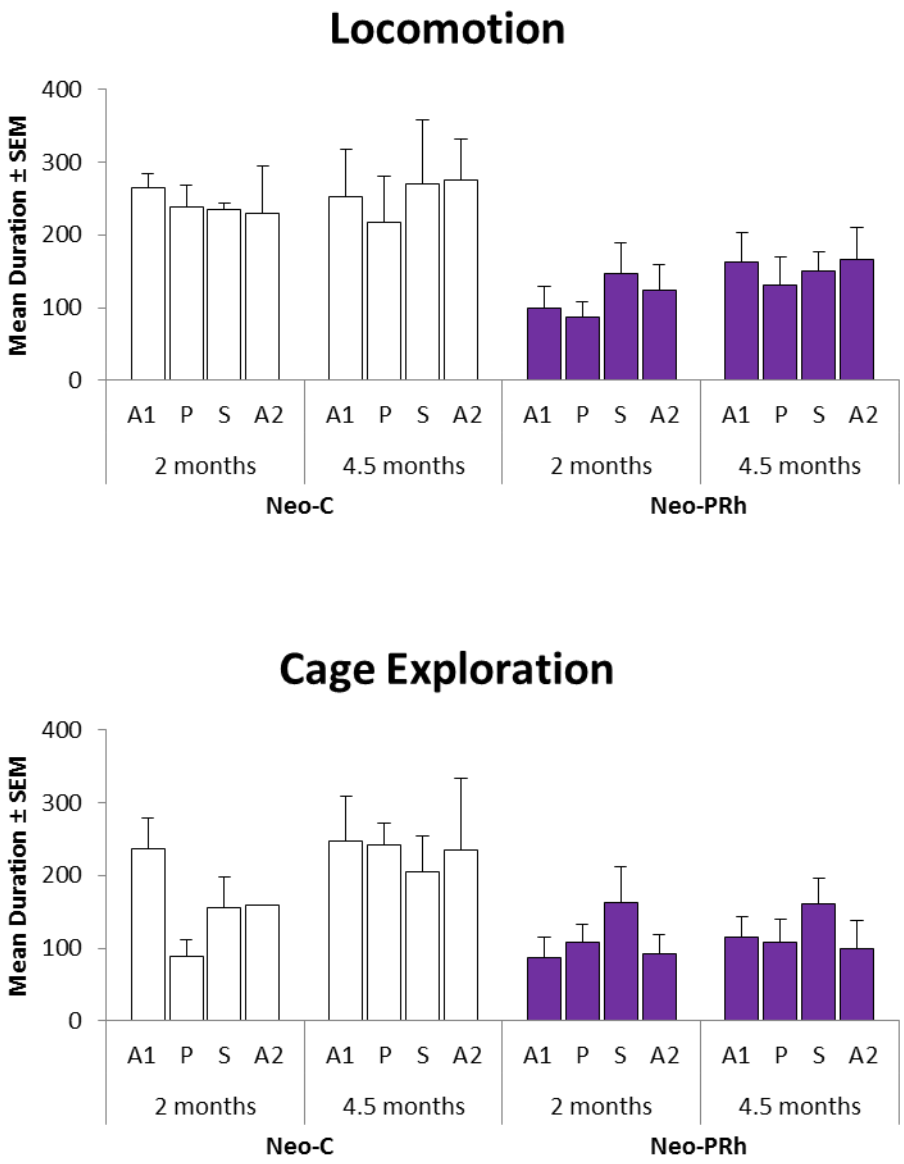
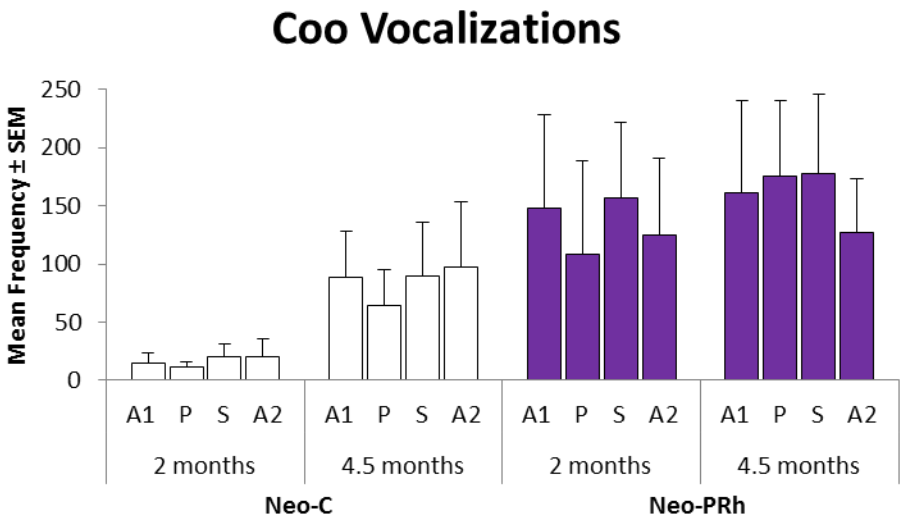

Locomotion
Mean Duration ± SEM
400
300
200
100
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh
Cage Exploration
Mean Duration ± SEM
400
300
200
100
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh

Figure 7


Coo Vocalizations
Mean Frequency ± SEM
250
200
150
100
50
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh

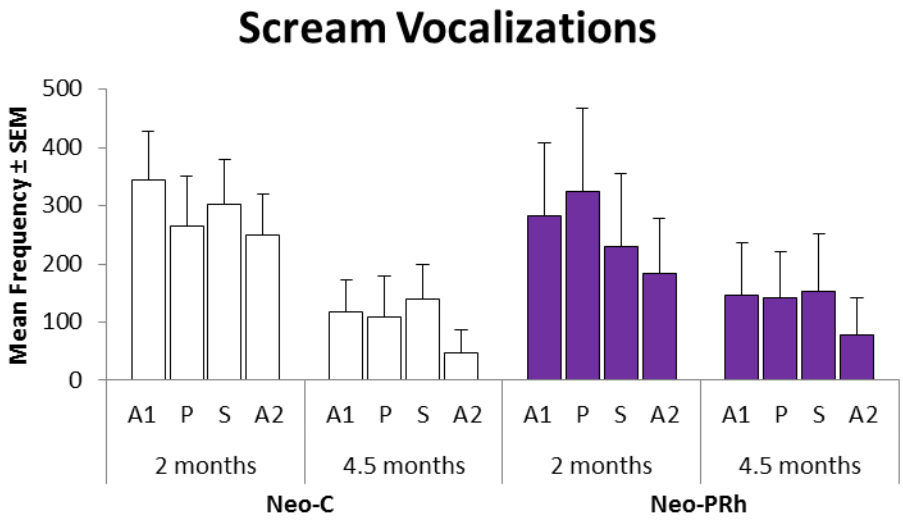

Scream Vocalizations
Mean Frequency ± SEM
500
400
300
200
100
0
A1 P S A2 A1 P S A2 A1 P S A2 A1 P S A2
2 months
4.5 months
2 months
4.5 months
Neo-C
Neo-PRh

Figure 8

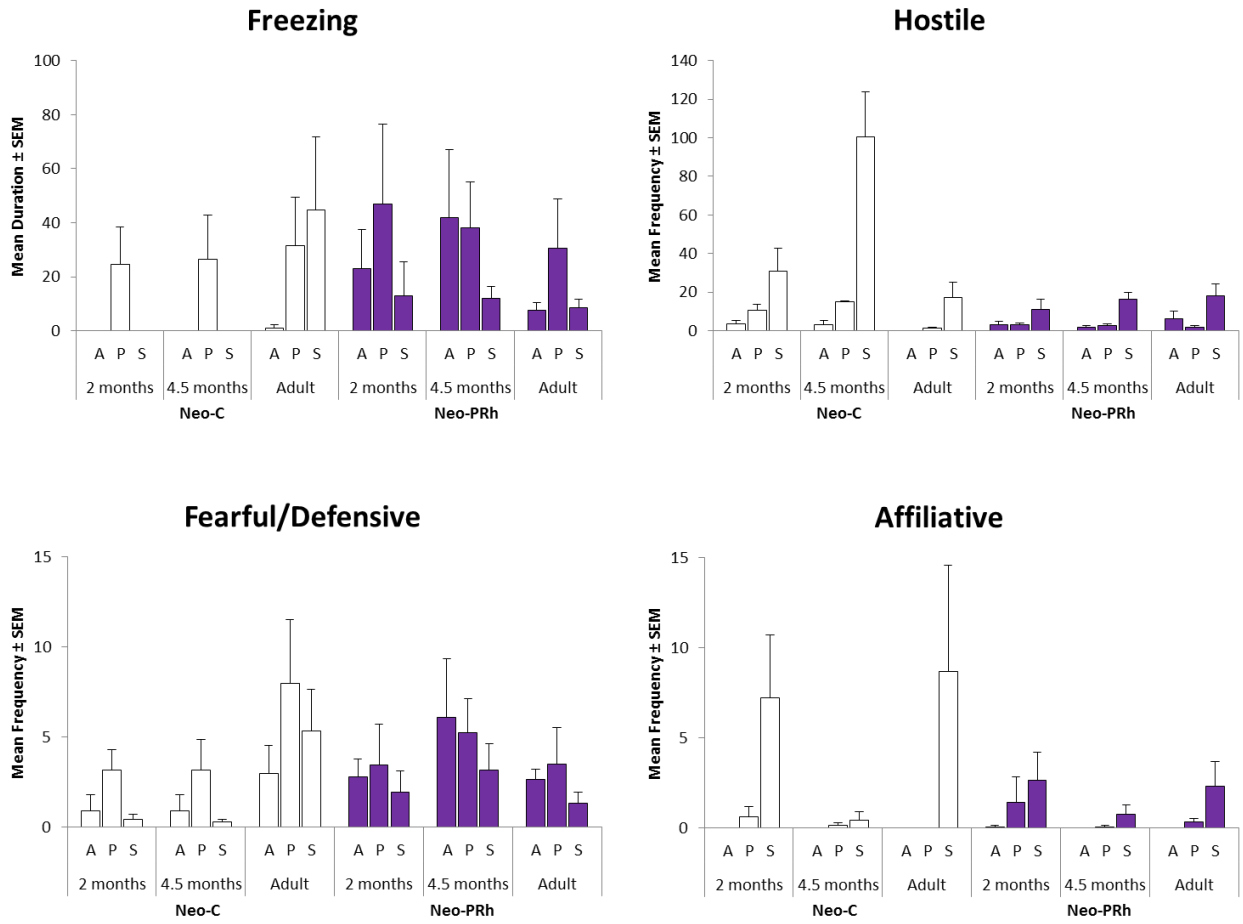

Freezing
Mean Duration ± SEM
100
80
60
40
20
0
A P S
2 months
4.5 months
Adult
Neo-C
Neo-PRh
Hostile
Mean Frequency ± SEM
140
120
100
80
60
40
20
0
Fearful/Defensive
15
10
5
Affiliative

Figure 9

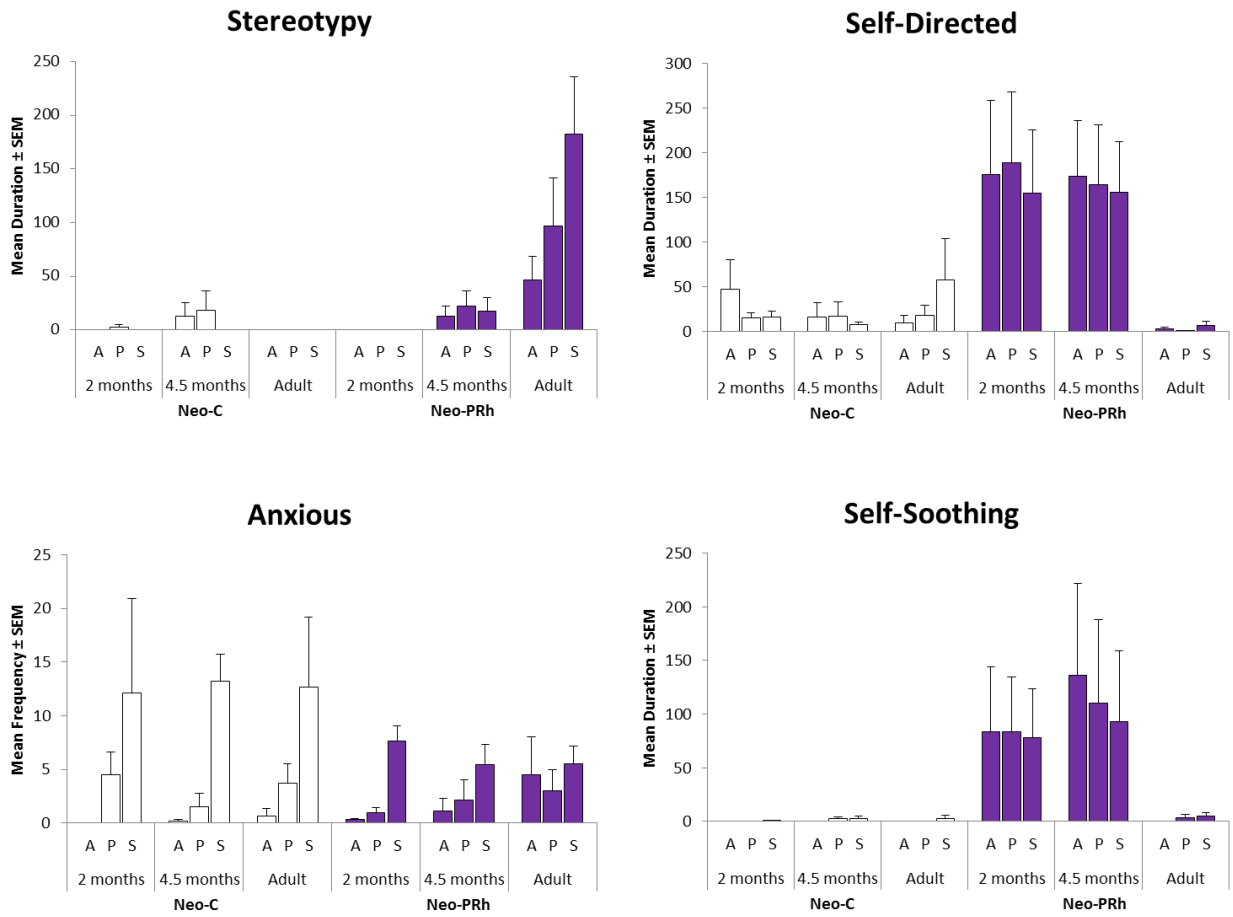

Stereotypy
Mean Duration ± SEM
250
200
150
100
50
0
A P S
2 months
4.5 months
Adult
Neo-C
Neo-PRh
Self-Directed
Mean Duration ± SEM
300
250
200
150
100
50
0
Anxious
Mean Frequency ± SEM
25
20
15
10
5
0
Self-Soothing
Mean Duration ± SEM

Figure 10

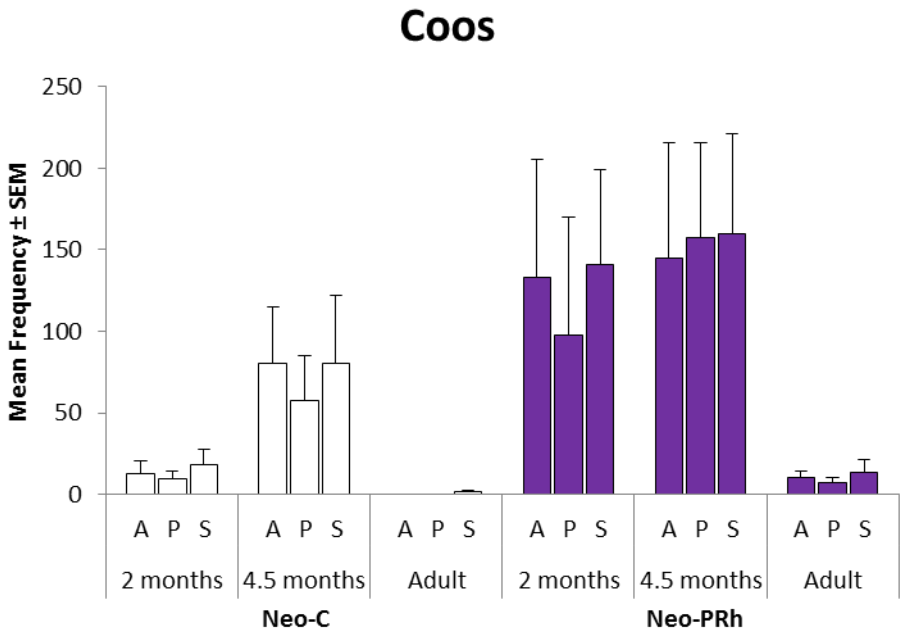

Coos
Mean Frequency ± SEM
250
200
150
100
50
0
A P S
2 months
4.5 months
Adult
Neo-C
Neo-PRh

Figure 11

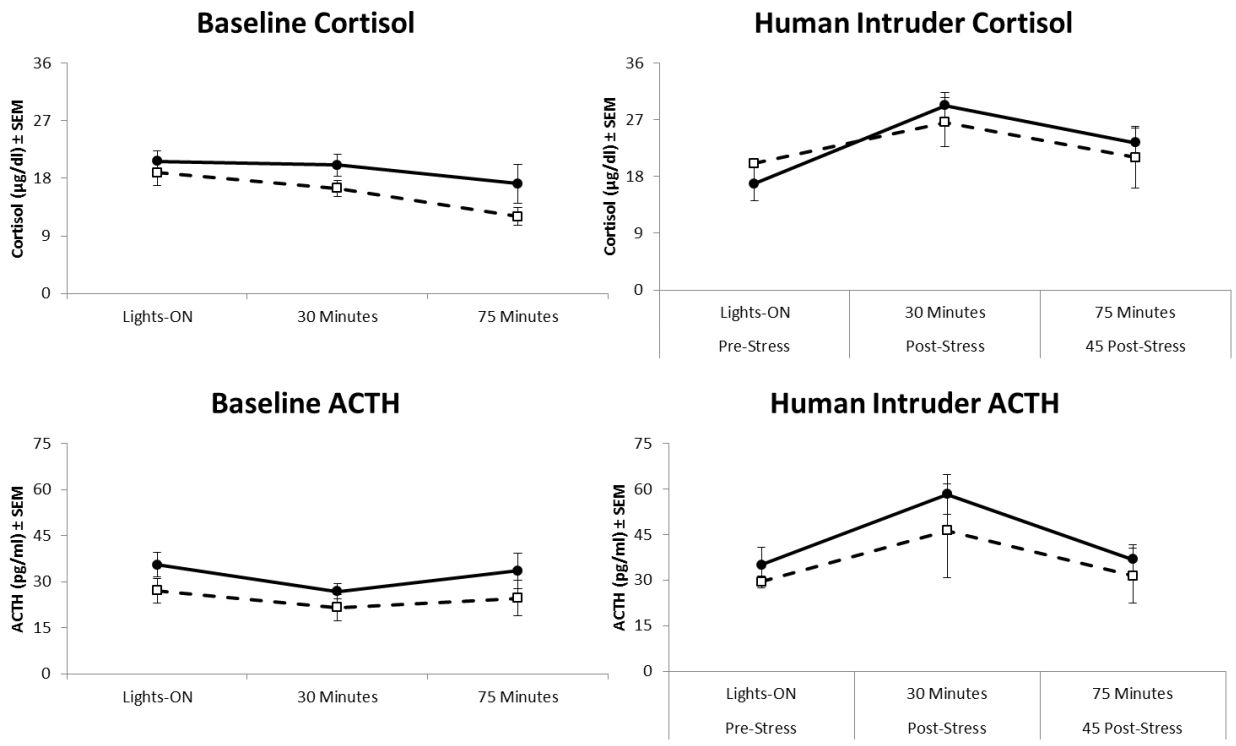

Baseline Cortisol
Cortisol (µg/dl) ± SEM
36
27
18
9
0
Lights-ON
30 Minutes
75 Minutes
Human Intruder Cortisol
Cortisol (µg/dl) ± SEM
36
27
18
9
0
Lights-ON
Pre-Stress
30 Minutes
Post-Stress
75 Minutes
45 Post-Stress
Baseline ACTH
ACTH (pg/ml) ± SEM
75
60
45
30
15
0
Lights-ON
30 Minutes
75 Minutes
Human Intruder ACTH
ACTH (pg/ml) ± SEM
75
60
45
30
15
0
Lights-ON
Pre-Stress
30 Minutes
Post-Stress
75 Minutes
45 Post-Stress

Figure 12

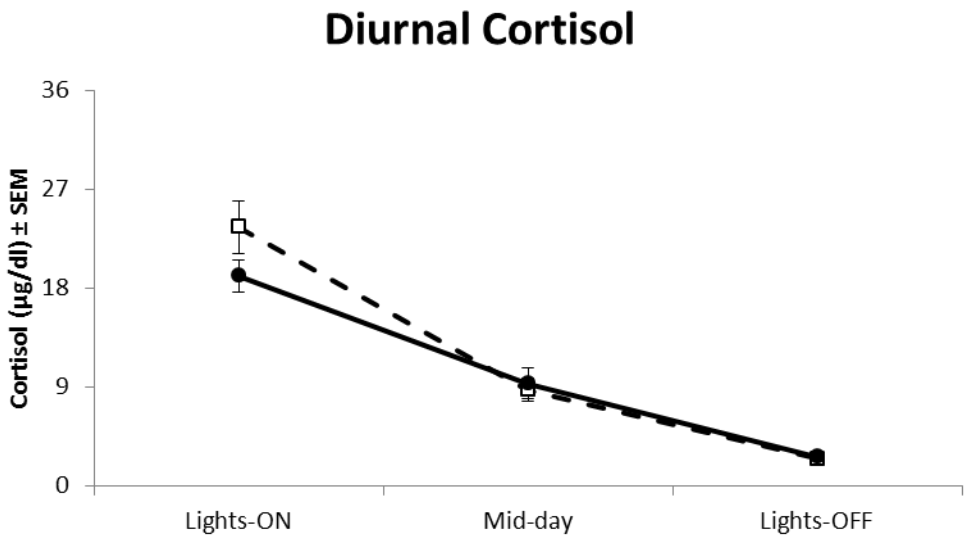

Diurnal Cortisol
Cortisol (µg/dl) ± SEM
36
27
18
9
0
Lights-ON
Mid-day
Lights-OFF

Table 1: Behaviors scored, how they are measured, and definitions

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

Category and specific behavior	Measurement	Brief Definition
Fearful defensive behaviors	Cumulative frequency	
Freeze	Frequency[a]	Silent, tense, motionless posture except slight head movement
Crouch	Frequency[a]	Whole body or just bent with head near floor
Withdrawl	Frequency	Quick, jerky motion away from intruder (jump back)
Hostile behaviors	Cumulative frequency	
Threat bark vocalization	Frequency	Short, low pitch, rasping, gutteral sound
Threat (Facial expression)	Frequency	Facial expressions including: open mouth (no teeth exposed), head-bobbing, or ear flapping
Cage Aggression	Frequency	Vigorously slaps, shakes, bites, or slams body against cage
Lunge	Frequency	A quick, jerky movement toward the intruder, maintaining eye contact
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 and 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)
Self-directed behaviors	Duration	i.e. sucking thumb, eye poke
Self-soothing behaviors	Cumulative duration	
Self-groom	Duration	Use of hands or mouth to pick through hair
Self-clasp	Duration	Non-manipulatory enclosing or holding of a body part
Self-Sex	Duration	Manipulation or licking of the genitalia
Affiliative behaviors	Cumulative frequency	
Grunt vocalization	Frequency	Deep, muffled, low intensity, almost gurgling sound
Lipsmack	Frequency	Rapid movement of pursed lips and smacking sound
Present	Frequency	Rigid posture (knees locked) with tail elevated and rump oriented toward intruder
Coo vocalizations	Frequency	Clear soft sound, moderate in pitch and intensity, sounds like "Whooooo"
Scream vocalizations	Frequency	High pitch, high intensity screech or loud chirp
Locomotion	Duration	Self-induced change of location, such as walking, climbing, or jumping
Cage Exploration	Duration	Tactile, oral, or visual inspection of the cage

Table 2: Percent of intended and unintended damage

Lesion assessments of hypersignal, intended and unintended damage to the PRh and ERh cortices.

	Intended Damage Perirhinal				Unintended Damage Entorhinal			
Cases	**L%**	**R%**	**X%**	**W%**	**L%**	**R%**	**X%**	**W%**
Neo-PRh-1	89.8	76.9	83.3	69.0	28.5	2.3	15.4	0.6
Neo-PRh-2	68.2	70.6	69.4	48.1	17.7	20.7	19.2	3.7
Neo-PRh-3	65.4	81.0	73.2	53.0	7.7	3.1	5.4	0.2
Neo-PRh-4	59.4	74.7	67.1	44.4	11.5	17.8	14.7	2.1
Neo-PRh-5	75.9	66.8	71.4	50.7	38.6	29.9	34.2	11.5
Neo-PRh-6	74.1	80.3	77.2	59.5	25.3	43.6	34.5	11.1
Average	***72.1***	***75.1***	***73.6***	***54.1***	***21.6***	***19.6***	***20.6***	***4.9***

L% - percent damage to the left hemisphere; R% - percent damage to the right hemisphere; X% - average damage to both hemispheres; W% - weighted damage to both hemispheres (W% = (L% x R%)/100).

Table 3: Statistical analyses for group Neo-C for all ages and behavioral categories

List of Condition main effects, Age main effects, and Age by Condition interactions for Group Neo-C during infancy, adulthood, and compared across all time periods for all behavioral categories

Neo-C Only		Main effect of condition					Main effect of age					Age X Condition Interaction				
Ages	**Behavior**	**df**	**F**	**η**	**p**	**Sig**	**df**	**F**	**η**	**p**	**Sig**	**df**	**F**	**η**	**p**	**Sig**
2 months, and 4.5 months	Freezing	3,8	6.46	0.71	0.02	s	1,8	0.12	0.01	0.74	ns	3,8	1.06	0.28	0.42	ns
	Fearful/Defensive	3,8	1.88	0.41	0.21	ns	1,8	0.17	0.02	0.69	ns	3,8	0.14	0.05	0.93	ns
	Hostile	3,8	14.93	0.85	0.001	s	1,8	6.64	0.45	0.03	s	3,8	1.35	0.34	0.33	ns
	Anxious	3,8	14.87	0.85	0.001	s	1,8	0.39	0.05	0.55	ns	3,8	1.82	0.41	0.22	ns
	Stereotypy	3,8	0.43	0.14	0.74	ns	1,8	2.34	0.23	0.17	ns	3,8	0.56	0.17	0.66	ns
	Self-Directed	3,8	0.20	0.07	0.89	ns	1,8	3.21	0.29	0.11	ns	3,8	0.12	0.04	0.95	ns
	Affiliative	3,8	12.61	0.83	0.002	s	1,8	7.23	0.48	0.03	s	3,8	5.20	0.66	0.03	s
	Self-Sooth	3,8	1.48	0.36	0.29	ns	1,8	1.91	0.19	0.20	ns	3,8	0.59	0.18	0.64	ns
	Coos	3,8	0.08	0.03	0.97	ns	1,8	9.86	0.55	0.01	s	3,8	0.09	0.03	0.96	ns
	Screams	3,8	0.60	0.18	0.63	ns	1,8	15.64	0.66	0.004	s	3,8	0.56	0.17	0.66	ns
	Locomotion	3,8	0.07	0.02	0.98	ns	1,8	0.01	0.001	0.92	ns	3,8	0.46	0.15	0.72	ns
	Cage Exploration	3,8	1.51	0.36	0.28	ns	1,8	4.60	0.37	0.06	ns	3,8	1.53	0.36	0.28	ns
Adults	Freezing	2,6	8.29	0.73	0.02	s										
	Fearful/Defensive	2,6	0.56	0.16	0.60	ns										
	Hostile	2,6	4.27	0.59	0.07	ns										
	Anxious	2,6	14.16	0.83	0.005	s										
	Stereotypy	2,6	1.00	0.25	0.42	ns										
	Self-Directed	2,6	1.01	0.25	0.42	ns										
	Affiliative	2,6	7.24	0.71	0.03	s										
	Self-Sooth	2,6	1.00	0.25	0.42	ns										
	Locomotion	2,6	0.38	0.11	0.70	ns										
	Cage Exploration	2,6	1.17	0.28	0.37	ns										
2 months, 4.5 months, and Adults	Freezing	2,6	13.01	0.81	0.001	s	2,12	9.09	0.60	0.04	s	4,12	4.10	0.58	0.03	s
	Fearful/Defensive	2,6	3.78	0.56	0.09	ns	2,12	4.70	0.44	0.03	s	4,12	0.25	0.08	0.91	ns
	Hostile	2,6	35.14	0.92	0.001	s	2,12	14.81	0.71	0.006	s	4,12	0.75	0.20	0.52	ns
	Anxious	2,6	78.47	0.96	0.001	s	2,12	0.10	0.02	0.91	ns	4,12	0.49	0.14	0.75	ns
	Stereotypy	2,6	0.56	0.16	0.60	ns	2,12	1.84	0.24	0.22	ns	4,12	0.58	0.16	0.61	ns
	Self-Directed	2,6	0.74	0.20	0.52	ns	2,12	0.64	0.10	0.55	ns	4,12	0.18	0.06	0.95	ns
	Affiliative	2,6	13.30	0.82	0.006	s	2,12	2.05	0.26	0.20	ns	4,12	1.74	0.37	0.25	ns
	Self-Sooth	2,6	4.33	0.59	0.07	ns	2,12	0.69	0.10	0.52	ns	4,12	0.45	0.13	0.77	ns
	Coos	2,6	0.97	0.24	0.43	ns	2,12	28.96	0.83	0.001	s	4,12	0.07	0.02	0.99	ns
	Locomotion	2,6	0.60	0.17	0.58	ns	2,12	11.27	0.65	0.002	s	4,12	2.71	0.48	0.08	ns
	Cage Exploration	2,6	2.13	0.42	0.20	ns	2,12	6.84	0.53	0.01	s	4,12	1.01	0.25	0.44	ns

Table 4: Statistical analyses comparing groups and ages for all behavioral categories

List of Condition main effects, Group main effects, Group by Condition interactions, Age main effects, and Age by Condition interactions during infancy, adulthood, and compared across all time periods for all behavioral categories. * indicates that when day 1 and day 2 were analyzed separately, these interactions were no longer observed.

Both Rx		Main effect of condition					Main effect of Rx					Rx X Condition Interaction					Main effect of age					Rx X Age Interaction				
Ages	Behavior	df	F	η	p	Sig	df	F	η	p	Sig	df	F	η	p	Sig	df	F	η	p	Sig	df	F	η	p	Sig
2 months and 4.5 months	Freezing	3,28	1.83	0.16	0.17	ns	1,28	5.38	0.16	0.03	s	3,28	1.37	0.13	0.27	ns	1,28	1.04	0.04	0.32	ns	1,28	0.44	0.02	0.51	ns
	Fearful/Defensive	3,28	0.74	0.07	0.54	ns	1,28	4.32	0.13	0.05	s	3,28	0.83	0.08	0.49	ns	1,28	0.75	0.03	0.40	ns	1,28	1.64	0.06	0.21	ns
	Hostile	3,28	29.40	0.76	0.001	s	1,28	15.94	0.36	0.001	s	3,28	3.21	0.26	0.04	s	1,28	2.68	0.09	0.11	ns	1,28	1.97	0.07	0.17	ns
	Anxious	3,28	14.93	0.85	0.001	s	1,28	0.88	0.03	0.34	ns	3,28	1.21	0.11	0.33	ns	1,28	0.75	0.03	0.39	ns	1,28	0.02	0.001	0.90	ns
	Stereotypy	3,28	0.66	0.07	0.58	ns	1,28	0.003	0.001	0.74	ns	3,28	0.42	0.04	0.74	ns	1,28	10.00	0.26	0.004	s	1,28	2.26	0.08	0.14	ns
	Self-Directed	3,28	0.14	0.01	0.94	ns	1,28	6.37	0.19	0.02	s	3,28	0.03	0.003	0.99	ns	1,28	1.74	0.06	0.20	ns	1,28	8.24	0.23	0.008	s*
	Affiliative	3,28	8.19	0.47	0.001	s	1,28	0.84	0.03	0.37	ns	3,28	0.90	0.09	0.45	ns	1,28	7.86	0.22	0.009	s	1,28	1.32	0.05	0.26	ns
	Self-Sooth	3,28	0.09	0.01	0.97	ns	1,28	7.81	0.22	0.009	s	3,28	0.004	0.001	1.00	ns	1,28	2.12	0.07	0.16	ns	1,28	0.17	0.01	0.68	ns
	Coos	3,28	0.11	0.01	0.95	ns	1,28	0.92	0.03	0.35	ns	3,28	0.04	0.01	0.99	ns	1,28	6.33	0.18	0.02	s	1,28	4.89	0.15	0.04	s
	Screams	3,28	0.29	0.03	0.83	ns	1,28	2.23	0.07	0.15	ns	3,28	0.08	0.01	0.97	ns	1,28	20.13	0.42	0.001	s	1,28	0.04	0.001	0.85	ns
	Locomotion	3,28	0.08	0.01	0.97	ns	1,28	9.32	0.25	0.005	s	3,28	0.07	0.01	0.98	ns	1,28	1.69	0.06	0.20	ns	1,28	1.84	0.06	0.19	ns
	Cage Exploration	3,28	0.46	0.05	0.71	ns	1,28	9.77	0.26	0.004	s	3,28	0.81	0.08	0.50	ns	1,28	2.20	0.07	0.15	ns	1,28	1.10	0.04	0.30	ns
Adult	Freezing	2,16	6.27	0.44	0.01	s	1,8	1.03	0.11	0.34	ns	2,16	3.54	0.31	0.05	s										
	Fearful/Defensive	2,16	0.68	0.08	0.52	ns	1,8	4.91	0.38	0.06	ns	2,16	0.70	0.08	0.51	ns										
	Hostile	2,16	9.42	0.54	0.002	s	1,8	0.001	0.001	0.99	ns	2,16	0.21	0.03	0.81	ns										
	Anxious	2,16	6.86	0.46	0.007	s	1,8	0.28	0.03	0.61	ns	2,16	1.24	0.13	0.32	ns										
	Stereotypy	2,16	2.97	0.27	0.08	ns	1,8	4.50	0.36	0.07	ns	2,16	1.53	0.16	0.25	ns										
	Self-Directed	2,16	3.39	0.30	0.09	ns	1,8	4.31	0.35	0.07	ns	2,16	0.28	0.03	0.66	ns										
	Affiliative	2,16	11.45	0.59	0.001	s	1,8	0.74	0.09	0.42	ns	2,16	2.21	0.22	0.14	ns										
	Self-Soothing	2,16	2.87	0.26	0.09	ns	1,8	0.83	0.09	0.39	ns	2,16	0.38	0.05	0.69	ns										
	Coos	2,16	0.33	0.04	0.63	ns	1,8	5.59	0.44	0.05	s	2,16	0.18	0.02	0.74	ns										
	Cage Exploration	2,16	1.91	0.19	0.18	ns	1,8	0.04	0.01	0.84	ns	2,16	1.20	0.13	0.33	ns										
	Locomotion	2,16	0.02	0.003	0.98	ns	1,8	0.57	0.07	0.47	ns	2,16	0.66	0.08	0.53	ns										
2 months, 4.5 months, and Adult	Freezing	2,21	2.58	0.20	0.10	ns	1,21	0.55	0.03	0.47	ns	2,21	1.36	0.12	0.28	ns	2,42	3.37	0.14	0.04	s	2,42	3.70	0.15	0.03	s
	Fearful/Defensive	2,21	1.36	0.11	0.28	ns	1,21	0.10	0.01	0.76	ns	2,21	0.95	0.08	0.40	ns	2,42	2.52	0.11	0.09	ns	2,42	4.14	0.17	0.02	s
	Hostile	2,21	29.00	0.73	0.001	s	1,21	8.19	0.28	0.009	s	2,21	3.00	0.22	0.07	ns	2,42	6.36	0.23	0.008	s	2,42	4.89	0.19	0.02	s*
	Anxious	2,21	28.05	0.73	0.001	s	1,21	1.83	0.08	0.19	ns	2,21	2.55	0.20	0.10	ns	2,42	0.65	0.03	0.48	ns	2,42	0.08	0.004	0.87	ns
	Stereotypy	2,21	0.25	0.02	0.78	ns	1,21	13.77	0.39	0.001	s	2,21	1.13	0.10	0.34	ns	2,42	5.63	0.21	0.02	s	2,42	8.06	0.28	0.005	s
	Self-Directed	2,21	0.36	0.03	0.70	ns	1,21	1.34	0.06	0.26	ns	2,21	0.04	0.004	0.96	ns	2,42	9.98	0.32	0.001	s	2,42	11.56	0.36	0.001	s
	Affiliative	2,21	11.16	0.52	0.001	s	1,21	1.09	0.05	0.31	ns	2,21	1.68	0.14	0.21	ns	2,42	3.85	0.16	0.03	s	2,42	0.62	0.03	0.54	ns
	Self-Soothing	2,21	0.40	0.04	0.67	ns	1,21	6.87	0.25	0.02	s	2,21	0.01	0.001	1.00	ns	2,42	2.90	0.12	0.10	ns	2,42	2.65	0.11	0.11	ns
	Coos	2,21	0.32	0.03	0.73	ns	1,21	3.70	0.15	0.07	ns	2,21	0.05	0.01	0.95	ns	2,42	20.29	0.49	0.001	s	2,42	1.52	0.07	0.23	ns
	Locomotion	2,21	0.03	0.003	0.97	ns	1,21	4.45	0.18	0.05	s	2,21	0.53	0.05	0.60	ns	2,42	2.06	0.09	0.14	ns	2,42	1.71	0.08	0.19	ns
	Cage Exploration	2,21	0.27	0.03	0.77	ns	1,21	1.76	0.08	0.20	ns	2,21	0.56	0.05	0.58	ns	2,42	5.02	0.19	0.02	s	2,42	1.17	0.05	0.31	ns

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