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Fear Learning, Conditioned Inhibition, and Extinction

In Adult Macaques

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Doctor of Philosophy

Psychology

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Fear Learning, Conditioned Inhibition, and Extinction In Adult Macaques

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An abstract of a dissertation 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

Doctor of Philosophy in Psychology

2010

Abstract

Fear Learning, Conditioned Inhibition, and Extinction

In Adult Macaques

By Andy M. Kazama

Our current understanding of the neural circuitry of fear responses supports an amygdalocentric model, in which the amygdala is critical for fear learning but the magnitude of fear responses can be down-regulated by either the hippocampus or the orbital frontal cortex when safety signals are detected. However, since much of this evidence comes either from correlative human neuroimaging studies and clinical populations suffering from non-selective brain damage, or from experimental studies in rodents that may not possess prefrontal regions homologous to those found in primates, it still is unclear whether these regulatory mechanisms are critical for safety signal learning. In addition, although the role of these brain structures in acquisition and regulation of fear learning was derived from lesions studies performed in adult animals, it is not known whether the same outcomes will occur when the same lesions will be incurred in early infancy when the brain is capable of significant structural and functional remodeling. . To this end, we tested 24 adult rhesus macaques that had received either neonatal sham-operations or neonatal amygdala, orbital frontal cortex, or hippocampal lesions in the first few days of in life (N = 6 in each group) in a fear-potentiated startle paradigm developed in rodents and humans. Animals were first trained to associate a stimulus (A+) with an aversive, but harmless, air-blast as measured by startle response (learned fear response). Next, animals learned to associate a second stimulus (B-) with the absence of an air-blast

(learned safety signal). Then, both stimuli were individually paired with a third stimulus (X) to form compound stimuli (AX+ or BX-). During a test phase, the effect of either air-blast alone, or all stimuli presented individually (A, B) and in combination (AX, BX, AB) on the magnitude of the potentiated startle was investigated. Finally, extinction of fear responses to the aversive stimuli (A- and AX-) was evaluated. As predicted, neonatal damage to the amygdala retarded fear learning but had no effect on safety signal learning, the modulation of fear, or extinction. However, neonatal damage to areas 11 and 13 of the orbital frontal cortex had no effects on any phase of the paradigm, suggesting that areas outside the middle orbital frontal cortex regions may be critical for down-regulating the amygdala during emotion regulation. Similarly, four of the six animals with early hippocampal damage had no difficulty in fear/safety signal learning, modulation or extinction. However, two animals with early hippocampal damage were severely impaired in their ability to learn either the fear or safety signals. The results seen in early hippocampal damage appear to be related to sparing both in emotion regulation as well as contextual learning and memory, thus it remains to be seen whether or not damage received late in life would affect fear/safety signal learning.

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Acknowledgments

The author would like to thank Jocelyne Bachevalier, Ph.D. for her guidance and patience. Thank you also to Mike Davis, Ph.D. for introducing me to the world of fear conditioning, and mentoring me on this specific project. Thank you also to Maria Alvarado, Ph.D. for her support and feedback, as well as to my wife and family for their continued encouragement. The author would also like to thank members of the Bachevalier Lab both past and present for helping with the daily care of the animals, intellectual stimulation, and friendship. Additionally, thank you to Darryl Neill, Ph.D., Leonard Howell, Ph.D., and Joe Manns, Ph.D. for serving on my committee and providing invaluable feedback throughout this project. Last, I would like to acknowledge the extreme sacrifice of the 24 monkeys who participated in this study, and whom I was blessed to care for from their birth.

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INTRODUCTION

Chronic Post-Traumatic Stress Disorder (PTSD) is characterized by excessive fear and anxiety resulting from a previously experienced traumatic event. The most recent reports suggest incidence rates of 5.2 million Americans and nearly a third of all Vietnam combat veterans (Kulka, et al., 1990; as cited in Gilbertson, et al., 2007). PTSD sufferers experience uncontrollable fear in response to a previously learned fearful cue (e.g. helicopter sound), even when surrounded by many cues that should signal safety (e.g. in the company of a loved one, far away from site of combat experience, etc.). Thus, understanding how fears are acquired and more importantly understanding how they can be regulated is critical in translating basic research to the treatment of human disorders associated with heightened fear responses, such as PTSD and anxiety disorders. Basic research in the search of the neural circuitry underlying fear learning and fear inhibition has consistently used classical fear conditioning and extinction paradigms. More recently, Davis and colleagues (2008) developed a cross-species paradigm that enable investigators to study the neural structures mediating fear conditioning, safety signal learning and extinction in rats, monkeys, and humans (including PTSD sufferers). Briefly, the AX+/BX- paradigm (described in detail in the Methods section) uses one cue (A+) to signal an aversive foot shock in rats, or an aversive air-blast in humans and monkeys, a second cue (B-) to signal safety, and a third neutral cue (X) so that the configuration of A+B- (Aversive/Safe) can be tested. Startle response amplitude in normal subjects is high to A, low to B and intermediate when A is combined to B, suggesting that subjects modulate their fear of A, based on the presence of the safety signal (B). Then, extinction can also be measured by repetitively presenting the aversive cue A in the absence of the aversive stimulus.

Using the AX+/BX- fear-potentiated startle paradigm in non-human primates, the proposed study will examine the neural underpinnings of fear learning, safety signal processing, and fear extinction in the hopes of better understanding the reasons why PTSD sufferers are unable to utilize safety signals from their environment to regulate their uncontrolled fear response. In addition, since evidence to date suggests that some individuals may have minor brain abnormalities present prior to being exposed to a traumatic event, and thus be predisposed to develop PTSD, this study will utilize an early lesion model on areas thought to play a role in emotion regulation. As discussed below, these areas are the amygdala, the orbital frontal cortex, and the hippocampus.

The neural system of fear learning and fear modulation

In humans, neuroimaging studies in normal subjects together with studies of patients suffering from PTSD or from traumatic brain injury have revealed several key brain areas involved in the emotional regulation of fear, with the primary focus being the hyper-excitation of the amygdala (for review see Shin, Rauch, & Pitman, 2006). Through its connections with hypothalamic and brainstem areas, the central nucleus of the amygdala has been repeatedly shown to mediate specific signs of fear and anxiety, including all aspects of the fight or flight response, such as increased heart-rate, cortisol, and an increase in acoustic startle response (Davis, 2000). Thus, over-excitation of the amygdala, especially during inappropriate circumstances, results in uncontrollable fear.

Although activation of the amygdala is central to the fear response (Davis & Whalen, 2001, LeDoux, 2000), other brain areas, including the hippocampus and ventromedial prefrontal cortex, are thought to play a critical role in both learning safety signals and using those signals to

functionally down-regulate the amygdala and reduce, or even extinguish, the fear response (see for reviews Quirk & Beer, 2006; Sotres-Bayon et al., 2006; Meyers & Davis, 2007; Corcoran & Quirk, 2007).

The following sections will briefly examine evidence for the involvement of each of these structures in fear learning and safety signal learning and have been organized by species given the known species differences in the neural substrate supporting these cognitive processes.

Human Studies

Amygdala

Many human neuroimaging studies have found activation of the amygdala correlating strongly with various aspects of the fear response, including during the early phases of fear learning (for review see Sehlmeyer et al., 2009), expression and recall of emotional memories (for review see Hamann, 2001), and the early phases of extinction (LaBar et al., 1997, 1998). Additionally, neuroimaging data suggest that hyperactivity in the amygdala is common for many anxiety disorders, including PTSD, social anxiety disorder, specific phobias and others (Rauch et al., 2000; Shin et al., 2004; Williams et al., 2006; Lorberbaum et al., 2004; Phan et al., 2006; Stein et al., 2002; Straube et al., 2004; Tillfors et al., 2001; Schienle et al., 2005; Veltman et al., 2004). Additionally, positive relationships exist between PTSD symptom severity and amygdala activation (Rauch et al., 2000; Protopopescu et al., 2005) and patients with PTSD show hyper vigilance towards their environment as well as heightened acquisition of conditioned fear in a laboratory setting (Shin et al., 2006; Orr et al., 2000; Peri et al., 2000). Finally, patients with traumatic brain injury, including the amygdala, have significantly less incidence of PTSD as compared to patients in whom the amygdala has been spared or healthy control subjects (Koenigs et al., 2007; Herskovits et al., 2002). Thus, based on the neuroimaging and lesion

studies, it appears that the amygdala is not only critical for the fear response, but additionally, hyper-excitation of the amygdala results in an exacerbation of the fear response, which is the hallmark symptom of PTSD.

Hippocampus

As with the amygdala, several human neuroimaging studies have noted hippocampal activity during fear learning, as well as during the modulation of emotion (for review see Sehlmeyer et al., 2009), and one study reported activation during Pavlovian extinction (Knight et al., 2004). Additionally, human studies examining the potential involvement of hippocampal dysfunction in PTSD have revealed several important findings. First, decreased hippocampal activity was found while PTSD patients experienced a symptomatic state (Bremner et al., 1999; Shin et al., 1999). Second, human patients with PTSD have shown decreased hippocampal volumes, compared to either trauma-exposed control subjects or trauma-unexposed healthy subjects. For instance, in a volumetric study using identical twins in which one sibling developed PTSD, Gilbertson and colleagues (2007) discovered that smaller hippocampal volumes were correlated with higher rates of PTSD. Additionally, identical twins with smaller than average hippocampal volumes had more difficulty in learning contextual cues, a function known to be dependent on the integrity of the hippocampus (Gilbertson, 2007). Third, fMRI studies have shown decreased hippocampal activation that correlated with PTSD symptom severity (Shin et al., 1999; Bremner et al., 1999). Conversely, Semple and colleagues (2000) reported elevated hippocampal activation in PTSD patients during baseline conditions, without decreases in hippocampal activation during symptom provocation. However, there are several

potential confounds in this later study, especially the fact that the PTSD patients had a history of cocaine and alcohol abuse.

Additional studies, which have looked at cognitive deficits in PTSD patients, have found evidence of possible hippocampal dysfunction, even going as far as to posit that PTSD should be classified as a memory disorder (for review, see Elzinga & Bremner, 2002). For instance, PTSD patients have been reported to have deficits in declarative memory, intrusive memories, fragmentation of memories, and trauma-related amnesia, all of which may indicate hippocampal dysfunction (Elizinga & Bremner, 2002). When tested in the laboratory setting, Vietnam veterans with PTSD scored significantly lower on the Wechsler Memory Scale, Auditory Verbal Learning Test, and showed significant decrements in retention of previously presented material following exposure to an intervening word list (Elizinga & Bremner, 2002).

In summary, the neuroimaging and cognitive evidence supports the hypothesis that dysfunction of the hippocampus is involved in PTSD and that the related deficits in contextual learning following hippocampal dysfunction may lead to a deficit in learning safety signals. Furthermore, some studies have suggested that small hippocampal volumes may predispose certain individuals towards developing PTSD (Koenigs et al., 2007).

Prefrontal cortex

Recent reports from human studies suggest that the ventromedial and later aspects of the prefrontal cortex may play a role in down-regulating the amygdala (for review see Davidson, 2002, Quirk and Beer, 2006), and that a dysfunction of the ventromedial prefrontal cortex may be responsible for some PTSD symptoms, particularly the inability to control the fear response long after the traumatic event has passed. Evidence supporting this claim includes: 1) Morphometric MRI studies showing decreased ventromedial prefrontal cortex volumes in PTSD

patients (Fennema-Notestine, Stein, Kennedy, Archibald, & Jernigan, 2002; Rauch, Shin, Segal, Pitman, Carson, McMullin, Whalen, & Makris, 2003), 2) Decreased activation in ventromedial prefrontal cortex in PTSD patients during negative trauma-unrelated narratives, combat pictures and/or sounds, fearful facial expressions, and performance of emotional Stroop interference tasks, as well as during a variety of symptom provocation paradigms (for review see Shin et al., 2006). 3) Neuroimaging studies demonstrating that ventromedial prefrontal cortex activation is negatively correlated with PTSD symptom severity (Williams, Kemp, & Felmingham, 2006, Shin, et al., 2004) and with the magnitude of the conditioned response during extinction in healthy subjects. 4) Finally, a clinical study by Roberts and colleagues (2004) reporting that non-selective frontal damage, which included ventromedial prefrontal cortex, was correlated with more facial expression of surprise and enhanced emotional responses when emotional cues were unexpected. Paradoxically, Koenigs and colleagues (2007) reported that damage to either the amygdala or ventromedial prefrontal cortex substantially reduced the occurrence of PTSD, although the damage was non-selective (all brain trauma caused by gunshot wounds to the head), and thus it is possible that the sparing of PTSD symptoms was not strictly due to damage to the ventromedial prefrontal cortex.

In summary, although the human evidence supports the hypothesis that the ventromedial prefrontal cortex is involved in the extinction of a learned fear and that hypoactivation of the ventromedial prefrontal cortex appears to play a role in PTSD, due to the non-selective nature of the brain injury in human subjects and the correlational nature of neuroimaging techniques, it is impossible to ascertain the exact nature of the ventromedial prefrontal cortex-amygdala interaction. Furthermore, a putative role of the ventromedial prefrontal cortex in safety signal learning and in the inhibition of learned fears has not been directly evaluated.

Summary

The human literature suggests that the amygdala plays an important role in fear learning, particularly in the early phases. However, other structures may play a greater role in learning safety-signals. Additionally, because of the correlational nature of the neuroimaging techniques and the non-specificity of the lesion studies, it is unclear whether or not the hippocampus and ventromedial prefrontal cortex are critical for regulating the fear response. Furthermore, their potential role in safety signal learning remains to be empirically evaluated.

Rodent Studies

Complementing human studies, which have given a tentative picture of the neuropathology involved in PTSD and detailed information concerning the cognitive deficits seen in this disorder, rodent models of fear learning and fear regulation have provided a detailed map of the neural pathways thought to be involved in PTSD.

Amygdala

Using various fear conditioning paradigms combined with either temporary or permanent lesion techniques, or pharmacological manipulations, the fear circuit has been described in great detail from stimulus input to response output (for review, see Davis, 1992, LeDoux, 1998). Like in humans, the rodent literature fully supports an amygdalocentric view of fear conditioning, as damage or temporary inactivation of the amygdala causes robust deficits of fear conditioning in rats (LeDoux, 1998). Unlike the primate amygdala, which is critical for the acquisition but not the expression of fear, the rodent amygdala, and in particular the lateral and central nuclei appear to be critical for both the acquisition and expression of fear (Davis, 2000). Although the basal nucleus of the amygdala has been implicated in extinction, results have varied depending upon whether the manipulation was performed before or after acquisition (Sotres-Bayon, Cain, &

LeDoux, 2006). Thus, the basal nucleus does not appear to be critical for extinction and, presumably, because of this limitation, its role in safety signal learning has yet to be evaluated.

Hippocampus

In rats, most studies examining damage to the hippocampus and contextual fear conditioning report a loss of contextual conditioning abilities, particularly as it pertains to spatial information (Phillips & LeDoux, 1992, Kim & Fanselow, 1992). In a recent study, Richmond and colleagues (1999) found that selective lesions to the ventral hippocampus, which is strongly connected to the amygdala, resulted in a loss of conditioned freezing while sparing spatial abilities. Additionally, both classical and novel anxiolytic compounds (both injected systemically and directly into the hippocampal formation) reduce hippocampal neuronal activity, resulting in behavioral inhibition (McNaughton & Corr, 2004). Thus, the hippocampus is clearly important for processing the context of the situation, but also for the regulation of emotion.

Nevertheless, other studies have reported no effects of hippocampal lesions on emotional regulation (Maren et al., 1997; Winocur, 1997). Holland and Bouton (1999) have theorized that, during simple tasks, and in the absence of a fully functional hippocampus, rats may use a simpler elemental strategy to associate a small number of contextual stimulus elements rather than a more detailed configural representation. These findings may parallel those of humans with PTSD, showing that simple triggers in the environment (e.g. sound of a helicopter) spark anxiety attacks, despite the presence of an environment rich in safety cues. To date no studies have directly evaluated the role of the hippocampus in safety signal learning.

Recently, Peters and colleagues (2010) found that Brain-Derived Neurotrophic Factor (BDNF) injected into infralimbic cortex (homologous to primate ventromedial prefrontal cortex)

mediated extinction. Importantly, a major BDNF input into infralimbic cortex is through the CA1 inputs of the hippocampus. Surprisingly, these injections of BDNF appeared to take the place of extinction training trials. In a second experiment, Peters and colleagues (2010) examined BDNF protein levels in rats that were quick learners as compared to rats that were slow learners. Higher levels of hippocampal BDNF were present in quick learners. Thus, it appears that, despite its direct connections to the basal nucleus of the amygdala, the hippocampus' primary down-regulatory action upon the amygdala may be indirectly through its BDNF output to the infralimbic/prefrontal cortex, which then acts upon the intercalated cells and/or the basolateral nucleus within the amygdala.

Prefrontal Cortex

As in humans, the rodent ventromedial prefrontal cortex is strongly connected to both the hippocampus and the amygdala (for review see Aggleton, 2000). The ability to extinguish a conditioned fear to a conditioned stimulus is impaired by damage to the medial prefrontal cortex (Morgan & LeDoux, 1995), and the infralimbic area of the prefrontal cortex in rats appears to be especially important for the inhibition of conditioned fear responses as electrical stimulation during extinction facilitates behavioral extinction. In an elegant study, Quirk and colleagues (2003) recorded electrical activity from neurons in the central nucleus of the amygdala, a nucleus thought to play a key role in the motor output of the fear response, while simultaneously stimulating the insula, which has major excitatory inputs to central nucleus. Because stimulation of the medial prefrontal cortex inhibited the responsiveness of neurons in the central nucleus, the authors concluded that the medial prefrontal cortex likely plays an important role in down-regulating the fear-response. Although theoretically, like the amygdala and hippocampus, the

medial prefrontal cortex is a strong candidate structure for safety signal learning, this proposal has not been directly evaluated.

Summary

In sum, using rodent models of fear conditioning, it has been shown that the amygdala is critical for fear learning, the hippocampus for contextual fear conditioning, and the ventromedial prefrontal cortex for the extinction of the fear response. However, although both the hippocampus and medial prefrontal cortex play critical roles in fear conditioning, their role in safety signal learning has yet to be evaluated. Finally, although the rat amygdala, and to a lesser degree the hippocampus, appears to be highly conserved across mammalian species, the rat frontal cortices are far less developed than in primates. Thus, given the greater similarities in brain structures and connectivity between monkeys and humans, the development of a non-human primate model of fear regulation may prove to be of greater benefit for elucidating the neural underpinnings of safety signal learning and extinction and for translating basic research to fear disorders in humans, such as PTSD and anxiety disorders.

Non-Human Primate Studies

Amygdala

Like humans and rats, the monkey amygdala appears to be critical for processing emotional information (for review, see Kalin & Shelton, 2003). Although several studies have shown that the monkey amygdala is critical for adaptively responding to threats (Aggleton, & Passingham, 1981; Kalin et al., 2001; Machado & Bachevalier, 2008; Izquierdo & Murray, 2004), only one study has specifically looked at its role in the acquisition of learned fear (Antoniadis et al., 2007), and no study has investigated its putative role in safety signal learning. Using a fear-potentiated startle paradigm adapted for monkeys, Antoniadis and colleagues (2007,

2009) found that selective ibotenic acid lesions of the amygdala blocked the acquisition, but not the expression of fear-potentiated startle, re-confirming the evidence seen in both humans and rats that the amygdala is critical for fear learning.

Hippocampus

There have been very few monkey studies that have investigated the role of the hippocampus in the expression and regulation of emotion. First, studies investigating the role of the hippocampus in defensive behaviors have found that lesions of the hippocampus blunted emotional reactivity in response to threatening stimuli (Machado & Bachevalier, 2008, Chudasama, Wright, & Murray, 2008). However, the ability to acquire a learned fear, measured with fear-potentiated startle, was not impacted by selective hippocampal damage (Antoniadis et al., 2007). Thus, a more thorough investigation into the role of the hippocampus in the regulation of the fear response is needed. Finally, there are currently no studies that have examined the role of the monkey hippocampus in safety signal learning and extinction.

Prefrontal Cortex

It has long been known that damage to the ventral surface of the prefrontal cortex (orbital frontal cortex) causes striking deficits in social cognition (Butter et al., 1970). Some have hypothesized that these deficits are due in part to an inability to modulate emotion-related behaviors (Kalin et al., 2007). Monkeys with damage to the orbital frontal cortex showed 1) abnormal aggressive behaviors (either increased or decreased) (Butter et al., 1970; Machado & Bachevalier, 2007); 2) loss of dominance status (Butter & Snyder, 1972); and 3) decreased threat-induced freezing and marginally decreased fearful responses to threatening stimuli (Kalin et al., 2007). Although these findings clearly show a deficit in emotion regulation, currently, no studies have directly looked at the potential role of the orbital frontal cortex in fear learning or

safety signal learning. Given that Brodmann areas 11 & 13 in particular are bi-directionally and heavily connected to the amygdala, we believe that these areas would be prime target candidate areas to modulate fear responses.

Summary

In sum, normal monkeys show behavioral patterns of fear inhibition similar to those found in both humans and rats in that they are able to modulate startle response based on cues signaling safety. Additionally, the neuroanatomical connections between the amygdala, hippocampus, and Brodmann areas 11 & 13 of the OFC have been well described in non-human primates, and fully support an amygdalocentric model of fear regulation. However, although the hippocampus and OFC are likely candidates, to date no studies have investigated their role in safety signal learning and the inhibition of the fear response.

Hypotheses

The purpose of the proposed study was to examine the neurobiological basis of safety signal processing in a the non-human primate animal model using the AX+/BX- fear-potentiated startle paradigm recently described by Winslow and colleagues (2008). These researchers found that, like healthy human subjects, normal monkeys are able to modulate their fear response, showing less startle amplitudes when the cue signaling the aversive air blast (A+) was combined with the safety signal (B-). Thus, using this paradigm, we proposed to assess the effects of neonatal selective lesions of the amygdala, hippocampus and orbital frontal cortex on fear conditioning, safety signal learning and fear extinction.

Based on the current literature, we hypothesize that:

1- Monkeys with neonatal amygdala damage will show impaired fear conditioning, and may not reach the remaining phases. However, should they show only retardation on the initial fear learning phase as compared to sham-operated, we would predict no impairments in safety-signal learning, or modulation. Finally, extinction would likely remain unaffected.

2- Monkeys with neonatal damage to areas 11 & 13 of the orbital frontal cortex will be unimpaired on basic fear learning, or safety-signal learning, but will show a lack of, or reduced, down-regulation of their fear response when a safety signal is available. Thus, they will take longer to extinguish the fear response when the aversive cue is no longer paired with the aversive air-blast. This prediction is largely based on data collected on the same animals using appetitive tasks and demonstrating that these selective orbital frontal cortex lesions altered the ability to modulate their responses to stimuli when their reward value was altered (Kazama et al., 2007).

3- Monkeys with neonatal damage to the hippocampus will be unimpaired on basic fear conditioning, but will have difficulty in learning to discriminate between the aversive cue and the cue signaling safety. Thus, these animals will be unable to use the safety-signal to modulate their startle during the transfer phase. Finally, these animals will also have difficulty in extinguishing to the aversive cue.

Fear learning, conditioned inhibition, and extinction in adult macaques: I- Effects of neonatal amygdala lesions

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Abstract

Fear conditioning studies have demonstrated the important role played by the amygdala in emotion processing. Recent neuroimaging research in humans has highlighted the centrality of the amygdala, especially during the early stages of fear conditioning, but also suggests that other structures may support fear learning during later stages. The limited research that has investigated the role of the amygdala in fear conditioning using monkeys has also suggested that the amygdala plays a critical role in the acquisition of fear, but that the fear memory and its expression can be supported by other structures. The current study sought to examine fear-learning abilities in adult monkeys who had received neonatal selective neurotoxic amygdala damage, as measured by fear-potentiated startle. Results suggested that although there was a significant impairment compared to control animals, amygdalectomized animals were able to eventually learn a simple stimulus/air-blast association. Because all animals were eventually able to learn this simple association, we then examined their ability to learn and apply a safety cue, as measured by the AX+/BX- Paradigm. Finally, we examined their ability to extinguish their fear to the previously learned aversive stimuli. We found that four of the six amygdalectomized animals were able to learn to discriminate between an aversive and safety cue, and that all of the animals that learned this distinction were able to modulate their fear-potentiated startle in the presence of the safety-cue. Finally, amygdalectomized animals were able to extinguish to the aversive cue as quickly as control animals. Taken together, these results suggest parallel, albeit slower fear processing outside the amygdala. In addition, in the absence of a fully functioning amygdala, this parallel processing sufficiently supports the ability to flexibly modulate the fear response. Finally, although human neuroimaging studies have reported amygdala activation

during the early stages of extinction, our findings suggest that this activation is not critical for the extinction of the learned fear.

Fear conditioning has proven to be an extremely robust, rapid, and precise experimental approach for studying the neurobiological substrates of fear. In particular, fear conditioning studies in rodents have demonstrated the important role played by the amygdala in the acquisition, retention and expression of fear (Davis, 1992; Fanselow & Ledoux, 1999; LeDoux, 2000; Maren, 2001; but see Falls & Davis, 1995). To establish a parallel between rodent and primate species in the role of the amygdala in fear conditioning, a recent study (Antoniadis et al., 2007) has used a fear-potentiated startle paradigm closely modeled after rodent studies (Winslow et al., 2002) to assess the role of the amygdala in conditioning and its expression in monkeys. As for rodents, complete bilateral neurotoxic amygdala lesions performed before fear training impaired fear conditioning (low fear-potentiated startle). By contrast, unlike rodents, when the amygdala lesions were performed after fear conditioning, monkeys expressed high fear-potentiated startle. These data suggest that in primates the amygdala plays a critical role in the acquisition of fear, but that the fear memory and its expression can be supported by other brain structures. Interestingly, similar conclusions were drawn from neuroimaging studies of fear conditioning in healthy human subjects when they were exposed to fear signals over long periods of time (for review, see Sehlmeyer et al., 2009). Robust amygdala activation was noted during the early stages of fear conditioning, but this activation decreased as conditioning proceeded longer. Instead, with longer time, the brain activation shifted from the amygdala to other structures, such as the anterior cingulate and insular cortices (Buchel et al., 1998; LaBar et al., 1998; Everitt & Robbins, 2005). These data suggested that the amygdala may be playing a key role only during early stages of fear acquisition, whereas other brain structures could maintain the conditioned-fear learning. Thus, similar to the lesion studies in monkeys, the retention and

expression of fear conditioning in humans appears to be supported by structures outside of the amygdala.

In summary, it is clear that the amygdala plays a critical role in fear learning across all species studied. However, what remains to be explored is whether the amygdala also plays a critical role in fear acquisition in early infancy. Gathering such information seems critical given that many neuropathological disorders in humans, such as anxiety, depression, and post-traumatic stress disorder have a strong developmental component and are associated with dysfunction of the amygdala (for review, see Machado & Bachevalier, 2003; Monk, 2008; Gillespie et al., 2009).

Previous developmental studies in rodents have indicated that early damage to the amygdala result in alterations of locomotor activity, social behaviors, stress-induced behaviors, pre-pulse inhibition, and acoustic startle response (Daenen et al., 2001; 2002 a, b; 2003; Wolterink et al., 2001), which were associated with decreased cerebral glucose utilization later in life (Mirjam et al., 2006). However, the effects of neonatal amygdala damage on simple fear learning have yet to be examined. Thus, in the present study, we examined the ability of adult monkeys with neonatal amygdala damage and their age-matched controls to associate a conditioned cue with an aversive, but painless, puff of air, using the fear-potentiated startle developed for nonhuman primates (Winslow et al., 2006; Antoniadis et al., 2007). Given that, similar to amygdala damage acquired in adulthood (Machado et al., 2008; Machado et al., 2007), neonatal amygdala damage altered the ability to flexibly modulate behavioral responses in an appetitive task (Kazama et al., 2007) and blunted emotional reactivity to fearful stimuli (Raper et al., 2009), suggesting little sparing of functions following amygdala lesions in infancy, we predicted that the neonatal amygdala lesions would also result in an impairment in the

acquisition of fear-potentiated startle. Interestingly, our results demonstrated that neonatal amygdala damage retarded, but did not abolish, fear acquisition. Given these unexpected results, we further investigated whether the neonatal amygdala lesions would alter safety-signal learning and their use to flexibly modulate fear-conditioned startle responses, as well as the extinction of fear-conditioned startle, using the AX+BX- Fear-Potentiated Startle Paradigm (Winslow et al., 2008). Preliminary data of this study have already been published in an abstract form (Kazama et al., 2010).

Methods

Subjects

Twelve adult rhesus macaques (Macaca mulatta) of both sexes, aged approximately six years and ranging from 4.5-8 kg participated in this study. All animals were acquired as newborns and were nursery-reared (see Goursaud & Bachevalier, 2007 for details) with daily contact with a human care-giver and peers until young adulthood, when they were separated into single cages that allowed visual exploration but limited physical contact among individuals. Animals received brain surgeries between 8-12 days of age that included sham-operations (Group Neo-C, 3 males and 3 females) and neurotoxic lesions of the amygdala (Group Neo-Aibo, 3 males and 3 females). Following surgeries, all animals were behaviorally tested to assess emotional reactivity (1-4 weeks, 2 & 5 months, 3 years of age), social interactions (3 & 6 months, 3 years of age) , goal-directed behaviors (3 months, 3, 4, & 5 years of age) and memory processes (6, 8, 9, & 18 months, 2 years of age) at different time points across development. The Animal Care and Use Committees of the University of Texas Health Science Center at Houston and of Emory University approved all neuroimaging, neurosurgical and behavioral testing procedures.

Procedures for neuroimaging, surgical, and estimation of lesion extent as well as rearing conditions have been described in details earlier (Nemanic et al., 2002; Kazama & Bachevalier, 2010; Goursaud & Bachevalier, 2006) and will be briefly summarized below.

Pre- and post-Surgical MRI scans

Just prior to surgery, animals were anesthetized with isoflurane gas (1-2% to effect), intubated with an endo-tracheal canulae to maintain sedation, and mounted in a stereotaxic non-ferromagnetic head holder. An intravenous drip solution containing 0.45% NaCl maintained hydration, and heart rate, respiration rate, blood pressure, body temperature and expired CO2 were monitored throughout the procedures. High resolution FSPGR (T-1) and Fluid-Attenuated Inversion Recovery (FLAIR) MRI scans were obtained in a GE sigma 1.5 Tesla Echo Speed scanner (GE Medical Systems, Milwaukee, WI) using a 3-inch head coil for all subjects. The T-1 images were used to precisely select and calculate coordinates of neurotoxin injection sites within the amygdala in all animals (Saunders, Aigner, & Frank, 1990; Málková et al., 2001; Nemanic et al., 2002).

Four to six injection sites spaced 2 mm apart in the Medial/Lateral and Dorsal/Ventral directions were centered within the amygdala to include all amygdaloid nuclei while sparing the adjacent cortical areas. These neuroimaging procedures were repeated 7-10 days after surgery for the experimental animals only and MR images were used to estimate lesion extent.

Surgery

At completion of the MRI procedures, animals were kept anesthetized and secured in the stereotaxic apparatus, and were immediately transported to the surgical suite. A local anesthetic (Marcaine 25%, 1.5m., s.c.) was injected along the incision line. Using aseptic surgical procedures, the skin was cut from the occiput to a point in between the two eyebrows and

retracted laterally together with the subcutaneous fascia. A small bone opening was performed above the amygdala bilaterally and small slits of the dura were made to allow the penetration of the injection needles. For sham-operations, the surgical procedures ended at this point and no injections were made. For amygdala lesions, injections of the neurotoxin, ibotenic acid (Biosearch Technologies, Novato, CA) 10/mg/ml in phosphate buffered saline, pH 7.0) were made simultaneously through two, 10 µl Hamilton syringes held in Kopf electrode manipulators (David Kopf Instruments, Tujunga CA). Each was lowered slowly to the injection target where 1.8 to 2.0 µl of ibotenic acid was slowly injected (0.2µl/minute) at each site. The needles were allowed to remain in place for an additional 3 minutes to allow diffusion of the drug before being retracted. After sham-operations or ibotenic acid injections were completed, the dura, subcutaneous fascia and skin were sutured in anatomical layers. The animals were then removed from the Isoflurane gas anesthesia and allowed to recover in an incubator ventilated with oxygen.

Pre- and Post-Surgical Treatment

Beginning 12 hours before surgery and ending on post-surgical day seven, all monkeys received treatments to control swelling (dexamethazone sodium phosphate, 0.4 mg/kg, s.c.) and minimize risk of infection (Cephazolin, 25 mg/kg, per os). Additionally, Acetaminophen (10mg/kg, p.o.) was administered four times a day for three days after surgery to relieve pain. A topical antibiotic ointment was also applied to the wound, daily.

Lesion Verification

Because all animals are currently used in additional behavioral studies, the extent of ibotenic acid lesions was assessed using both FLAIR and T1-W coronal MR images obtained 7 to 10 days after surgery, and comparing them to the pre-surgical MR images. Extent of hypersignals on FLAIR images (indicative of brain edema) were transposed onto drawings of

coronal sections from a normal two-week-old infant rhesus monkey atlas (J. Bachevalier, unpublished atlas) matched to the MR images. Estimated volume of edema for each brain area of each drawn coronal section was measured using Image J, and percent of estimated volume damage for each brain area was then calculated.

Behavioral testing

The animals were 4-6 years of age at the start of behavioral testing, which lasted approximately one month. All sessions were spaced 72 hours apart, and session length depended upon the stage of training (see below for details). During training, animals were neither food deprived nor water restricted, but were given additional treats during primate chair training as well as fresh fruit, daily. All methods below have previously been described (Winslow et al., 2008; Antoniadis et al., 2007, Winslow et al., 2007), and will be briefly summarized below.

Apparatus: During training, animals were seated in a non-human primate chair located in a sound attenuated chamber equipped with an automated system designed to deliver unconditioned and conditioned stimuli. The chair was mounted on a platform located above a load cell (Med Associates, St. Albans, VT). Animal startle produced displacement of the load cell (Sentran YG6-B-50KG-000) the output of which was amplified, digitized and stored on a computer.

Stimuli: Two unconditioned stimuli (US) were used. A 700 msec jet of air (100 PSI) generated by an air compressor located outside the chamber and projected at the face of the monkey via four air jet nozzles. A startle stimulus, which was a 50-msec burst of white noise (5 msec rise-decay time) of varying intensities (range: 95-120 dB) emitted by a white noise generator and delivered through the same speakers as the background noise. Three cues served either as an aversive conditioned stimulus (A), a safety conditioned stimulus (B) or a neutral

stimulus (X). The visual CS was a 4-sec light produced by four over-head 20-Watt halogen bulbs (combined 250 Lux) attached to the top of the test chamber. The auditory CS was a tone (80 dB, 4 sec, 5000 kHz) produced by an overhead speaker. The tactile CS was produced by a quiet computer fan that directed gentle airflow onto the monkey's head. The CS assignments as cues A, B or X were pseudo-random and counter-balanced across groups. Thus, some animals received the light as the aversive CS, whereas others received the tone as aversive CS, and so forth.

Acoustic Startle Response:

To evaluate any potential effects of lesion on acoustic startle, the animals were placed in the apparatus and exposed to two separate days of 60 trials each, which were composed of baseline activity without startle stimuli (10 trials), and of startle responses to noises of varying decibel intensities (95, 100, 110, 115, & 120 dB; 10 trials each). All trials were pseudo-randomly intermixed throughout each session. Animals were then tested for pre-pulse inhibition before moving on to the AX+/BX- paradigm and these data will be published elsewhere.

Pre-training: Prior to the conditioning phase, the animals were habituated to the three conditioned cues to assess any unconditioned effects of the cues on the startle response prior to conditioning. First, animals received two separate days of 30 trials each during which the to-be-conditioned cues (light, tone, or airflow from quiet fan) and their combinations (light/tone, light/airflow, tone/airflow) were presented in the absence of the startle noise. Then, animals were given days of 60 trials, consisting of 30 trials with the startle noise alone (95dB) and 30 trials in which the 95dB startle noise was paired with one of the to-be-conditioned cues or their combinations for 5 trials each pseudo-randomly ordered. Within each of the cue-startle trials, the startle stimulus was presented 4 sec after the onset of the CS. These pre-training sessions were

repeated for each monkey until presentation of the cue that served as the safety signal (cue B) for that animal produced less than a 30% increase in startle amplitude compared to noise alone presentations.

A+ Training Phase:

The purpose of this phase was to train the animal, using Pavlovian fear conditioning procedures, to associate a cue A with an aversive air-blast. These A+ air-blast trials occurred four times per 28-trial session, and were always scheduled such that one occurred at the beginning and one at the end of each session. The remaining two pairings were psuedorandomly intermixed within the remaining 24 startle test trials so that animals could not predict when cue A would be followed by an air-blast as opposed to a startle noise. The startle stimulus or air-blast was presented 4 sec after the onset of cue A. The remaining 24 trials consisted of 4 trial-types, i.e. Noise Alone at 95dB, Noise Alone at 120dB, Cue A with 95dB Noise, Cue A with 120dB Noise) and were presented pseudo-randomly 6-trial each per session. Animals received A+ Training for a minimum of two sessions, and until their percent Fear-Potentiated Startle (%FPS) was 100% above their pre-training startle to cue A+. %FPS was defined as: [Mean startle amplitude on CS test trials – mean startle amplitude on startle noise alone test trials)/mean startle amplitude on noise burst alone test trials] X 100.

A+/B- Training Phase: The purpose of this phase was to train the animal to associate a second cue (B) with the absence of an air-blast, thus this cue was termed the safety-signal. Animals received 40-trial sessions composed of: twelve trials in which the safety cue (B) was presented with both startle noise intensities (95dB and 120dB, 6 trials each) but never paired with the air-blast US, four trials in which the A+ continued to be paired with the air-blast (according to the schedule described previously) ,twelve trials in which the A cue was paired

with the startle noise (95 dB and 120 dB, 6 trials each); and twelve trials of startle noise alone (95 dB and 120 dB, 6 trials each). Animals received A+/B- Training for a minimum of two sessions, and until a difference of 100% FPS was obtained between the two cues.

AX+/BX- Training Phase:

Previous conditioned inhibition training in primates had found that the presentation of the transfer cue (AB) was treated not as a compound cue consisting of the aversive and safety cues, but rather as a completely novel third cue. Thus, the purpose of this phase was to train the animal to discriminate compound cues using a third neutral cue (X), which was presented in combination with either the A or B cues. This phase included 40-trial sessions constructed similarly to A+/B- Training. The only difference was that both the aversive cue (A) and the safety cue (B) were presented in combination with the neutral cue (X), yielding compound cues AX+ and BX-. As with the A+/B- Training, animals received the AX+/BX- Training for a minimum of two sessions, and until there was a difference of 100% FPS between the two compound cues.

AB Testing/Transfer Test: In this probe test of conditioned inhibition, animals were tested to determine whether the presence of the safety signal (B) would reduce the anxiety (and thus %FPS) to the aversive cue (A) when both were presented simultaneously (AB). This 48-trial probe session, presented 72 hours after the last AX+/BX- Training session consisted of all trial types, including two A+ air-blast pairings intermixed within (a) Noise Alone trials (95 dB and 120 dB, 6 trials each), (b) 95 dB and 120 dB cue pairings (A, B, AX, BX, 5 trials each per noise intensity); and (c) 95 dB and 120 dB AB compound cue (5 trials per noise intensity). All trials were pseudo-randomly intermixed.

Extinction: Finally, all animals were presented with multiple 12-trial sessions of either the 95 dB startle stimulus elicited alone or in the presence of cues A and AX (4 trials of each type) to evaluate fear extinction. Training was completed when the animal returned to its pre-training startle amplitude.

Data Analysis

Throughout the different phases, the startle amplitudes were recorded via the Med Associates software and amplified via the load cell. The main parameter of interest was the percent fear potentiated startle (FPS) as defined above. If in the course of training, an animal's % FPS steady declined with no improvement over an extended period, that animal was given a maximum score of 15 sessions. This criterion was determined after training one animal for 15 days without successful conditioning).

Data analysis included three parts. First, we used a Geisser-Greenhouse corrected repeated measures ANOVA to compare the acoustic startle responses to the varying intensities (95, 100, 110, 115, & 120 dB) across groups. Second, we assessed the animal's ability to associate and discriminate between the aversive and safety cues (A, B, AX, BX) using a "sessions to criterion parameter". Because our control animals learned the task at floor (e.g. 2 sessions per phase), and thus had no variability, we again used non-parametric statistics to investigate any group differences (Mann-Whitney U). Third, since previous reports (Winslow, Noble, & Davis, 2008) indicated that startle values are not normally distributed, the transfer test data were transformed with a logarithmic base 10 transformation and group comparisons were made with repeated measures ANOVAs.

Results

Lesion extent:

The extent of bilateral amygdala damage in all cases averaged 62.5 % (see Table 1, and Figure 1 for a representative case), and always included the central, medial, accessory basal, and dorsal areas of the basal nuclei. For three cases (Neo-Aibo -1, -4 and -6), the damage was substantial and symmetrical and the remaining three cases (Neo-Aibo -2, -3 and -5) had more substantial amygdala damage on the right hemisphere (61.1 % to 77.6 %) than on the left hemisphere (33.0 % to 42.0 %). Finally, extent of unintended damage to the perirhinal and entorhinal cortical areas, anterior portion of the hippocampus, and striatum were negligible for nearly all cases with the exception of Neo-Aibo-1 and -4 that had slight unilateral damage to the caudate nucleus ventrally.

Acoustic Startle Response

Because the baseline startle response of two animals in Group C (cases Neo-C-2 and Neo-C-6) exceeded the amplitude recorded by the load cells across this phase, these two animals were dropped from the study. As illustrated in Figure 2, both sham-operated and animals with neonatal amygdala lesions demonstrated greater startle responses as the intensity of the startle noise increased (Greenhouse-Geisser corrected Repeated Measures ANOVA: $F(1,5) = 7.176$, $p = .019$). In addition, although the effect of Group and the Group by Startle amplitude interactions did not reach significance [$F = 0.144$ and $F = 0.999$, all $ps > .05$, respectively], startle responses across almost all noise intensities were greater in animals with amygdala lesions than in sham-operated controls.

Fear Learning (A+ Training)

The number of sessions each animal took for the A+ conditioning phase is given in Table 2. All animals acquired the conditioning responses to the A cue, although animals with neonatal amygdala damage took more sessions, requiring an average of 5.5 sessions as compared to 2 sessions for sham-operated controls (Mann-Whitney U, $p = .022$).

To investigate A+ conditioning across sessions, the average log-transformed fear-potentiated startle per session for both groups is illustrated in Figure 3. Immediately during the first session, control animals showed higher fear-potentiated startle to the A+ conditioning trials as compared to animals with neonatal amygdala lesions, although this difference failed just short of significance ($t = 2.00$, $p = .08$). However, by the second session when fear-potentiated startle responses of sham-operated controls reached criterion performance (100% over their baseline startle to cue A), fear-conditioned startle responses of animals with neonatal amygdala lesions did not improve and differed significantly from those of controls ($t = 2.8$; $p = .02$). Slight increases in fear-potentiated startle begin at the fourth session and reached criterion by Session 6.

Fear/Safety Signal Discrimination Learning (A+B-, AX+BX- Training)

Because both A+B- and AX+BX- phases were theoretically similar in nature, sessions from these two phases were combined for the analyses (see Table 2, Figure 4). Although animals with neonatal amygdala lesions required more sessions (average: 10.5) than controls (average: 4) in this phase, this difference did not reach significance (Mann-Whitney U, $p > .05$). However, as shown in Table 2, four of the six animals in Group Neo-Aibo learned to discriminate the aversive cues from the safety cues as quickly as control animals (Mann-Whitney U, $p > .05$), but the remaining two (cases Neo-Aibo-1 and Neo-Aibo-4) with the most extended lesions never learned this discrimination. The lack of discrimination learning in these two Neo-

Aibo animals can be attributed to an extinction of fear-potentiated startle to the aversive cues (A, AX) despite their reinforcement, where Neo-Aibo-1 and Neo-Aibo-4 scored -72% and 7.1% FPS, respectively, to the aversive cues on their last day of training.

Modulation of fear in the presence of the safety signal (AB probe trial)

Only the four amygdala animals that learned to discriminate between the aversive and safety cues were tested for conditioned inhibition. A repeated ANOVA including Group and Trial Types (i.e. A, B, X, AX, BX, and AB) as main factors and repeated measures for the last factor was performed on log-transformed %FPS measures. As seen in Table 3 and Figure 5, there were no differences between the two groups ($F(1,8) = 0.041$, $p > .05$) and no interaction between the two factors ($F(4,8) = 0.954$, $p > .05$), although the Trial Type factor reached significance ($F(4, 8) = 7.168$, $p < .001$). Thus, both the sham-operated animals and animals with neonatal amygdala damage had significantly greater startle to the aversive cues (A, AX) compared to either the safety cues (B, BX) (t-tests, all *ps* < .05) or the transfer cue (AB) (t-tests, all *ps* < .05).

Extinction

Number of sessions that each animal required to extinguish their fear to the A+ cue is given in Table 2. Both groups extinguished very quickly to repeated presentations of the fearful cues (A-, AX-) in the absence of the US, requiring an average of 3.0 sessions for Group Neo-Aibo and 3.5 sessions for Group Neo-C.

Discussion

In summary, our results demonstrated that acoustic startle response was not altered by neonatal damage to the amygdala. However, the same damage retarded but did not completely abolish the acquisition of a learned fear. After acquisition of the fear signal, neonatal damage to the amygdala did not impact the ability to discriminate a fear signal from a safety one, or to use the safety signal to reduce the fear response. Finally, the extinction of a learned fear does not appear to be amygdala-dependent. These conclusions will be discussed in turn below.

Baseline Acoustic Startle

We found that neonatal damage to the amygdala resulted in normal baseline acoustic startle. Both groups showed increased startle in response to increased intensity, although startle responses in animals with neonatal amygdala lesions were slightly but not significantly different from those of sham-operated controls. This slight increase was mostly due to larger individual variability in startle amplitude in animals with neonatal amygdala lesions. This variability, however, cannot be explained by extent of the amygdala lesions since increased lesion size did not correlate with increased startle amplitude ($r = -.655$, $p > .05$. Thus, consistent with the neurocircuitry described in the rodent literature, a fully functioning amygdala appears to be necessary only when emotional information is used to modulate the baseline acoustic startle response, presumably through its connections to the nucleus reticularis pontis caudalis (for review see Davis, 2007). Although our results are consistent with the rodent findings, they differ slightly with those of Antoniadis and Colleagues (2007) who reported that monkeys that had acquired their amygdala lesions in adulthood had slightly, but significantly, higher acoustic startle response at almost all noise amplitude tested. One important difference between the two

studies relates to greater individual variability in startle response in animals of the present study as compared to those in the Antoniadis et al.'s study, though the individual variation in our amygdala animals was independent of lesion size. Other factors include rearing conditions and the age of lesion. In any case in both studies the increased in startle amplitude after amygdala lesions either in adulthood or in infancy is relatively minimal.

Fear Learning

Neonatal damage to the amygdala did impair fear learning abilities, but did not totally abolish these abilities. Thus, only one animal in Group Neo-Aibo acquired the initial fear signal in 2 sessions as did control animals, the other five animals required more sessions to reach acquisition criterion. In fact, in most of the animals, learning was absent in the first few testing sessions but increased progressively from sessions 4 to 8. Thus, our findings complement those of Antoniadis and colleagues (2007) since both studies demonstrated that fear conditioning is severely affected during the first phases of learning. However, our data extend those of Antoniadis and colleagues (2007) in showing that with additional training animals with amygdala lesions can indeed acquire fear to a stimulus. Nevertheless, the different outcomes of the two studies may be explained by differences in both the time of insult as well as the lesion size. In the present study, the amygdala damage occurred within 7-10 days of age, and animals were tested around six years of age. Thus, it is possible that compensatory mechanisms could have occurred during maturation and allowed some other structures to compensate in the absence of a functional amygdala. While we cannot rule out this possibility completely, it seems unlikely since these same animals were impaired on other tasks known to be amygdala dependent (Reinforcer Devaluation Paradigm, Approach/Avoidance Paradigm, Kazama, et al., 2008,

Glavis-Bloom et al, 2008, Kazama et al, 2007, Raper et al., 2009). As regards to the lesions size, amygdala lesions in Group-Neo-Aibo averaged 63% as compared to 85% in the Antoniadis et al.'s study. Thus, it is possible that more complete lesions of the amygdala could have resulted in greater impairment. However, even the animal with the most extended damage to the amygdala (> 74% in case Neo-Aibo-1, see Table 1) was able to learn this association over time. In addition, in all six animals the amygdala damage included the central, medial, accessory basal, and dorsal areas of the basal nuclei, which are the main amygdala nuclei known to support fear learning abilities in rodents (Davis, 2007). Thus, it seems unlikely that extent of damage in our study may have resulted in the sparing of fear conditioning ability. Alternatively, the larger amygdala lesions in the Antoniadis et al.'s study may have also altered fibers of passage since ibotenic acid lesions can alter fibers (Coffey et al., 1988) and fibers coursing through the amygdala arise from the temporal cortical areas some of which are known to be critical for fear conditioning, such as the perirhinal cortex (for review, see Davis et al., 1993). Overall, our findings suggest that, although the amygdala plays an important role in fear learning, other areas are able to carry this function, especially when longer training is provided. This conclusion is in fact in line with some recent neuroimaging studies in humans.

When healthy human subjects are exposed to fear over longer periods of time, robust amygdala activation was noted during the early stages of fear conditioning, but this activation decreased as fear conditioning training proceeded longer (for review, see Sehlmeyer et al., 2009). More importantly, with longer training time, brain activation shifted from the amygdala to other structures, such as the medial prefrontal, anterior cingulate and insular cortices (Buchel et al., 1998; LaBar et al., 1998; Knight et al., 2004; LaBar et al., 1998). Thus, the amygdala may be playing a key role during early stages of fear acquisition, whereas the maintenance of this

learning may be supported outside of the amygdala. This pattern of results has been interpreted as the amygdala processing fear signals, and then sending the fear association onto these other structures as a fear memory. However, these results may also suggest parallel, as opposed to, serial processing. Thus, rather than the amygdala sending emotional valence information on to other structures that come online later in fear acquisition, other, as yet unknown, structure(s) may be processing emotional valence in parallel, and simply require longer time to form the stimulus-fear association. A major question still remains as to where exactly fear associations are being generated in the absence of a fully functional amygdala? The human neuroimaging data suggest at least three possible candidates, the medial prefrontal cortex, the anterior cingulate gyrus, and the insular cortex (Buchel et al., 1998; LaBar et al., 1998; Everitt & Robbins, 2005). In rodents, the prime candidates are the medial prefrontal cortex, which has been shown to encode fear learning (Laviolette, Lipske, & Grace, 2005), or the bed nucleus of the stria terminalis (BNST), which has also been shown to modulate anxiety (for review see, Winslow, Noble, & Davis, 2007) and could potentially compensate in the absence of a functional amygdala. As was previously noted, our amygdala damage encompassed the more dorsal aspects of the amygdala, which are known to be connected to these candidate areas (see Aggleton & Saunders, 2000), thus it is unlikely that any sparing of lesion resulted in the fear-learning within the amygdala to be passed on to these candidate areas. Alternatively, information about stimuli could be sent to these alternative areas via the perirhinal cortex (Bachevalier & Mishkin, 1986; Bachevalier, Parkinson, & Mishkin, 1985; Goulet, Doŕe, Murray, 1998), which could provide a parallel route by which stimuli could gain emotional valence. This alternate route could enable the animals with neonatal amygdala lesions to slowly acquire fear conditioning.

Finally, the retardation of fear conditioning after neonatal amygdala lesions contrasts with the normal appetitive learning demonstrated in the same animals. Thus, animals with neonatal amygdala lesions learned as rapidly as the sham-operated controls stimulus-reward tasks, such as Object Discrimination Reversal (both at 3 months and 3 years of age, Kazama & Bachevalier, 2002; 2006), and Concurrent Discrimination (Kazama & Bachevalier, 2002; Kazama, Kazama, O'Malley, & Bachevalier, 2007; Kazama, Glavis-Bloom, & Bachevalier, 2007). Taken together, these results imply that the amygdala may be more critically involved in processing aversive than appetitive associations.

Safety Signal Learning

We found that most of our animals with neonatal amygdala damage were quickly able to discriminate between the aversive cue and the safety signal, suggesting that the amygdala may not be critical for safety signal learning abilities. To date, this is the first study to examine the role of the monkey amygdala in acquiring safety signals (also called conditioned inhibition). Although four of the six animals with neonatal amygdala lesions had no difficulties in learning to distinguish between an aversive cue and a safety signal, it does appear that there was a qualitative difference between how these animals learned as compared to normal animals. Typically, control animals reacted fearfully to the addition of the safety cue, but quickly learned that a safety cue would never be followed by an air blast and thus decreased their startle to the safety cue. In contrast, amygdala-operated animals showed very little initial startle to the safety cue to begin with. Thus, four of these animals, which maintained high startle to the aversive cue, demonstrated normal aversive/safety cue discrimination, whereas the other two progressively decreased their fear reactivity to the aversive cue and eventually failed to learn the

discrimination. Thus, in these two animals the presence of the safety cue seems to have blunted their reaction to the aversive cue. Nevertheless, even in the four animals that showed aversive/safety cue discrimination, it is not yet clear whether they had learned anything about the safety signal since they did not respond to it even at the beginning of training. Thus, for all animals with neonatal amygdala lesions, it is difficult to determine the degree to which they had learned to associate the safety cue with the absence of the air-blast, as opposed to demonstrate an inherent lack of fear. This bias towards safety or lack of fear in all amygdala cases is in fact a hallmark symptom of amygdala damage and has been noted in many species including humans (Bechara et al., 1995; Adolphs, Tranel, & Damasio, 1998; Tranel et al., 2006). Nevertheless, because the four animals with neonatal amygdala lesions were able to use the safety cue to modulate their fear reactivity to the aversive cue (probe trials) suggests that indeed these animals had learned something about the safety cues.

While there is much evidence suggesting that aversive associations are guided by the amygdala, basic appetitive associations may be striatal dependent (Schiller et al., 2008). For instance, in a recent human neuroimaging study, Schiller and colleagues (2008) conditioned subjects to associate one cue with a mild shock, and a second cue with no shock. Although higher amygdala activation was noted during the aversive cue, greater striatal activation was found in the presence of the safety cue. They then reversed the reinforcement contingencies, observing a shift in neural activity from the amygdala for fearful cues, to areas of the ventral prefrontal cortices and striatum during the safety cue (Schiller et al, 2008). It is interesting to note that the two animals that failed to learn the aversive/safety signal discrimination both had unintended damage to ventral aspects of the striatum, though unilaterally, that could have affected the learning of the safety cue. In conclusion, although it is difficult to know exactly

how quickly the amygdalectomized animals learned the positive valence of the safety cue, their ability to flexibly modulate their startle response to the aversive cues in the presence of the safety cues does suggest that other structure may be critical for safety signal learning.

Flexible Modulation of Fear

Animals with neonatal amygdala damage were able to use a safety cue to modulate their fear-potentiated startle. These results complement a study by Falls and Davis (1995), demonstrating that amygdala-operated rats were also spared in their ability to apply a safety signal to a reacquired fearful stimuli. These data suggest that conditioned inhibition may be amygdala independent and question a major, but until now, untested assumption of the amygdalocentric model of the fear response, which serves as the basis for models of PTSD and other anxiety disorders (Rauch, Shin, & Phelps, 2006). Generally, the amygdalocentric model holds that the fear response is tempered by areas outside the amygdala, such as the hippocampus and prefrontal cortices. While this is supported by virtually all relevant human neuroimaging data reported to date, these studies carry an inherent caveat of neuroimaging activation, which is that activation is unable to determine whether or not a structure is critical for behavior. Thus, given that in animals with neonatal amygdala lesions, the areas processing safety signals could not act upon a functional amygdala, they must have exerted their modulation via connections to other areas along the startle pathway. As has been previously mentioned, the exact areas where these modulatory processes might take place are still largely unknown.

Extinction

Just as we found no evidence of amygdala involvement in conditioned inhibition, we also did not find any amygdala involvement in fear extinction. Thus, animals with neonatal amygdala lesions extinguished their fear response to the aversive cue as rapidly as did the sham-operated controls. These data may explain recent human neuroimaging data (LaBar et al., 1997, 1998) and electrophysiological studies in rodents (Quirk, 1997) demonstrating that amygdala activation is limited to the early phases of fear extinction. Until now, it was difficult to determine whether this early amygdala activation pertained to decrease in arousal to the fearful cue, or whether it was critical to a re-learning process. Thus, given that amygdala-operated animals actually extinguished their fear to the aversive cue even slightly faster than control animals, the data suggest that early amygdala activation is more likely representing an arousing effect of the aversive cue rather than a re-learning process.

Finally, the normal extinction after selective neonatal amygdala lesions suggests that other structures may support this process. Currently, the neuroimaging (LaBar et al., 1998; Milad et al., 2007; Kalisch et al., 2006) and rodent models (Quirk et al., 2003) have indicated that the medial prefrontal cortex and/or hippocampus may be critical for extinction, although additional studies are required to more directly explore the critical brain areas involved in the extinction of learned fear.

Conclusions

In conclusion, the rodent, human, and monkey literature all support the critical contribution of the amygdala during the early phases of fear conditioning, and leads to quick, robust responses to potentially threatening stimuli. Indeed, quickly learning the emotional valence of potentially dangerous stimuli in the environment is highly adaptive across species.

Likewise, when the fear processing system is affected by neuropathology or psychological trauma, the maladaptive fear response can have devastating consequences. In this study, we have found that, although the amygdala was necessary for quick fear learning, other structures are capable of fear learning in the absence of a functional amygdala at least in infancy. These results help explain human neuroimaging studies that have found decreased amygdala activity in the later stages of fear conditioning, and point to parallel, albeit slower fear processing outside the amygdala. In addition, in the absence of a fully functioning amygdala, this parallel processing sufficiently supports the ability to flexibly modulate the fear response. Finally, although human neuroimaging studies have reported amygdala activation during the early stages of extinction, our findings suggest that this activation is not critical for the extinction of the learned fear.

Acknowledgements:

This work was supported by grants from the National Institute of Mental Health (MH-58846, MH-086947, and MH-047840), the National Institute of Child Health and Human Development (HD-45471), Yerkes base grant (RR-00165), and the Autism Speaks Predoctoral Fellowship Grant. We thank the University of Texas Health Science Center at Houston veterinary and animal husbandry staff for expert animal care, Roger E. Price and Belinda Rivera for the care and handling of animals during the MR imaging procedures, and Edward F. Jackson for assistance in neuroimaging techniques.

Figure legends

Figure 1. Intended lesion and extent of amygdala damage in a representative case (Neo-Aibo-1). Intended damage is shown in gray on coronal sections through the anterior-posterior extent of the amygdala of an infant macaque brain atlas (left column), hypersignals caused by edema resulting from cell death are present in the FLAIR MR images (middle column), and reconstructed extent of hypersignals is shown in gray on corresponding drawing of coronal sections of a normal brain (right column). Asterisks point to areas of unintended damage to the ventral striatum and the pes hippocampus on the left (see levels +3 to +5). Arrows indicate slight sparing of tissue within the amygdala mostly on the left. Abbreviations: A – amygdala; amts – anterior medial temporal sulcus; ERh – entorhinal cortex; ; H – hippocampus; ls – lateral sulcus; ots – occipital temporal sulcus; PRh – perirhinal cortex; rs – rhinal sulcus; sts – superior temporal sulcus; TE, temporal cortical area and TH/TF – cytoarchitectonic fields of the parahippocampal gyrus as defined by von Bonin and Bailey (1947).

Figure 2. Mean (± SEM) percent of acoustic startle response to differing sound intensities (95 dB, 100 dB, 110 dB, 115 dB, & 120 dB) for sham-operated controls (Neo-C; n = 4) and animals with neonatal amygdala lesions (Neo-Aibo; n = 6).

Figure 3. Log-transformed %FPS per session during the A+ Training Phase for sham-operated controls (Neo-C, circles) and for animals with neonatal amygdala lesions (Neo-Aibo; squares). The horizontal dotted line represents criterion of 100% FPS.

Figure 4. Mean (± SEM) sessions to reach criterion in learning the aversive cue A+ and in safety signal learning (A+B- and AX+BX-) in sham-operated animals (Neo-C, white bars) and animals

with neonatal amygdala lesions (Neo-Aibo; black bars). Note that animals in group Neo-C had no variance in the number of sessions for both phases.

Figure 5. Mean (± SEM) percent fear-potentiated startle, as expressed by log-transformed, for each cue in sham-operated controls (Neo-C; white bars) and animals with neonatal amygdala lesions (Group Neo-Aibo; black bars). For both groups, aversive cues (A, AX) were significantly different from safety cues (B, BX) (all $p < .05$), and the aversive cues were also significantly different from the transfer cue (AB) (all ps $< .05$).

Table 1: Extent of intended and unintended damage in Group A-ibo

Cases	**Amygdala**				**Hippocampal Formation**			
	L	R	Avg	W	L	R	Avg	W
Neo-Aibo-1	89	59.8	74.4	53.2	5.1	3.1	4.1	0.2
Neo-Aibo-2	42	77.6	59.8	32.6	0	0.8	0.4	0
Neo-Aibo-3	33	61.1	47.1	20.2	0	0	0	0
Neo-Aibo-4	62.1	90	76	55.9	1.9	3	2.4	0.1
Neo-Aibo-5	41.2	66.6	53.9	27.5	0	0	0	0
Neo-Aibo-6	52.1	75.6	63.8	39.3	5.6	10.3	8	0.6
X	**53.2**	**71.8**	**62.5**	**38.1**	**2.1**	**2.9**	**2.5**	**0.1**

Data are the estimated percentage of damage as assessed from MR (post-surgical T1) images. L: percentage of damage to the left hemisphere; R: percentage of damage to the right hemisphere; Avg: average of L and R; W = (L x R)/100 [weighted index as defined by Hodos and Bobko (1984)]; X: group mean.

Table 2: Sessions per learning stage

Group	A+	A+B-	AX+BX-	Combined Safety Learning	Extinction
Neo-C-1	2	2	2	4	5
Neo-C-3	2	2	2	4	5
Neo-C-4	2	2	2	4	2
Neo-C-5	2	2	2	4	2
X	**2**	**2**	**2**	**4**	**3.5**
Neo-Aibo-1	7	2	15	17	NA
Neo-Aibo-2	8	2	2	4	2
Neo-Aibo-3	6	2	2	4	6
Neo-Aibo-4	4	15	15	30	NA
Neo-Aibo-5	2	2	2	4	2
Neo-Aibo-6	6	2	2	4	2
X	**5.5**	**4.2**	**6.3**	**10.5**	**3**

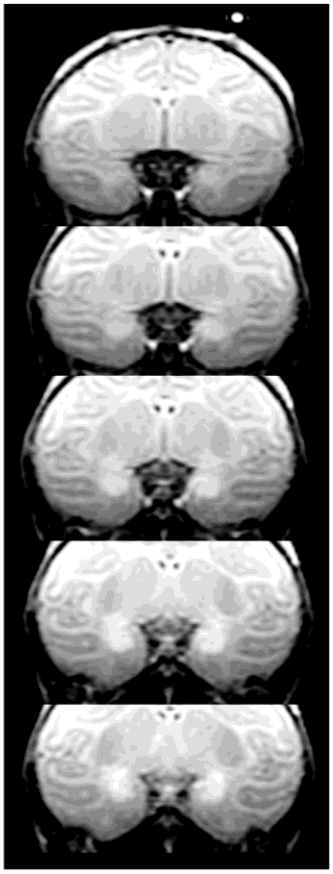
Data are the total number of sessions to reach criterion performance for the initial fear learning (Stage A+), the safety signal learning stages (A+B-, AX+BX-; Combined Safety Learning is the summed scores of the two safety signal learning stages), and the extinction stage. The X scores in bold are the group means per stage.

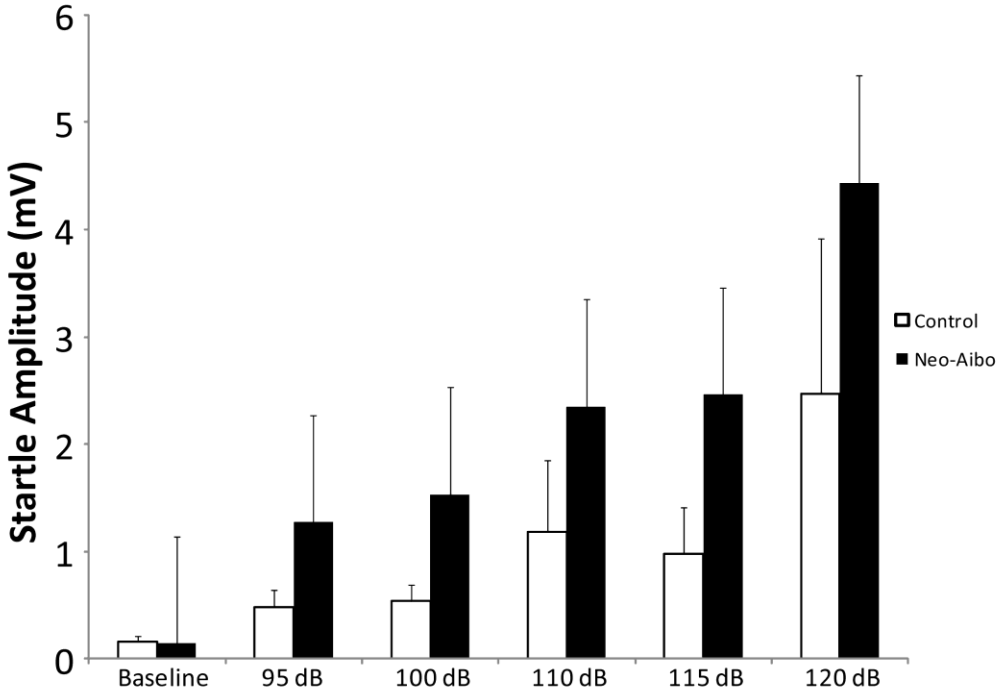
Table 3: Log-Transformed % Fear-Potentiated Startle

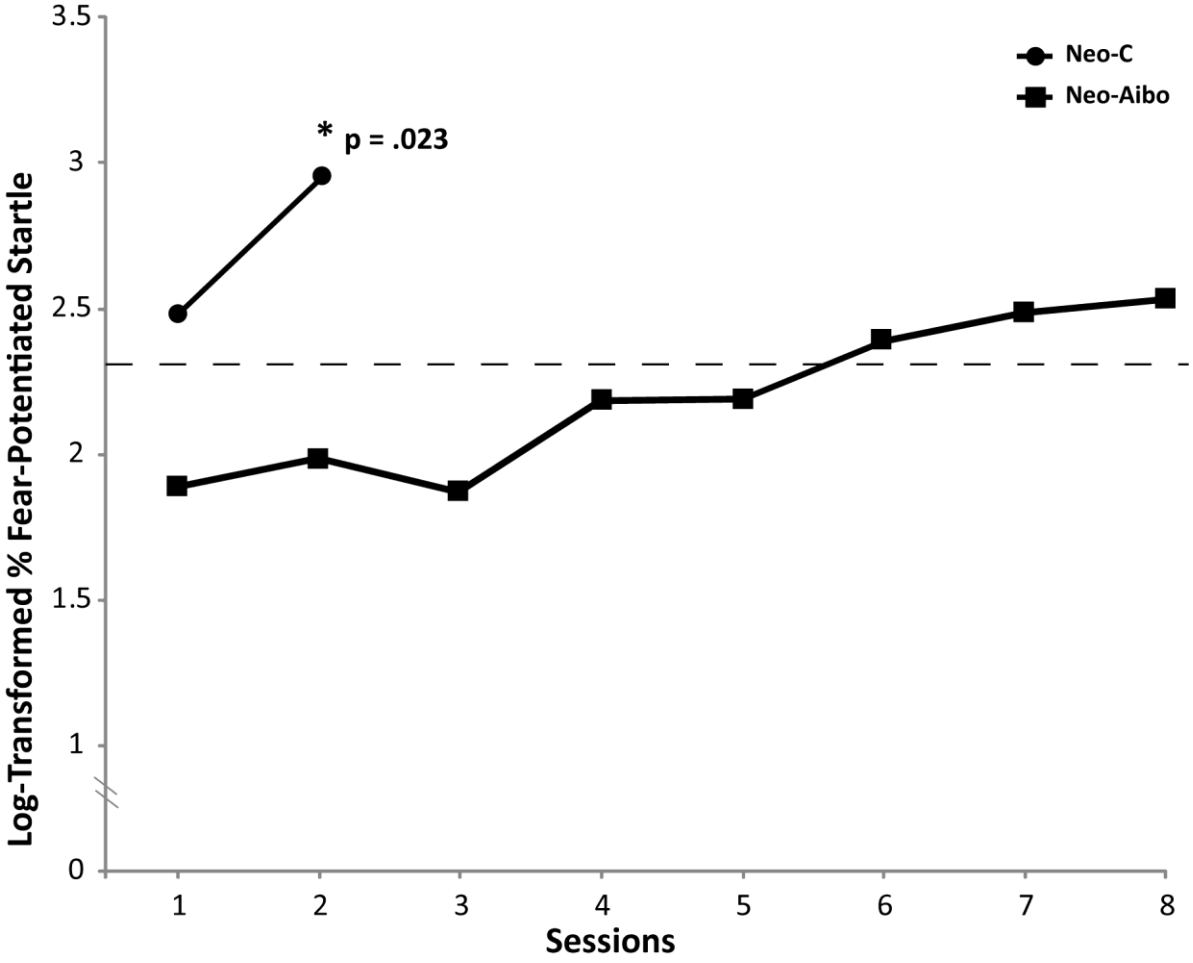
Group	**A**	**B**	**AX**	**BX**	**AB**
Neo-C-1	3.35	2.07	3.57	2.35	1.9
Neo-C-3	2	1.48	1.77	1.27	1.85
Neo-C-4	3.58	2.46	3.8	2.51	3.54
Neo-C-5	2.56	1.64	1.36	1.23	2.04
X	**3.17**	**2.06**	**2.91**	**2.03**	**2.49**
Neo-Aibo-1	Failed				
Neo-Aibo-2	2.14	1.87	2.11	1.71	2.04
Neo-Aibo-3	2.51	1.95	2.8	2.54	2.49
Neo-Aibo-4	Failed				
Neo-Aibo-5	2.87	2.25	2.51	2.01	2.6
Neo-Aibo-6	2.46	1.86	2.34	2.1	1.63
X	**2.5**	**1.98**	**2.44**	**2.09**	**2.19**

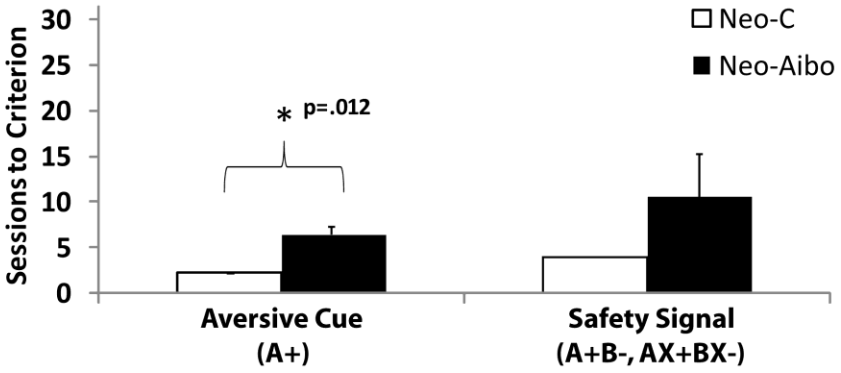
Data are the Log-Transformed %FPS amplitudes taken during the transfer test. Each individual score was obtained from the very first time the animal experienced that cue at the optimal decibel level (95dB or 120dB) for that particular animal. The X scores in bold are the group means per stage.

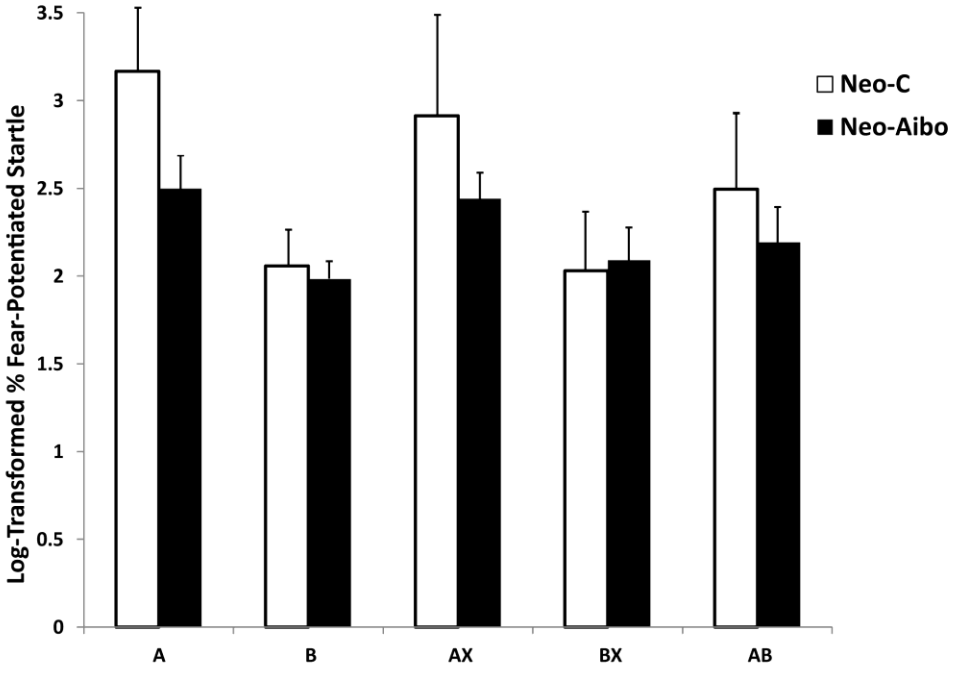
Neo-Aibo-1

Intended Lesion Extent	1-Week Post Surgical FLAIR MRI	Reconstructed Lesion Extent
+7		
+6		
+5		
+4		
+3		


Startle Amplitude (mV)
0
1
2
3
4
5
6
Baseline
95 dB
100 dB
110 dB
115 dB
120 dB
Control
Neo-Aibo


3.5
3
2.5
2
1.5
1
0
Log-Transformed % Fear-Potentiated Startle
* p = .023
Neo-C
Neo-Aibo
1
2
3
4
5
6
7
8
Sessions


Sessions to Criterion
30
25
20
15
10
5
0
* p=.012
Neo-C
Neo-Aibo
Aversive Cue
(A+)
Safety Signal
(A+B-, AX+BX-)


Log-Transformed % Fear-Potentiated Startle
3.5
3
2.5
2
1.5
1
0.5
0
A
B
AX
BX
AB
Neo-C
Neo-Aibo

Fear learning, conditioned inhibition, and extinction in adult macaques: II- Effects of neonatal damage to areas 11 & 13 of the orbital frontal cortex

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Abstract:

The amygdalocentric model of fear regulation posits that while the amygdala is central to the fear response, areas outside the amygdala such as the hippocampus, and prefrontal areas, are responsible for down-regulating the fear response when safety-signals are present in the environment. Previous nonhuman primate studies have implicated the role of the orbital frontal cortex in behavioral flexibility. Given this lack of regulation of behavioral responses to appetitive stimuli, and given the strong bidirectional connections shared with the amygdala; in the present experiment we asked whether animals with neonatal lesions to areas 11, 13, and insular area will also show impairment in the flexible modulation of fear in response to Pavlovian conditioned fear learning, using the AX+/BX- fear-potentiated startle paradigm to measure conditioned inhibition and extinction. Given that these neonatal OFC lesions resulted in a pattern of impairment in appetitive tasks similar to that seen in animals that had received the same lesions in adulthood, we predicted that the neonatal lesions will also alter the inhibitory control of conditioned fear responses. However, despite impairments on other cognitive tasks, suggesting no sparing of function, we found no impairment in animals with early OFC lesions either in their ability to learn the fear/safety-signals, or flexibly modulate their fear measured by either conditioned inhibition or extinction. Thus, despite its strong connections with the amygdala, it appears that the nature of these connections may relate more to appetitive processes.

Recent advances in our understanding of the processes involved in behavioral and emotional regulation of aversive stimuli have indicated the critical role that the prefrontal-amygdala interactions play in these processes. Thus, behavioral and emotional regulation of negative stimuli, either through cognitive re-appraisal or suppression in humans, or through extinction in humans and rats, activates orbital frontal cortex (OFC), or inhibits the amygdala. This led many researchers to hypothesize that the OFC network, and perhaps more specifically ventromedial areas 25 and 32, mediates a top-down control of prepotent aversive responses stored in the amygdala (Levesque et al., 2003; Ohira et al., 2006; Phan et al., 2005; Shin et al., 2005; see also Quirk et al, 2006 for review). Yet, contrary to the large literature in rodents and the emerging neuroimaging findings in humans, much less is known of the role of the nonhuman primate OFC in the regulation of aversive conditioned responses.

Most of the nonhuman primate studies on the role of the OFC in behavioral inhibition and extinction have generally used appetitive tasks (object reversal, go/nogo, reinforcer devaluation or extinction of instrumental responses) and have shown that lesions of the OFC yielded inflexible or inappropriate responses to stimuli that are no longer rewarded (Mishkin, 1964; Teitelbaum, 1964; Butter, 1969; Chudasama & Robbins, 2003; Izquierdo et al., 2004) or when their reward value have changed (Izquierdo et al., 2004), and impaired the extinction of unrewarded instrumental responses (Izquierdo & Murray, 2005). Thus, the OFC was thought to play a role in flexibly guiding behavior when outcomes change (Murray et al., 2007; Schoenbaum et al., 2007). In these previous studies the lesions were extended and included different OFC sectors, such as the middle areas 11 and 13, the rostral area 10, the ventromedial areas 14 and 25, and, in some instances, the lateral areas 12/47 (Iversen & Mishkin, 1970; Jones & Mishkin, 1972; Meunier et al., 1997; Izquierdo et al., 2004). The one exception was the earlier

study of Butter (1969) who demonstrated that severe discrimination reversal deficits after lesion restricted to the lateral area 12, but not after damage limited to either the most anterior medial areas 10 and 11 or the most posterior medial areas 13 and insular area. This finding suggested that not all OFC subfields are critical for the regulation of behavioral inhibition (see also Wallis, 2001). With advances in the primate anatomical organization of OFC sectors and their connections (Barbas, 2007; Price, 2007), more recent studies have began to specifically investigate the lateral orbital network (Petrides & Pandya, 1984; Carmichael & Price, 1994; Price, 2007; Barbas, 2007), which possesses unique and segregated inputs into two main subdivisions of the amygdala (lateral and central nuclei) that could potentially modulate behavioral inhibition (Ghashghaei and Barbas, 2002; Barbas et al., 2003; Arana et al., 2003; Sugase-Miyamoto and Richmond, 2005; Wellman et al., 2005; Paton et al., 2006). For instance, adult monkeys with damage limited to lateral OFC areas 11, 13 and insular area display normal fear responses during an approach/avoidance paradigm (Machado, Kazama, & Bachevalier, 2009), but were unable to flexibly modulate tension-related behaviors when confronted with a human intruder presenting different levels of threat (Machado & Bachevalier, 2008). Furthermore, the same animals were able to switch their response away from unrewarded choices (Kazama and Bachevalier, 2008), but were unable to flexibly modulate their responses when reward value of objects had been manipulated by a reinforcer devaluation procedure (Machado & Bachevalier, 2007a). This inability to flexibly modulate emotional and behavior responses likely contributed to their abnormal behaviors when socially interacting with peers (Machado & Bachevalier, 2006). These data suggested that the lateral orbital sector is not critical for inhibiting prepotent, unrewarded, responses, but rather flexibly regulates appetitive responses when reward or emotional value of stimuli has changed. Interestingly, this conclusion holds true

even when the lesions of the lateral orbital fields were damaged early in infancy. Thus, as with the adult lesions, animals with neonatal lesions of areas 11, 13 and insular area could normally switch their response to unrewarded stimuli (Kazama et al., 2008), but were unable to flexibly switch their response away from objects that had been devalued through a satiation procedure (Kazama et al., 2007). Given this lack of regulation of behavioral responses to appetitive stimuli, in the present experiment we asked whether the same animals with neonatal lesions to areas 11, 13, and insular area will also show impairment in the flexible modulation of fear in response to Pavlovian conditioned fear learning, using the AX+/BX- fear-potentiated startle paradigm (Winslow et al., 2002, 2007) to measure conditioned inhibition and extinction. Given that these neonatal OFC lesions resulted in a pattern of impairment in appetitive tasks similar to that seen in animals that had received the same lesions in adulthood (Machado & Bachevalier, 2007, Machado et al., 2009, Raper et al., 2009, Kazama et al., 2008), we predicted that the neonatal lesions will also alter the inhibitory control of conditioned fear responses. Preliminary data of this study have already been published in an abstract form (Kazama et al., 2010).

Methods

Subjects

Eleven adult rhesus macaques (*Macaca mulatta*) of both sexes (5 males, 6 females), ranging from ~4.5-8 kg, participated in this study. All animals were acquired as newborns from MD Anderson Cancer Center Science Park (Bastrop, TX) and were surrogate-nursery-reared (see Goursaud & Bachevalier, 2007 for details) in the primate nursery at MD Anderson Cancer Center (Houston, TX). All animals received extensive daily contact with both human caregivers and their peers. They were first hand fed a diet of infant Similac formula (Abbot Laboratories) and, starting around 8 months of age, they were fed jumbo primate chow (Lab Diet #5037, PMI

Nutrition International Inc., Brentwood, MO) and fresh fruit daily. Between 8-12 days of age, six monkeys (3 males and 3 females) received sham-operations (Group Neo-C) and five monkeys (2 males and 3 females) received aspiration lesions of areas 11 and 13 of the OFC (Group Neo-Oasp). Following surgeries, all animals underwent extensive behavioral and cognitive examination to assess emotional reactivity (1-4 weeks, 2 & 5 months, 3 years of age), social interactions (3 & 6 months, 3 years of age), goal-directed behaviors (3 months, 3, 4, & 5 years of age) and memory processes (6, 8, 9, & 18 months, 2 years of age). All procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Texas Health Science Center at Houston and Emory University.

Surgical procedures

All procedures have already been described in details in an earlier report (Goursaud & Bachevalier, 2006) and will be briefly summarized below. Animals were first anesthetized using isoflurane gas (1-2% to effect). An intravenous drop solution containing 0.45% NaCl was given to maintain hydration and heart rate, respiration, blood pressure, body temperature, and expired CO2 were recorded during the entire Magnetic Resonance Imaging and the surgical procedures. The animal's head was then secured in a non-ferric stereotaxic head holder and placed at the center of a GE sigma 1.5 Tesla Echo Speed scanner (GE Medical Systems, Milwaukee, WI). The pre-surgical brain imaging included a 3D T1-weighted fast spoiled gradient (FSPGR)-echo sequence (TE = 2.6 ms, TR = 10.2 ms, 25° flip angle, contiguous 1 mm sections, 12 cm FOV, 256 x 256 matrix) obtained in the coronal plan that was used to precisely visualize the position of the orbital frontal sulci that were used as landmarks for the surgical removal of areas 11 & 13 (Machado and Bachevalier, 2006).

Following the MRI scans, animals were kept anesthetized in the stereotaxic apparatus and brought immediately to the surgical suite where they were prepared for the surgical procedures that were performed under aseptic conditions. For all surgical procedures, a local anesthetic (Marcaine 25%, 1.5m., s.c.) was injected along the midline skin incision from the occiput to a point in between the orbital ridges. The skin and underlying connective tissue were retracted laterally to expose the bone that was then open.

For the sham-operations, a small craniotomy was performed in both hemispheres just in front of bregma and the dura was then cut, but no aspiration lesions or neurotoxin injections were performed. For the orbital frontal cortex lesion, the bone was opened as a crescent just above each supra-orbital ridge to gain access to the frontal lobe surface. With the use of a surgical microscope, the frontal lobe was gently retracted and the olfactory striae and the medial and lateral orbital frontal sulci located. The orbital frontal cortex lesions were restricted to areas 11 and 13 which were gently aspirated with 21 & 23 gauge aspirating probes and an electro-cautery. The anterior border of the lesions were a line joining the anterior tip of the lateral and medial orbital sulci, and the posterior border ended at the location where the olfactory striae begun to turn laterally. Laterally, the lesion ended at the medial lip of the lateral orbital sulcus and, medially, at the lateral border of the stria olfactory. Within these borders, the lesion included most of areas 11 and 13 and a small anterior portion of Ia (anterior insula).

After the surgical procedures, the wound was sutured in anatomical layers, the animals were then removed from the Isoflurane gas anesthesia and allowed to recover in an incubator ventilated with oxygen. Treatments were started 12 hours before surgery and continued on until post-surgical day seven. All monkeys received both pre and post-surgical antibiotic treatments (Cephazolin, 25 mg/kg, per os) to reduce the chance of infection as well as dexamethazone

sodium phosphate (0.4 mg/kg, s.c.) to control post-surgical swelling. Additionally, a topical antibiotic ointment/anesthetic and Acetaminophen (10mg/kg, p.o.) was administered four times a day for three days after surgery to relieve pain and hasten recovery.

Lesion Verification

The extent of aspiration lesions was assessed using MRI techniques performed 7 to 10 days after surgery as described previously (Machado and Bachevalier, 2006). The animals were again anesthetized with Isoflurane, fixed into the stereotaxic apparatus and scan to obtain a 3D T1-weighted fast spoiled gradient-echo sequence FSPGR scans. The postsurgical structural coronal images were compared to those obtained pre-surgically to estimate the extent of orbital frontal tissue damaged. Coronal MR images through the frontal lobe were matched to corresponding drawings of coronal histological sections of an infant monkey brain (approximately two-weeks old; J. Bachevalier, unpublished data). Loss of neural tissue observed on the MR images was drawn onto the corresponding drawings, which were then imported into ImageJ® to measure the surface area (in pixels^2) of damage within the left and right orbital frontal cortex (including areas 11, 13, 12, 14, 25, and Ia). For each area, estimated percent volume damaged was then calculated by dividing the total volume of damage for the right and left hemisphere by the normal volume of an area obtained from the normal one-week old monkey brain. Percent reduction was then calculated using the following formula: [100-total ROI volume remaining/average ROI volume in normal one-year-old monkey]*100).

Behavioral testing

Training began when the animals were 4-6 years of age and lasted approximately one month. All inter-session intervals were 72 hours, and session length depended upon the stage of training (see below for details). Animals were given their normal daily chow, water, and fresh

fruit daily, as well as additional treats during primate chair training. All methods have previously been described in detail (Winslow, Noble, & Davis, 2008; Antoniadis, Winslow, Davis, & Amaral, 2007, Winslow, Parr, & Davis, 2007; Kazama, et al., 2010), and will be described briefly below.

Apparatus: Animals were seated in a non-human primate chair located in a sound attenuated chamber equipped with an automated system designed to deliver unconditioned and conditioned stimuli. The chair was positioned above a load cell (Med Associates, St. Albans, VT). Movements initiated by the animals produced displacement of the load cell (Sentran YG6-B-50KG-000), the output of which was amplified, and analyzed via the Med Associates Primate Startle Software (Med Associates, St. Albans, VT).

Stimuli: Two unconditioned stimuli (US) were used. A 500 msec jet of compressed air (100 PSI) generated by an air compressor located outside the chamber and projected at the face of the monkey via four air jet nozzles. A startle stimulus, which was a 50 msec burst of white noise (5 msec rise-decay time) of varying intensities (range: 95-120 dB) emitted by a white noise generator and delivered through the same speakers as the background noise. Three cues served as either an aversive conditioned stimulus (A), a safety conditioned stimulus (B) or a neutral stimulus (X). The visual CS was a 4 sec light produced by 4 overhead halogen bulbs producing a combined 250 Lux, attached to the top of the test chamber. The auditory CS was an 80 dB, 4 sec, 5000 kHz tone produced by an overhead speaker. The tactile CS was produced by a quiet computer fan that directed gentle airflow onto the monkey's head. The CS assignments as cues A, B or X were pseudo-random and counter-balanced across groups. Thus, some animals received the light as the aversive CS, whereas others received the tone as aversive CS, and so forth.

Acoustic Startle Response: To evaluate any potential effects of lesion on acoustic startle, the animals were placed in the apparatus and exposed on two separate days of 60 trials each, which were composed of baseline activity without startle stimuli (10 trials), and of startle responses to noises of varying decibel intensities (95, 100, 110, 115, & 120 dB; 10 trials each). All trials were pseudo-randomly intermixed throughout each session. Animals were then tested for pre-pulse inhibition before moving on to the AX+/BX- paradigm (Heuer, et al., 2010).

Pre-training: Prior to the conditioning phase, the animals were habituated to the three conditioned cues to assess any unconditioned effects of the cues on the startle response prior to conditioning. First, animals received two separate days of 30 trials each during which the to-be-conditioned cues (light, tone, or airflow from quiet fan) and their combinations (light/tone, light/airflow, tone/airflow) were presented in the absence of the startle noise. Then, animals were given days of 60 trials, consisting of 30 trials with the startle noise alone (95dB), and 30 trials in which the 95dB startle noise was paired with one of the to-be-conditioned cues or their combinations for 5 trials each pseudo-randomly ordered. Within each of the cue-startle trial the startle stimulus was presented 4 sec after the onset of the CS. These pre-training sessions were repeated for each monkey until presentation of the cue that was assigned to serve as the safety signal (cue B) for that animal produced less than a 30% increase in startle amplitude compared to noise alone presentations.

A+ Training Phase: The purpose of this phase was to train the animal, using Pavlovian fear conditioning procedures, to associate a cue (A+) with an aversive air-blast. These A+ air-blast trials occurred four times per 28-trial session, and were always scheduled such that one occurred at the beginning and one at the end of each session. The remaining two pairings were pseudo randomly intermixed within the 24 startle test trials across sessions so that animals could

not predict when cue A would be followed by an air-blast as opposed to a startle stimulus. The startle stimulus or air-blast was presented 4 sec after the onset of cue A. The remaining 24 trials consisted of 4 trial-types (Noise Alone 95dB, Noise Alone 120dB, Cue A 95dB Noise, Cue A120dB Noise) and were presented pseudo-randomly 6 trials each per session. Animals received A+ Training for a minimum of two sessions, and until their percent Fear-Potentiated Startle (% fear-potentiated startle) was 100% above their pre-training startle to the A cue. The % fear-potentiated startle was defined as:[Mean startle amplitude on CS test trials – mean startle amplitude on startle noise alone test trials)/mean startle amplitude on noise burst alone test trials] X 100.

A+/B- Training Phase: The purpose of this phase was to train the animal to associate a second cue (B) with the absence of an air-blast (B-), thus this cue was termed the safety-signal. Animals received 40-trial sessions composed of six trials in which the safety cue B was presented with both startle noise intensities (95dB and 120dB) but never paired with the air-blast US; four trials in which the A continued to be paired with either the air-blast (according to the schedule described previously – A+) or both startle noise intensities (95 dB and 120 dB, 6 trials each); and startle noise alone trials (95 dB and 120 dB, 6 trials each). Animals received A+/B- Training for a minimum of two sessions, and until a difference of 100% fear-potentiated startle was obtained between the two cues.

AX+/BX- Training Phase: Previous conditioned inhibition training in humans using the typical design (A+/AB-) found that B, the safety signal, did not transfer to another cue that had not previously been put in compound with A and instead AB- was probably treated not as a compound cue consisting of the aversive and safety cues, but rather as a completely novel third cue (Grillon and Ameli, 2001).

Thus, the purpose of this phase was to train the animal to discriminate compound cues using a third neutral cue (X), which was presented in combination with either the A+ or B- cues. This phase included 40-trial sessions constructed similarly to A+/B- Training. The only difference is that both the aversive cue (A+) and the safety cue (B-) were presented in combination with the neutral cue (X), yielding compound cues AX+ and BX-. As with the A+/B- Training, animals received the AX+/BX- Training for a minimum of two sessions, and until there was a difference of 100% fear-potentiated startle between the two compound cues.

AB Testing/Transfer Test: Animals were tested for conditioned inhibition (i.e. transfer) in a single session within 72 hrs after the last AX+/BX- training session to examine the potential inhibitory effects of B on A. This 48-trial probe session consisted of all trial types, including two A+ air-blast pairings intermixed within (a) 95 dB and 120 dB Noise Alone trials (6 trials each), (b) 95 dB and 120 dB cue pairings (A, B, AX, BX, 5 trials each per noise intensity), and (c) 95 dB and 120 dB AB compound cue (5 trials per noise intensity). Hence, when trained in this way transfer of fear on the AB test trial could not be accounted for by configural learning. All trials were pseudo-randomly intermixed.

Extinction: Finally, all animals were presented with successive 12-trial sessions of the 95 dB startle stimulus elicited alone (4 trials) or in the presence of cues A and AX to evaluate fear extinction (4 trials of each type). Training was completed when the animal returned to its pre-training startle amplitude.

Data Analysis

Throughout the different phases, the startle amplitudes were recorded via the Med Associates software and amplified via the load cell. If in the course of training, an animal's % fear-potentiated startle showed a steady decline and no improvement over an extended period,

that animal was given a maximum score of 15 sessions (a criterion that was determined in a previous study, Kazama et al., 2010).

Data analysis included three parts. First, we used a Geisser-Greenhouse corrected repeated measures ANOVA to compare the acoustic startle responses to the varying intensities (95, 100, 110, 115, & 120 dB) across groups. Second, we assessed the animal's ability to associate and discriminate between the aversive and safety cues (A, B, AX, BX) using a "sessions to criterion" measure. Because all our control animals learned the task at floor (e.g. 2 sessions per phase), and thus had no variability, we again used non-parametric statistics to investigate any group differences (Mann-Whitney U). Third, because previous reports (Winslow, Noble, & Davis, 2008) indicated that startle values are not normally distributed; we transformed the transfer test data using a logarithmic base 10 transformation comparing both groups using repeated measures ANOVAs.

Results

Lesion Extent

The extent of bilateral OFC damage for Group Neo-Oasp was very complete, symmetrical, and averaged 90.5% for middle areas 11 and 13 (see Table 1). A representative case of the ORB lesions is illustrated on Figure 1. Damage to cortical areas adjacent to areas 11 and 13 included anterior portion of the inferior agranular area (insular area; averaging 48.3%, bilaterally), lateral area 12 (ranging from 3.4% to 25.6%, bilaterally) and medial area 14(ranging from 8.5% to 19.4%, bilaterally).

Acoustic Startle Response

Because the baseline startle response of two animals in the control group (cases Neo-C-2 and Neo-C-6) was greater than the maximum amplitude of the load cell, these two animals were

dropped from the study. As illustrated in Figure 2, both sham-operated and animals with neonatal OFC lesions demonstrated greater startle responses as the intensity of the startle noise increased (Greenhouse-Geisser corrected Repeated Measures ANOVA: $F(1,5) = 7.7$, $p = .02$). In addition, although the effect of Group and the Group by Startle amplitude interactions did not reach significance [$F = 2.37$ and $F = 0.155$, all $ps > .05$, respectively], startle responses across almost all noise intensities were lower in animals with OFC lesions than in sham-operated controls.

Fear Learning (A+ Training)

All animals, regardless of group learned to associate Cue A+ with the air-blast very quickly. Control animals all performed at floor, completing this stage in the minimum two sessions, whereas animals in group Neo-Oasp took an average of 3.4 sessions (Mann-Whitney U, $p > .05$, Table 2, Figure 3).

Fear/Safety Signal Discrimination Learning (A+B-, AX+BX- Training)

Because both A+B- and AX+BX- phases were theoretically similar in nature, data for these 2 phases were combined for the analyses (see Table 2, Figure 3). Although one animal, Neo-Oasp-5 developed very high baseline startles and had to be dropped at the AX+BX- training phase, all remaining animals, regardless of group, learned to differentiate between the aversive and safety cues in the minimum two days per stage with no variability between animals (Mann-Whitney U, $p > .05$).

Modulation of fear in the presence of the safety signal (AB probe trial)

For the four control animals and four OFC animals that learned to discriminate between the aversive and safety cues, a repeated measures ANOVA was used to assess differences between the log-transformed % fear-potentiated startle to the various cues (i.e. A, B, AX, BX, and

AB). As seen in Table 3 and Figure 4, there were no differences between the two groups ($F(1,8) = .011$, $p > .05$), and no interaction between the two factors ($F(4,8) = .852$, $p > .05$). However, both the control group (Neo-C) and animals with early OFC damage (Neo-Oasp) had significantly greater startle to the aversive cues (A, AX) compared to either the safety cues (B, BX; t-tests, all *ps* < .05) or the transfer cue (AB; t-tests, all *ps* < .05).

Extinction

As seen in Table 2, both groups extinguished very quickly to repeated presentations of the fearful cues (A-, AX-) in the absence of the US, averaging less than four sessions to return to baseline levels of startle ($p > .05$).

Discussion

The results demonstrated that neonatal damage to the lateral orbital network (areas 11 & 13 and insular area) had no effects on (a) baseline acoustic startle, (b) fear conditioning and safety signal learning, (c) conditioned inhibition and extinction. These results will be discussed in turn, followed by an explanation on how the timing of the lesions could have led to the results obtained.

Baseline Acoustic Startle

Neonatal damage to the orbital areas 11 and 13 resulted in a negligible decrease in baseline acoustic startle responses as compared to sham-operations. Animals in both groups increased their startle in responses to increased noise intensity, but animals with neonatal OFC damage did show slightly lower startle amplitudes across all intensities. These findings parallel the lack of effects of selective ventromedial prefrontal lesions on baseline acoustic startle in rodents (Sullivan & Gratton, 2002).

Fear Learning

Neonatal damage to the orbital network also spared fear learning abilities. All animals, regardless of group learned to associate the A+ cue with the aversive air-blast in the minimum two training sessions. Although this lack of group difference could be explained by a potential ceiling effect, we have previously reported significant deficits using this simple association after neonatal lesions of the amygdala (See Kazama, et al., 2010), and thus, the paradigm appears to be able to detect gross fear learning deficits in monkeys and has previously been shown to be sensitive in rodents (Davis, 1992). We cannot rule out, however, the possibility of a subtle learning deficit as we have previously observed spared stimulus-reward association abilities on

small stimuli sets (5 object pairs), but a severe impairment on large stimuli sets (60 object pairs) (Kazama, O'Malley, & Bachevalier, 2007; Kazama, Glavis-Bloom, & Bachevalier, 2008). Thus, perhaps these animals would do poorly if given many different cues to associate with a learned fear. Importantly, this deficit on large stimuli sets has only been observed in animals with early OFC damage, and is not seen with similar damage received in adulthood (Machado & Bachevalier, 2007; Izquierdo & Murray, 2004).

The normal fear learning after lesions of the prefrontal cortex are also consistent with rodent data (for review, see Sotres-Bayon & Quirk, 2010), but contrast with the fear conditioning deficits found after ventromedial prefrontal cortex damage in humans (Bechara et al., 1999), or after more generalized frontal-temporal damage as a result of Frontal-Temporal Dementia (Hoefer et al., 2008). Given that the OFC damage in human patients included prefrontal areas lying close to the middle line that were not included in our study, it is likely that the different outcomes could be accounted by damage to these more medial orbital fields. Future studies will need to investigate the role of the medial orbital network in fear conditioning in monkeys. Thus, despite the heavy bi-directional connections between the orbital/sensory network to the amygdala, an area shown to be important for fear learning, it appears that the nature of these connections may have little to do with supporting fear learning abilities.

Safety Signal Learning

We found no evidence that areas 11 & 13 of the OFC play a role in safety signal learning. To date, this is the first study to examine the role of the monkey OFC in acquiring safety signals. As with the fear learning phase, the extremely fast discrimination learning in both control and lesion groups resulted in somewhat of a potential ceiling effect. Thus, although it is possible that if the task were made more difficult we might be able to detect a group difference, this simple

discrimination appears to be unaffected by early damage to the orbital network. It should be noted that, although animals with neonatal damage to the OFC had no difficulty with this discrimination, some animals with early selective damage to the hippocampus were unable to discriminate between the aversive and safety cues or those that did took an extremely long time to reach criteria on AX+/BX- (manuscript in prep). Furthermore, the lack of discrimination between a comparable aversive and safety cue have also been noted in patients suffering from PTSD using the AX+/BX- Paradigm (Jovanovic et al., 2009). Notably, previous work has demonstrated functional dysregulation within the orbital frontal cortex in patients with PTSD (Schuff, et al., 2010; Milad et al., 2009), although the degree to which this dysfunction stems from the lateral OFC network as opposed to the more medial aspects of the OFC is unknown.

In rodents, several areas appear to be involved in safety-signal learning; however, given the complexity of the data, it appears that more than one structure may be involved in this process. For instance, similar to the current study, selective damage to the ventral pre-frontal cortex did not disrupt safety-signal learning (Gewirtz et al., 1997). However, selective lesions to perirhinal (Falls et al., 1997), auditory thalamus (Heldt and Falls, 1998), and nucleus accumbens (Josselyn et al., 2005) also failed to disrupt safety-signal processing. Finally in rodents, the hippocampus appears to be involved in safety-signal learning, although the fact that damage to the hippocampus did not abolish the ability to re-acquire conditioning suggests that it may not be critical for this process (Heldt et al., 2002).

Given the convincing evidence suggesting that fear learning is amygdala-dependent (Davis, 1992; LeDoux, 2000), and that basic appetitive associations are dependent on the striatum (Schiller et al., 2008), it is not too surprising that the lateral OFC network is not critical for safety signal learning. Perhaps the most convincing argument for why we observe a lack of

effect comes from the human neuroimaging. Using a fear conditioning reversal paradigm, Schiller and colleagues (2008) paired one cue with a mild shock, while a second cue was paired with safety (no shock). Upon reversal of the reinforcement contingencies, neural activity shifted from the amygdala for the fearful cue, to areas of the ventromedial prefrontal cortex and striatum as the cue now became associated with safety (Schiller, et al., 2008). Notably, there was an absence of neural activity modulation in the sensory/orbital network during both contingencies. Thus, the present results support the human neuroimaging in positing that damage to the ventromedial OFC network may cause deficits in safety signal processing, whereas damage to the lateral orbital network is more disruptive to reward processing, and possibly higher order emotion-related behaviors (but see Gewirtz et al. 1997). This hypothesis would also be consistent with the known neuroanatomical findings indicating that the ventromedial OFC send more projections to the amygdala than it receives, whereas the lateral OFC receives more projections from the amygdala than it sends (Barbas, 2007). Thus, ventromedial OFC may be in a better position to regulate amygdala activity and this information might then be sent to the lateral OFC for further higher-order processing.

Flexible Modulation of Fear

Just as we found no evidence for a lateral orbital network's involvement in fear or safety-signal learning, this orbital area did not contribute to fear modulation. Both animals with neonatal OFC lesions and the sham-operated controls exhibited anxious behavior (high fear-potentiated startle) in the presence of the aversive A cue, low anxiety in the presence of the safety cue (B), and importantly, mild anxiety, when for the first time, the two cues were presented together (AB). Although Group Neo-Oasp did have a relatively lower fear-potentiated startle to the AX cue during the probe test than Group Neo-C, this group difference did not reach

statistical significance. The lower fear-potentiated startle in Group Neo-Oasp was largely driven by one case (see Table 3, Neo-Oasp-1), who startled less to the AX cue, than to the safety cue (B). Although Case Neo-Oasp-1 did have relatively more unintended damage to area 12 (see Table 1), a Pearson correlation matrix did not reveal any significant interactions between lesion extent of the various sub-regions of the OFC (both intended and unintended) and the ability to modulate fear-potentiated startle (all ps > .05).

Extinction

There was also no evidence of impaired ability to extinguish to the aversive cues (A-, AX-) after neonatal OFC damage. These findings complement appetitive-related findings wherein both early and late selective damage to the sensory/orbital network resulted in a sparing of reversal learning abilities (Kazama & Bachevalier, 2002, 2008), indicating that these animals are able to inhibit responses to cues that have become unrewarded. Again, this sparing is contrasted by the severe flexible decision-making deficits seen in the reinforcer devaluation paradigm (Kazama et al., 2007, Machado & Bachevalier, 2007; Izquierdo & Murray, 2004). As compared to rodent and humans, most of the studies on the role of the OFC in extinction and behavioral inhibition in nonhuman primates have generally used appetitive tasks, such as extinction of instrumental responses or object reversal and go/nogo tasks. Although medial and lateral OFC damage has been found to result in extinction deficits, selective damage to areas 11 & 13 have only resulted in deficits in the flexible modulation of behavior during the reinforcer devaluation task.

Conclusions

The present findings demonstrate that selective neonatal lesions to the lateral OFC network had no negative impact on fear learning, conditioned inhibition or extinction. However,

it is important to raise a potential account for this lack of effects of neonatal OFC lesions. It has long been demonstrated that brain lesions incurred in infancy may result in significant sparing of functions (Kennard, 1936, Goldman, 1976). Thus, it is possible that the animals sustaining damage to areas 11 & 13 of the OFC in infancy were able to compensate by engaging other brain areas not normally mediating fear/safety-signal learning and fear modulation. We believe that this alternative explanation is unlikely given that the same animals with neonatal OFC lesions have been shown to be severely impaired in emotion regulation and flexibly regulating appetitive responses when emotional and reward value of stimuli has changed. Thus, as compared to sham-operated controls, animals with neonatal OFC lesions showed emotion-related behavioral abnormalities in that they are more socially withdrawn during dyadic interactions with age-matched control animals (Payne et al., 2007) and displayed blunted fear reactivity to fearful stimuli as assessed by the Approach/Avoidance Paradigm (Raper et al., 2009). In addition, animals with neonatal OFC lesions were also retarded in discriminating pairs of objects when large stimuli sets were used (60 object pairs; Kazama, O'Malley, & Bachevalier, 2007; Kazama, Glavis-Bloom, & Bachevalier, 2008) and were unable to flexibly switch their response away from objects that had been devalued through a satiation procedure (Kazama et al., 2007). Thus, the evidence suggests that the lateral OFC network may not be required for the modulation or the extinction of basic fear responses but is rather implicated in fear modulation in situations involving higher-order processing, such as during complex or ambiguous social interactions. Future studies will need to assess whether the same outcomes will follow damage to the lateral OFC network in adult monkeys. In addition, given that in humans and rodents the visceromotor OFC network, consisting of lateral prefrontal areas 12 and ventromedial prefrontal areas 14 and 25, appears to be critical for both appetitive and aversive extinction (for review see Barbas 2007,

and Price, 2007). We have begun to study the effects of selective damage to the visceromotor network on both conditioned inhibition and extinction processes.

Figure legends

Figure 1. Intended lesion and representative case for Neo-Oasp. Intended damage is shown in gray on coronal sections through the orbital frontal cortex of an infant macaque brain atlas in the left column. Structural MR images are shown in the middle column. The lack of gray matter on the ventral surface indicates where the aspiration lesion took place. The estimated reconstructed lesion extent is shown in the right column. Arrows point to areas of unintended damage or sparing. Abbreviations: mos – medial orbital sulcus; los – lateral orbital sulcus; numbers refer to Brodmann areas (Brodmann, 1909).

Figure 2. Mean Acoustic Startle Response to differing sound intensities (95 dB, 100 dB, 110 dB, 115 dB, & 120 dB) by group. White bars represent the control group averages, while black bar represent group Neo-Oasp average startle. Error bars represent the SEM for each group.

Figure 3. Average sessions to criterion per stage of learning by group. White bars represent group means for Neo-C and the black bars represent Group Neo-Oasp. Animals in group Neo-C had no variance in the number of sessions per stage, and the individual scores within Group Neo-Oasp are represented by the animal's ID as seen in Tables 1 and 2.

Figure 4. Average log-transformed fear-potentiated startle by cue. White bars represent group means for Neo-C and the black bars represent Group Neo-Oasp. Although there was no significant effect of group, all aversive cue types were significantly different from all safety cues, and both cue types were significantly different from the transfer cue (AB), with the exception of

Group Neo-Oasp, cue AX, which was not significantly different from the safety cues ($p > .05$, all other $ps < .05$).

Acknowledgements:

This work was supported by grants from the National Institute of Mental Health (MH-58846, MH-086947, and MH-047840), the National Institute of Child Health and Human Development (HD-45471), Yerkes base grant (RR-00165), and the Autism Speaks Predoctoral Fellowship Grant. We thank the University of Texas Health Science Center at Houston veterinary and animal husbandry staff for expert animal care, Roger E. Price and Belinda Rivera for the care and handling of animals during the MR imaging procedures, and Edward F. Jackson for assistance in neuroimaging techniques.

Table 1: Extent of intended and unintended damage in Group O-asp

Cases	**Areas 11 & 13**				**Area 12**			
	L	R	Avg	W	L	R	Avg	W
Neo-Oasp-1	86.8	83.1	85.0	71.6	40.2	11.0	25.6	4.4
Neo-Oasp-2	81.0	97.8	89.4	79.6	9.3	1.4	5.4	0.1
Neo-Oasp-3	96.4	91.2	93.8	88.0	22.3	21.6	22.0	4.8
Neo-Oasp-4	85.7	94.8	90.2	81.2	2.8	4.0	3.4	0.1
Neo-Oasp-5	90.4	98.0	94.3	88.6	18.5	22.8	20.6	4.2
X	**88.1**	**93.0**	**90.5**	**81.8**	**18.6**	**12.2**	**15.4**	**2.7**

Cases	**Area 14**				**Ia**			
	L	R	Avg	W	L	R	Avg	W
Neo-Oasp-1	8.0	10.2	9.1	0.8	11.6	3.4	7.5	0.4
Neo-Oasp-2	31.9	6.8	19.4	2.2	78.5	57.7	68.1	45.3
Neo-Oasp-3	18.7	11.6	15.1	2.2	16.5	13.8	15.1	2.3
Neo-Oasp-4	9.7	12.6	11.2	1.2	82.5	64.6	73.6	53.3
Neo-Oasp-5	6.5	11.0	8.5	0.7	87.0	67.8	77.4	59.0
X	**15.0**	**10.4**	**12.7**	**1.4**	**55.2**	**41.5**	**48.3**	**32.1**

Data are the estimated percentage of damage as assessed from MR (post-surgical T1) images. L: percentage of damage to the left hemisphere; R: percentage of damage to the right hemisphere; Avg: average of L and R; W = (L x R)/100 [weighted index as defined by Hodos and Bobko (1984)]; X: group mean. Areas 11, 12, 13 and 14: cytoarchitectonic subregions of the macaque frontal lobe and Ia: agranular insular areas as defined by Carmichael and Price (1994).

Table 2: Sessions per learning stage

Group	A+	A+B-	AX+BX-	Combined Safety Learning	Extinction
Neo-C-1	2	2	2	4	5
Neo-C-3	2	2	2	4	5
Neo-C-4	2	2	2	4	2
Neo-C-5	2	2	2	4	2
X	**2**	**2**	**2**	**4**	**3.5**
Neo-Oasp-1	2	2	2	4	3
Neo-Oasp-2	2	2	2	4	5
Neo-Oasp-3	5	2	2	4	3
Neo-Oasp-4	5	2	2	4	2
Neo-Oasp-5	3	2	dropped		
X	**3.4**	**2**	**2**	**4**	**3.25**

Data are the total number of sessions to reach criterion performance for the initial fear learning (Stage A+), the safety signal learning stages (A+B-, AX+BX-; Combined Safety Learning is the summed scores of the two safety signal learning stages), and the extinction stage. The X scores in bold are the group means per stage.

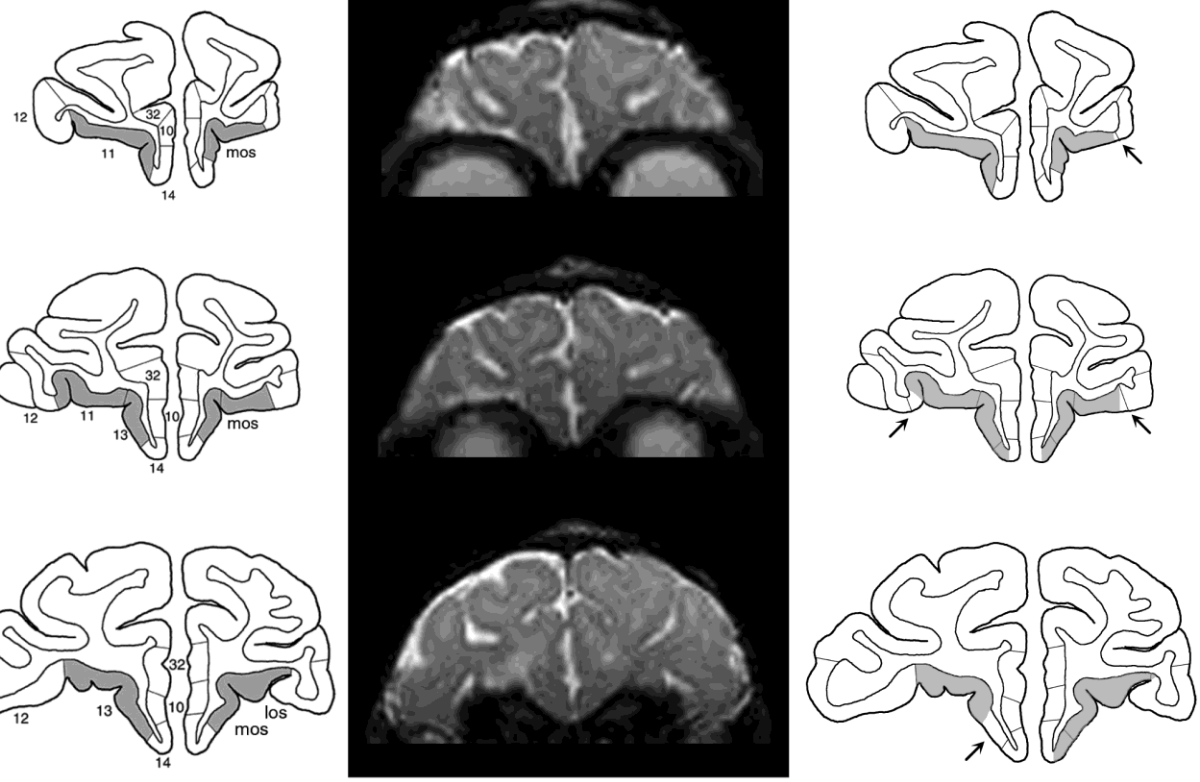
Table 3: Log-Transformed % Fear-Potentiated Startle

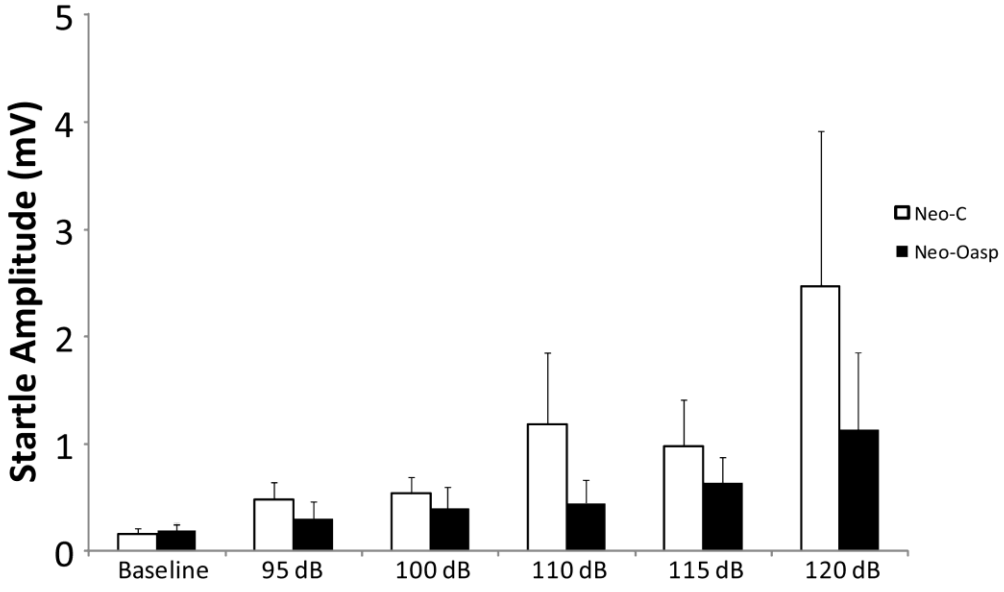
Group	**A**	**B**	**AX**	**BX**	**AB**
Neo-C-1	3.35	2.07	3.57	2.35	1.9
Neo-C-3	2	1.48	1.77	1.27	1.85
Neo-C-4	3.58	2.46	3.8	2.51	3.54
Neo-C-5	2.56	1.64	1.36	1.23	2.04
X	**3.17**	**2.06**	**2.91**	**2.03**	**2.33**
Neo-Oasp-1	3.33	1.99	1.82	2.53	3.03
Neo-Oasp-2	3.05	2.51	2.47	1.80	2.66
Neo-Oasp-3	2.08	2.20	2.44	1.90	1.96
Neo-Oasp-4	2.71	2.28	2.31	1.97	2.29
Neo-Oasp-5	Dropped				
X	**2.79**	**2.25**	**2.26**	**2.05**	**2.49**

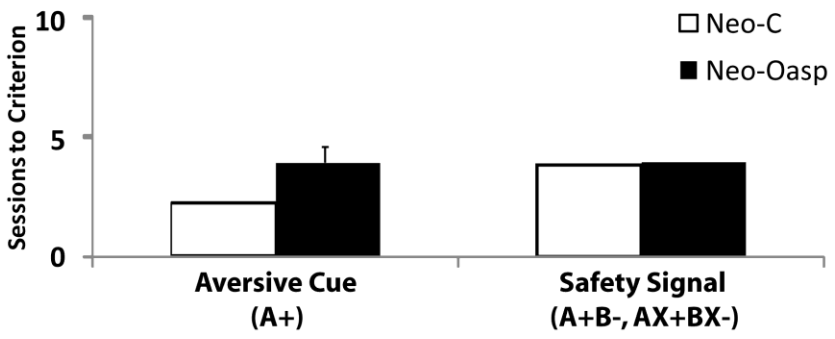
Data are the Log-Transformed % fear-potentiated startle amplitudes taken during the transfer test. Each individual score was obtained from the very first time the animal experienced that cue at the optimal decibel level (95dB or 120dB) for that particular animal. The X scores in bold are the group means per stage.

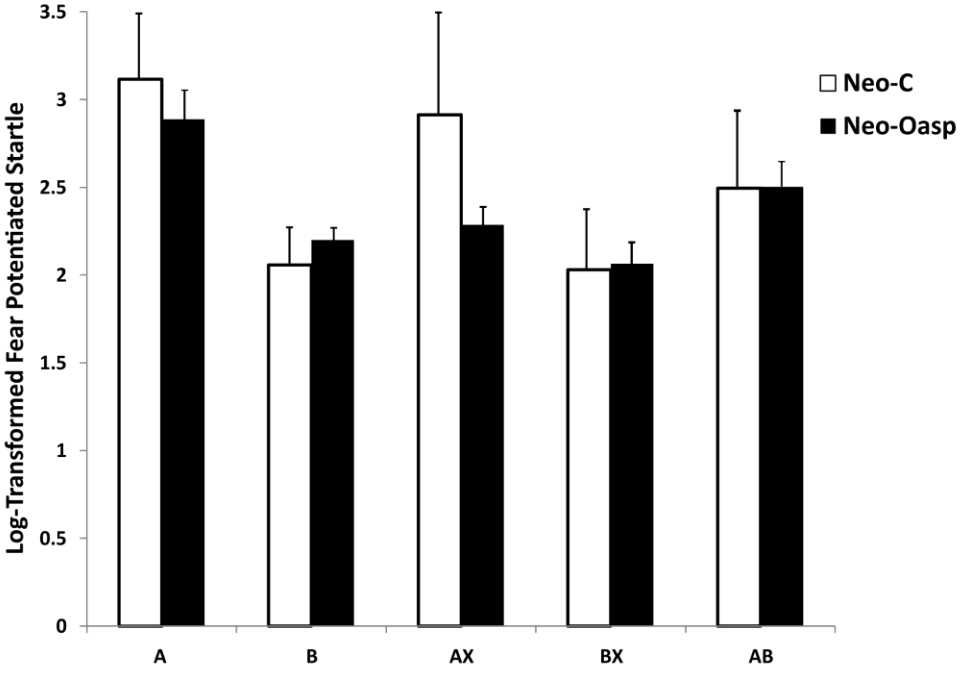
Neo-Oasp-4

Intended Lesion Extent	1-Week Post Surgical Structural MRI	Reconstructed Lesion Extent




Startle Amplitude (mV)
5
4
3
2
1
0
Baseline
95 dB
100 dB
110 dB
115 dB
120 dB
Neo-C
Neo-Oasp


Sessions to Criterion
10
5
0
Neo-C
Neo-Oasp
Aversive Cue
(A+)
Safety Signal
(A+B-, AX+BX-)


Log-Transformed Fear Potentiated Startle
3.5
3
2.5
2
1.5
1
0.5
0
A
B
AX
BX
AB
Neo-C
Neo-Oasp

Fear learning, conditioned inhibition, and extinction in adult macaques: III- Effects of neonatal hippocampal damage

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Abstract:

Evidence from recent human neuroimaging suggests that while the amygdala is central to the fear response, areas outside the amygdala such as the hippocampus, and prefrontal areas, are responsible for down-regulating the fear response when safety-signals are present in the environment. To date, there have been very few studies directly examining the role of the hippocampus in safety-signal learning and the flexible modulation of emotion. To this end, we proposed to examine the role of the hippocampus in non-human primates using an early lesion model and assessed fear/safety-signal learning, conditioned inhibition, and extinction using the AX+/BX- Fear-Potentiated Startle Paradigm. With the exception of two animals that sustained inadvertent damage to both the dorsomedial and ventral striatum, the data showed that the hippocampus is not necessary for either fear/safety-signal learning, or the down-regulation of the fear response, either during conditioned inhibition or extinction. However, given that these same animals had previously demonstrated some sparing of function as compared to animals with damage received in adulthood, both on an emotion regulation task (Human Intruder Paradigm) and contextual learning; and given the evidence from humans, rodents, and monkeys that hippocampal dysfunction correlates with severe deficits in contextual learning abilities necessary for AX+/BX- learning; it is possible that the lack of impairment observed may relate to the timing of the lesions and will have to be further investigated.

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The regulation of fear reactivity is a process that allows us to control the value and intensity of fear we express given the situational contexts and social norms. Disruption of this process, however, leads to excessive and pervasive fear that interferes with normal functioning and has been associated with several human disorders, such as anxiety disorders and Post-Traumatic Stress Disorder (PTSD). Studies on the neural network supporting fear regulation have implicated the medial prefrontal cortex as a likely candidate (see for review Quirk & Beer, 2006; Sotres-Bayon et al., 2006; Shin et al., 2006; Elzinga & Bremner, 2002). More recently, however, the role of the hippocampus in this regulatory process has also been proposed (Ji & Maren, 2007, Heldt & Falls, 2003). These advances stem from the impaired contextual fear memory (but not tone fear memory) following selective hippocampal lesions (Phillips & LeDoux, 1992; Kim & Fanselow, 1992; Maren, Aharonov & Fanselow, 1997) and in mutant mice with deficient LTP in the hippocampus (Abeliovich et al., 1993; Bourtchudale et al., 1994; Huerta et al., 2000). In addition, recent studies suggest that the hippocampus is critical for contextual modulation of fear expression and extinction recall (see for reviews Kim & Jung, 2006; Corcoran & Quirk, 2007). Richmond and colleagues (1999) demonstrated that selective lesions to the ventral hippocampus, which is strongly connected to the amygdala, resulted in a loss of conditioned freezing while sparing spatial abilities. Additionally, both classical and novel anxiolytic compounds (both injected systemically and directly into the hippocampal formation) reduced hippocampal neuronal activity, resulting in behavioral inhibition (McNaughton & Corr, 2004). Thus, in rodents, the hippocampus is clearly important for processing the context of the situation in which aversive or safety cues occur, but also for the regulation of emotion.

Further evidence of a role of the hippocampus in emotional and fear regulation is provided by the study of patients suffering from PTSD. At the root of PTSD symptoms is the inability to use cues available in one's safe environment to modulate fear. Thus, when experiencing a "trigger" cue related to a traumatic event, an individual suffering from PTSD will respond with an uncontrollable fear response, in spite of obvious cues in the environment that signal safety. This uncontrollable fear response indicates that safety cues in the environment may not be used appropriately to modulate a learned fear response. Interestingly, decreased hippocampal volumes were reported in PTSD patients, compared to either trauma-exposed control subjects or trauma-unexposed healthy subjects (Gilbertson, et al., 2007; Bremner et al., 1995). In a volumetric study using identical twins in which one sibling developed PTSD, Gilbertson and colleagues (2007) reported that smaller hippocampal volumes were correlated with higher rates of PTSD and resulted in greater difficulty in learning contextual cues, a function known to be dependent on the integrity of the hippocampus (Gilbertson, 2007). Decreased hippocampal activation was also found while PTSD patients experienced a symptomatic state, and was highly correlated with symptom severity (Bremner, et al., 1999; Shin, et al., 1999). Finally, PTSD patients have deficits in declarative memory, intrusive memories, fragmentation of memories, suggestive of hippocampal dysfunction (for review, see Elzinga & Bremner, 2002).

There have been only a few studies that have selectively investigated the role of the hippocampus in the expression and regulation of emotion and fear in monkeys. Recent findings have shown that damage to the hippocampus in adult monkeys results in blunted emotional reactivity towards threatening stimuli (Machado & Bachevalier, 2008, Chudasama, Wright, & Murray, 2008), although Pavlovian fear learning was spared (Antoniadis et al., 2007).

All together the data thus far suggest a critical role of the hippocampus in fear regulation, although more thorough investigations into the role of the hippocampus in safety signal learning and emotional regulation is needed. To this end, the present study investigated the effects of selective neonatal damage to the hippocampus in acquisition of fear, safety-signal learning and modulation, and extinction, using the AX+BX- Fear-Potentiated Startle Paradigm (Winslow et al., 2002, 2007). Preliminary results have appeared in abstract format (Kazama et al., 2010)

Methods

Subjects

Twelve adult rhesus macaques (Macaca mulatta) of both sexes (6 males, 6 females) and approximately six years of age and ranging from ~4.5-8 kg participated in this study. All animals received surgical brain procedures between 8-12 days of age and included: six monkeys with sham-operations (Group Neo-C, 3 males and 3 females), and six monkeys that received neurotoxic lesions of the hippocampus (Group Neo-Hibo, 2 males and 4 females). Following surgeries, all animals were behaviorally tested to assess emotional reactivity, social interactions, goal-directed behaviors and memory processes at different time points across development. All procedures were approved by the Animal Care and Use Committees of the University of Texas Health Science Center at Houston and of Emory University and details on the rearing conditions and neuroimaging and neurosurgical procedures can be found in already published papers (Goursaud & Bachevalier, 2006; Zeamer, Heuer & Bachevalier, 2010).

Pre-Surgical MRI

Just prior to surgery, high resolution FSPGR scan (T-1: TE = 2.6 ms, TR = 10.2 ms, 25° flip angle, contiguous 1 mm sections, 12 cm FOV, 256 X 256 matrix) and three Fluid-Attenuated Inversion Recovery scans (FLAIR: TE = 140 ms, TR =10,000 ms, inversion time = 2200 ms,

contiguous 3-mm sections, 12 cm FOV, 256 X 256 matrix, offset by 1mm posteriorly) were obtained using a GE sigma 1.5 Tesla Echo Speed scanner (GE Medical Systems, Milwaukee, WI) and a 5-cm surface coil. For animals of Group Neo-Hibo, the high-resolution structural images were used to precisely select and calculate coordinates for neurotoxin injection sites within the hippocampus (Saunders, Aigner, & Frank, 1990; Málková et al., 2001; Nemanic et al., 2002) for each animal and both types of images served as a baseline for quantifying lesion extent, post-surgically.

Using the MR ear bars and the midline sinus coordinates as referents, three dimensional stereotaxic coordinates (A/P, M/L, D/V) on the coronal T-1 images for each injection site per hippocampus. For the posterior two-thirds of the hippocampal formation, 5-6 injection sites were selected every 1.0 mm and centered within the body of the hippocampal formation. For the most anterior portion, where the uncus was clearly visible, two injection sites were selected, one situated laterally, and the other located more medially within the uncus.

Surgery

Following neuroimaging procedures, all animals remained anesthetized and were immediately transported in the surgical suite where they were prepared for surgical procedures, which were performed under aseptic conditions. Animals were placed on a heating pad to prevent hypothermia and administered an intravenous drip solution containing 0.45% NaCl to maintain hydration. A local anesthetic (Marcaine 25%, 1.5m., s.c.) was injected along the anterior-posterior midline incision to reduce pain. The skin and underlying connective tissue were retracted to expose the bone. Small craniotomies were made above the hippocampus of each hemisphere and small slits were made in the dura. For the hippocampal lesions, using two Hamilton syringes held by Kopf electrode manipulators (David Kopf Instruments), the

neurotoxin, ibotenic acid (Biosearch Technologies, Novato, CA) was simultaneously injected in both the left and right hippocampi at each of the 7-8 sites selected (total of 3.2-5.4 µl, 10mg/ml in PBS, pH 4.0, rate of 0.2 µl/30sec). Sham-operations consisted of opening of the skull and dura, but no injections were performed. During the surgical procedures, vital signs (heart and respiration rates, expired CO2, and temperature) were monitored until the monkey fully recovered from anesthesia. After ibotenic acid injections or sham-operations the dura, galea and skin were sutured separately. The animals were then removed from the Isoflurane gas anesthesia and allowed to recover in an incubator ventilated with oxygen.

Animals received pre- and post-surgical treatments that began 12 h prior to surgery and lasted for 7 days. These treatments included dexamethazone sodium phosphate (0.4 mg/kg, s.c.) to control swelling and Cephazolin (25 mg/kg, per os) to minimize risk of infection. Additionally, Acetaminophen (10mg/kg, p.o.) was administered four times a day for three days after surgery to relieve pain. A topical antibiotic ointment was also applied to the wound, daily.

Lesion Extent

The extent of ibotenic acid lesions was assessed using MRI techniques using both FLAIR and 3D T1-weighted fast spoiled gradient-echo sequence FSPGR scans performed 7 to 10 days after surgery, using the same scan parameters than the pre-surgical MR sequences, and were already described in details in two previous publications (Goursaud & Bachevalier, 2006; Zeamer et al., 2010). The extent of intended hippocampal damage as well as extent of damage to adjacent areas are given for each case of Group Neo-Hibo in Table 1. Figure 1 illustrated the lesion extent of one representative case (Neo-Hibo-3) as identified from hypersignals (indicative of brain edema resulting from cell death) on the MR images.

Behavioral testing

All methods have been previously described in detail (Kazama, Davis, & Bachevalier, Manuscript in prep) and included the acoustic startle response, pretraining, training to the aversive conditioned cue (A+), simultaneous training to the aversive and safety conditioned cues (A+/B-), training to the compounds AX+/BX-, AB transfer testing, and extinction. All training was run over the course of one to two months, and occurred when the animals were approximately 4-6 years of age. All phases were made of sessions differing in lengths (see below for details) but which were spaced 72 hours apart. All animals received their normal daily diet of primate chow (Purina, St. Louis), fresh fruit, and water *ad libitum*, in addition to additional treats during primate chair training.

Apparatus:

In all phases, monkeys were seated in a non-human primate chair positioned in a sound attenuated chamber outfitted with an automated system that delivered unconditioned and conditioned stimuli. The chair was positioned on a platform connected to a load cell (Med Associates, St. Albans, VT). Movements from the monkey produced displacement of the load cell, and this output was then amplified (Tedea Huntley model 1040), digitized and transferred to a computer.

Stimuli:

There were two unconditioned stimuli (US): (a) a 700 msec jet of compressed air (100 PSI) generated by an air compressor from outside the chamber and projected via four air jet nozzles directed the air flow at the face of the monkey, and (b) a startle stimulus consisting of a 50 msec burst of white noise (5 msec rise-decay time) emitted through the same speakers as the background noise and which varied in intensity (range: 95-120 dB).

There were three conditioned stimuli/cues (CS) identified as cues A, B, and X. First, the visual CS was a 4 sec light generated by an 8 W fluorescent bulb (100 µsec rise time, 700 foot lamberts) attached to the top of the test chamber. Second, an auditory CS was produced by a white noise generator and bandpass-filtered, with both the low and high passes set at 2 kHz (24 dB/octave attenuation), at an intensity of 65 dB. Third, a tactile CS was generated by a quiet computer fan that produced a gentle airflow directly onto the monkey's head. The cue assignments were pseudo-random and counter-balanced across groups such that some animals received the tone as the aversive CS, whereas others received the light as an aversive CS, and so forth.

Acoustic Startle Response:

This phase evaluated the animals' acoustic startle response for the purposes of detecting any effects of lesion on baseline acoustic startle and began when the animals were fully accustomed to be restrained in the primate chair within the startle box. Animals were placed in the apparatus and exposed to two days of 60-trial sessions each, which composed equally of baseline activity trials (10 trials), and startle noises of varying decibel intensities (95, 100, 110, 115, & 120 dB; 10 trials each), all pseudo-randomly ordered throughout each session. Animals were then tested for pre-pulse inhibition before moving on to the AX+/BX- paradigm (data published in Heuer, Kazama, Davis & Bachevalier, in preparation).

Pre-training:

This phase habituated the animals to any unconditioned effects of the cues on startle intensity prior to conditioning. Animals first received two 30-trial sessions, in which only the to-be-conditioned cues (tone, light, airflow from quiet fan, and combinations of the three) were presented without the startle noise. Next, animals were given a minimum of two 60-trial

sessions consisting of 30 noise alone trials (95dB), and 30 cue-noise trials where the 95dB startle noise was paired with one of the to-be-conditioned cues (5 trials per cue, psuedorandomly ordered). Within each trial when the CS came on the startle stimulus was presented 4 sec after its onset. Pre-training sessions were repeated for each monkey until presentation of what would become the safety cue produced less than a 30% increase in startle amplitude compared to noise alone presentations.

A+ Training Phase:

During this phase, animals were trained to associate the assigned aversive cue A with the presentation of the air-blast (A+). These A+ air-blast trials occurred four times per 28-trial session, and were scheduled so that one pairing appeared at the beginning and one at the end of each session. The remaining two pairings were intermixed with startle test trials within the 28 trials and their placement varied irregularly across sessions to make them unpredictable to the monkey. When cue A appeared the startle stimulus or air-blast was presented 4 sec after the onset of cue A. Within each 28-trial session, each of the other 4 trial-types (Noise Alone 95dB, Noise Alone 120dB, Cue A+ 95dB Noise, Cue A+ 120dB Noise) were presented pseudo-randomly 6 trials each per session. Animals received A+ Training for a minimum of two days, and until their % Fear-Potentiated Startle (FPS) was 100% above their pre-training startle to the A+ cue.

A+/B- Training Phase:

During this phase, animals received 40-trial sessions composed of 95 dB and 120 dB Noise Alone trials (6 trials each); six trials with Cue B-presented with both startle noise intensities but never paired with the air-blast US; four trials with Cue A+ paired with the air-blast (according to the schedule described previously), as well as with both startle noise intensities (6

trials each). Animals received A+/B- Training for a minimum of two days, and until there was a difference of 100% FPS between the two cues.

AX+/BX- Training Phase:

These 40-trial sessions were constructed very similar to A+/B- Training. However, the remaining cue X was presented simultaneously with either A+ or B- throughout these training sessions to yield compound cues AX+ and BX-. As with the A+/B- Training, animals received the AX+/BX- Training for a minimum of two days, and until there was a difference of 100% FPS between the two cues.

AB Testing/Transfer Test:

Animals were tested for latent inhibition (i.e. transfer) in a single session occurring 72 hrs after the last AX+/BX- training session to examine the potential inhibitory effects of B on A. This 48-trial probe session consisted of all trial types, including: two A+ air-blast reminder trials intermixed with both 95 dB and 120 dB Noise Alone trials (6 trials each), 95 dB and 120 dB cue pairings (A, B, AX, BX, 5 trials each per noise intensity), and 95 dB and 120 dB AB compound cue (5 trials per noise intensity). All trials were pseudo-randomly ordered.

Extinction:

During the final phase, all animals were presented with multiple 12-trial sessions of the 95 dB startle stimulus elicited alone or in the presence of cues A and AX (4 trials of each type) to evaluate fear extinction. Training was completed when the animal returned to its pre-training startle amplitude.

Data Analysis

During each phase, startle amplitudes were recorded via the Med Associates software and amplified via the load cell. The primary parameter was the percent fear potentiated startle (FPS)

defined as: [Mean startle amplitude on CS test trials – mean startle amplitude on startle noise alone test trials)/mean startle amplitude on noise burst alone test trials] X 100. If in the course of training, an animal's % FPS showed a steady decline and no improvement over an extended period, that animal was given a maximum score of 15 sessions (which was determined after testing Neo-Hibo-5 out to 15 days with no improvement).

Non-parametric Mann-Whitney U test was used to compare the acoustic startle responses to the varying intensities (95, 100, 110, 115, & 120 dB) across groups due to the heterogeneity of the startle responses between groups. The animal's ability to associate and discriminate between the fearful and safety cues (A+, B-, AX+, BX-) was assessed using a "sessions to criterion parameter". Because control animals learned the task in the minimum number of sessions (e.g. 2 sessions per phase) resulting in no variance within the group, group differences were again determined with non-parametric statistics (Mann-Whitney U). Given that startle values were not normally distributed as previously reported (Winslow, Noble, & Davis, 2008), the transfer test data were transformed using a logarithmic base 10 transformation and analyzed with repeated measures ANOVAs.

Results

Lesion Extent

A full description of the lesion extent is available for all cases in Table 1 and representative cases can be seen in Figures 1 and 2. The hippocampal damage for Group Neo-Hibo varied from 3.9% to 87.4%. The damage was extensive and bilateral in two cases (Neo-Hibo-2,-3), extending throughout the entire length of the hippocampi. Case Neo-Hibo-1 had mostly unilateral damage to the left side, although this unilateral damage extended throughout the hippocampus. Neo-Hibo-6 received very slight damage (8%) to the left hippocampus, and

although his damage was slight, a one year post-surgical volumetric scan revealed a 20% reduction in hippocampal volume in this case. Thus, due to his consistent performance with the rest of his group on a number of other behavioral measures, this case was included in the analysis. Finally, two other cases (Neo-Hibo-4 and -5) sustained extensive lesions to the right hippocampi (67 and 84% respectively), but more moderate damage to the left hippocampi (20%). However, for both cases, the damage sustained to the left hippocampus was located laterally within the body of the hippocampus but spanned the entire length of the hippocampus. Thus, although the most medial portion of the body and the uncus were not impacted by the lesions, the CA1-CA3 subfields were largely damaged throughout. In addition, in these two cases, inadvertent damage resulting from the penetration of the injection needles was identified in portions of both the dorsomedial and ventral aspects of the striatum, situated dorsal to the hippocampus. This damage was not apparent in the remaining four animals in the group (Neo-Hibo-1, -2, -3, & -6; see Figure 2). Unintended damage to structures surrounding the hippocampus was very slight in all cases (see Table 1).

Acoustic Startle Response

As previously reported by Heuer and colleagues (Heuer et al., 2010), the baseline startle response of two animals in Group C (cases Neo-C-2 and Neo-C-6) was greater than the maximum amplitude of the load cell. Thus, these two animals were dropped from the study. As illustrated in Figure 3, the magnitude of the startle responses increased with progressive increases in the amplitude of the startle noise in both sham-operated controls and animals with neonatal hippocampal lesions (Startle amplitude effect: $F_{G\text{-}G}(1,5) = 8.2$, $p = .01$). In addition, although the effect of Group and the Group by Startle amplitude interactions did not reach significance [F = .482 and F = 0.438, all *ps* > .05, respectively], average startle responses across

all noise intensities were slightly higher in animals with hippocampal lesions than in sham-operated controls. This lack of group effect did not result from variations in lesion extent in Group Neo-Hibo since a Pearson Correlation Matrix did not reveal any significant correlations between the extent of damage (both intended and unintended) and startle amplitude (all ps > .05).

Fear Learning (A+ Training)

Most animals, regardless of group learned to associate Cue A+ with the air-blast very quickly and the group difference did not reach significance (Mann-Whitney U, $p > .05$, Table 2, Figure 4). Control animals all performed at floor, completing this stage in the minimum two sessions, and all animals but two in Group Neo-Hibo as well as controls (2 sessions). From the remaining two animals of Group Neo-Hibo, one (Neo-Hibo-2) reached criterion in 5 sessions and the other (Neo-Hibo-4) was unable to learn and training was stopped after 15 A+ training sessions.

Fear/Safety Signal Discrimination Learning (A+B-, AX+BX- Training)

Because both A+B- and AX+BX- phases were theoretically similar in nature, all sessions were combined for the analyses (see Table 2, Figure 4). Again control animals learned the discrimination in the minimum number of sessions (4 sessions) as well as four of the six Neo-Hibo animals (Group effect: Mann-Whitney U, $p > .05$). However, the remaining two animals of Group Neo-Hibo (Neo-Hibo-4 and Neo-Hibo-5) were unable to learn these discriminations in the maximum of sessions to criterion (30 sessions) and thus failed this phase.

Modulation of fear in the presence of the safety signal (AB probe trial)

Data for only the four animals of Group Neo-Hibo that were able to discriminate between the aversive and safety cues were compared to those of the four control animals. As shown in Table 3 and Figure 5, although the two groups did not differ (group effect: $F(1,8) = .023$, $p >$

.05), both groups had significantly greater startle to the aversive cues (A, AX) compared to either the safety cues (B, BX) (t-tests, all *ps* < .05) or the transfer cue (AB) (t-tests, all *ps* < .05).

Extinction

Both groups extinguished very quickly to repeated presentations of the fearful cues (A-, AX-) in the absence of the US (Table 2 and Figure 4, requiring 3.5 sessions for Group Neo-C and 4.5 sessions for Group Neo-Hibo (Mann-Whitney U, Z = -.619, p > .05).

Discussion

In summary, early damage to the hippocampus resulted in normal baseline acoustic startle responses, and in the majority of animals normal fear learning, safety signal learning, fear modulation, and extinction. However, two of the four hippocampalectomized animals had very severe deficits during the training phases, and thus were not able to be tested for transfer or extinction. Discussion on the factor influencing individual variation in the results as well as on the role of the hippocampus in emotional regulation will be discussed below.

Baseline Acoustic Startle

Neonatal damage to the hippocampus resulted in a negligible increase in baseline acoustic startle responses as compared to sham-operations. Animals in both groups increased their startle in response to increased noise intensity, but animals with neonatal hippocampal damage did show slightly elevated startle amplitudes across all intensities, although this group difference did not reach significance. These findings parallel the lack of effects of selective early ventral hippocampal lesions in rats (Lipska et al., 1995) or selective hippocampal lesions in adult monkeys (Antoniadis et al., 2007) on acoustic startle.

Individual variations in fear learning and regulation after neonatal hippocampal damage

Animals with neonatal hippocampal damage showed important individual variation across all phases of the task. Thus, whereas four animals performed normally in both the fear acquisition, AX+/BX- learning, conditioned inhibition as well as extinction, the remaining two animals (cases Neo-Hibo-4 and -5) had severe learning impairments through all phases of the task. Given the individual variation in the hippocampal lesion size (see Table 1), it is possible that more complete damage to the hippocampus in these latter two cases may have resulted in more severe deficits. However, this does not seem to be the case. As shown in Table 1, cases Neo-Hibo-4 and 5 had extensive hippocampal damage to the right hemisphere but less extended damage to the hippocampus on the left, although this restricted damage included the CA1 and CA2 fields and spanned the entire length of the hippocampus, thus disrupting significantly the functioning of the trisynaptic circuit. Thus, dysfunction of the hippocampus in these two cases that performed poorly appears as important as that found in two other cases (Neo-Hibo-2 and -3) that had extensive bilateral hippocampal lesions (average: 67.6% and 87.4%, respectively) but performed normally. Thus, extent of hippocampal damage may not be the common denominator for this individual difference.

Another potential explanation for this individual difference may relate to damage outside of the hippocampus. Examination of the post-surgical structural MRIs of all six animals in Group Neo-Hibo revealed that, although none of the cases had substantial damage to structures adjacent to the hippocampus (posterior amygdala, entorhinal, perirhinal and parahippocampal cortex; average: 5% or less), cases Neo-Hibo-4 and -5 were the only two cases of the group that demonstrated hypersignals above the hippocampus indicating fairly significant damage caused by the passage of the needles during the neurotoxin injection. The hypersignals indicated

ischemic infarct that extended through the striatum, and included more specifically both the dorsomedial striatum as well as the ventral aspects of the putamen and nucleus accumbens. This unintended damage could have had a significant impact on performance on the AX+/BX- paradigm, given that the striatum has been implicated in fear learning. For instances, striatal structures increased their activity during avoidance learning in humans and are known to be critical for Pavlovian fear learning in rodents (for review, see Delgado et al., 2009). Thus, it is possible that this unintended damage to the striatum in these two specific animals, either alone or in combination with the hippocampal lesions, may explain their more severe deficits during the learning phases. Although this explanation will remain to be empirically tested, if proven to be correct, our current findings will suggest that selective neonatal hippocampal lesions in monkeys do not alter fear learning, safety signal learning, conditioned inhibition and extinction.

Role of the hippocampus in fear learning and regulation

Learning aversive or safety signals: The sparing of fear conditioning in the present study complements recent findings demonstrating that selective lesions of the hippocampus in adult monkeys also spared the ability to acquire a learned fear, measured with the same fear-potentiated startle paradigm as used in the present study (Antoniadis et al., 2007). It also parallels the rodent studies reporting normal fear conditioning to a cue (tone) after hippocampal lesions, at least as far as the context in which the cue is presented is made irrelevant for normal performance (Quinn et al., 2002; Esclassan et al., 2008). Further, examination of the brain structures during Pavlovian fear conditioning in humans have not indicated increased activity in the hippocampus, although other fear-related structures, such as the amygdala, anterior cingulate, insula, and parahippocampal gyrus, do appear to be activated during the same tasks (Reinhardt et

al., 2010; Tabbert et al., 2010). Thus, damage to the hippocampus in infancy does not affect Pavlovian fear conditioning. This conclusion is strengthened by additional recent findings indicating that all animals with neonatal hippocampal lesions showed normal fear avoidance responses to fearful stimuli (Raper et al., 2009), a result consistent with that reported by Prather and colleagues (2001) and with that found after the same lesions in adulthood (Machado, et al., 2009).

Similarly, with the exception of the two previously mentioned hippocampal cases, the remaining four animals with neonatal damage to the hippocampus learned the safety-signal normally. To date, the majority of studies, both in humans (Goh et al., 2004; Dolan & Strange, 2002; Burgess et al., 2001) and rodents (Smith & Mizumori, 2006; Anagnostaras et al., 2001; Kim & Fanslow, 1992; but see Gewirtz et al., 2000), have determined that the hippocampus plays an important role in contextual learning. However, it could be argued that learning to pair one cue with an air-blast, and another cue with the lack of an air-blast does not require contextual learning, and thus would not be hippocampal dependent (for review, see Holland & Bouton, 1999). Therefore, if safety-signal learning in this task is independent of context, one might predict a lack of impairment following hippocampal damage; a prediction consistent with the current findings.

Fear Modulation: All four hippocampal animals that learned both the fear cues (A+, AX+) and the safety cues (B-, BX-) were able to use the safety cue to flexibly modulate their fear to the A+ cue when, for the very first time, it was presented in conjunction with the B- cue (AB). These results do not seem to support the view that the role of the hippocampus in contextual learning makes it a major contributor to the amygdalocentric model of the fear

response (Rauch, Shin, & Phelps, 2006). In this model, fearful cues lead to excitation of the amygdala, whereas contextual safety cues, purportedly processed by the hippocampus, inhibit the amygdala and thus down-regulate the fear response. This proposal has received support from many clinical studies indicating that patients suffering from PTSD have a variety of cognitive deficits, including contextual learning impairments (for review, see Maren, 2001; Rauch et al., 2006; Shin et al., 2006) and are impaired on the AX+/BX- discrimination learning phase (Jovanovic & Ressler, 2010; Jovanovic et al., 2009; Jovanovic et al., 2006). However, one reason that might explain the lack of deficits in conditioned inhibition following neonatal lesions may relate to the timing of the lesions. This proposal is substantiated by two recent findings from our laboratory on the same animals demonstrating that the animals with neonatal hippocampal lesions showed significant functional sparing. First, all animals in Group Neo-Hibo performed normally on an incidental contextual recognition task (contextual visual paired comparison; Glavis-Bloom et al., 2010), a task known to be impaired when the hippocampal lesions are performed in adulthood (Bachevalier & Nemanic, unpublished data). Second, they also displayed normal regulation of emotional responses when faced with different levels of threat (Bachevalier and O'Malley, unpublished data), fear modulation responses that are also known to be impacted by hippocampal damage incurred in adulthood (Machado & Bachevalier, 2008). Thus, the evidence so far suggests that hippocampal lesions in adulthood, but not those performed in infancy, may result in impaired conditioned inhibition. Nevertheless, the effects of hippocampal lesions in adult monkeys on safety signal learning and conditioned inhibition will need to be directly tested given that in rodents McNish and colleagues (1997) have reported normal contextual fear as measured by fear-potentiated startle after damage to the rodent dorsal hippocampus.

Further research will be required to determine what structures are being recruited to carry these processes in the absence of a functioning hippocampus in early infancy. Several possibilities will be offered below.

Extinction: Just as we found no evidence of impaired conditioned inhibition during the AX+/BX- fear-potentiated startle paradigm after neonatal hippocampal damage, we also did not find any impact of these early lesions on the ability to extinguish responses to the aversive cue. These results are consistent the normal performance of these same animals in object discrimination reversal (ODR); a task thought to measure response inhibition (Kazama, Glavis-Bloom, Bachevalier, 2008).

These results substantiate previous rodent studies reporting normal extinction after selective damage to the hippocampus (Gewirtz et al., 1997; but see different results when freezing instead of startle is used as the behavioral response, McNish et al., 1997; McNish et al., 2000), as well as normal corticotrophin-releasing factor-enhanced startle (Lee and Davis, 1997).

Role of Development

The major issue regarding the lack of effects of early hippocampal damage on fear regulation is the potential for recovery of function. Although the specific mechanisms are yet unknown, we do know that five of these animals are able to process contextual information during a recognition task (Glavis-Bloom et al., 2010), and are able to modulate their fear reactivity when faced with threat signals differing in magnitude (Bachevalier & O'Malley, unpublished data), two abilities that are known to be when damage to the hippocampus occurs in adulthood (Bachevalier & Nemanic, unpublished data; Machado & Bachevalier, 2008). Furthermore, in cases of concurrent discrimination learning and object discrimination reversal, these same animals appear to show facilitated learning, making significantly less errors than their

sham-operated controls (Glavis-Bloom, Kazama, and Bachevalier, 2008). Thus, it is possible that these animals have engaged some compensatory mechanism(s) to learn, modulate, and extinguish their fear of an aversive cue in the absence of a functioning hippocampus. There are two distinct possibilities: 1) Given the presence of sparing of tissue in all cases, the remaining hippocampus was able to carry this function or 2) these functions were able to be processed outside of the hippocampus.

The first possibility is recovery of function within the remaining hippocampus. As previously mentioned, we did not find any significant correlation in the amount of damage and performance. The lack of correlation was present not only for the AX+/BX- paradigm, but also for two contextual learning tasks (Glavis-Bloom et al., 2010). However, the hippocampus is a structure that has been shown to be highly plastic, and of course, one of the first areas where neurogenesis is present in adulthood (for review see, Deng et al, 2010). Thus, given this plasticity, the remaining hippocampus could reorganize itself, particularly if the damage was done early in life. There is at least an fMRI study in one case of selective early hippocampal damage due to ischemic infarct in humans, in Patient Jon, who may substantiate this possibility. This patient, as well as age-matched controls were instructed to remember autobiographical events prior to the scanning session. They were then asked to recollect the same events and to provide specific information for these events while being examined in an fMRI study. In terms of performance, Jon showed some marked difficulties remembering autobiographical information, in that certain memories appear to lack contextual information; his memory for certain events appeared to be spared. Interestingly, during the recollection of events in which his memory was spared, Jon had increased activation of the remaining intact hippocampus despite the presence of extensive (50%) and bilateral hippocampal damage (Maguire et al.,

2001). The second possibility is that in the absence of a functional hippocampus, areas outside the hippocampus have adapted to carry these functions. Peters and colleagues (2010) found that, although the hippocampus is directly connected to the amygdala via the basal nucleus, the major route by which the hippocampus down-regulates the amygdala (at least during extinction) appears to be through the hippocampus' indirect BDNF-related connections via the infralimbic cortex (medial prefrontal cortex in monkeys). Given the many connections to the medial prefrontal cortex, in the absence of a functional hippocampus, several alternative avenues exist, including rhinal cortices, and parahippocampus (Höistad and Barbas, 2008), by which contextual information might reach the medial prefrontal cortex and be utilized to modulate the fear response.

Conclusions

In conclusion, results from the current experiment suggest that early damage to the hippocampus does not impair either fear/safety-signal learning or the flexible modulation of fear either in conditioned inhibition or extinction. However, given that early lesions resulted in significant sparing of function relative to animals with damage received in adulthood, both in terms of contextual learning and memory, as well as the flexible modulation of emotion, further research is necessary to determine whether adult lesions of the hippocampus will result in similar sparing during the AX+/BX- paradigm. Additionally, we found that in two monkeys, inadvertent damage to the striatum resulted in a profound loss of fear/safety-signal learning abilities. Future studies may investigate the role of these structures in safety signal learning and conditioned inhibition.

Figure legends

Figure 1. Intended lesion and extent of hippocampal damage in a representative case (Neo-Hibo-3). Intended damage is shown in gray on coronal sections through the anterior-posterior extent of the hippocampus of an infant macaque brain atlas (left column), hypersignals caused by edema resulting from cell death are present in the FLAIR MR images (middle column), and reconstructed extent of hypersignals is shown in gray on corresponding drawing of coronal sections of a normal brain (right column). Arrows indicate unintended damage.

Figure 2. Coronal FLAIR and structural MR images of representative cases Neo-Hibo-5 and Neo-Hibo-2. FLAIR MR images (left) demonstrate edema caused by cell death within the hippocampus, while structural MR images (right) demonstrate damage or lack thereof to striatal areas.

Figure 3. Mean (± SEM) percent of acoustic startle response to differing sound intensities (95 dB, 100 dB, 110 dB, 115 dB, & 120 dB) for sham-operated controls (Neo-C; n = 4) and animals with neonatal hippocampal lesions (Neo-Hibo; n = 6).

Figure 4. Mean (± SEM) sessions to reach criterion in learning the aversive cue A+ and in safety signal learning (A+B- and AX+BX-) in sham-operated animals (Neo-C, white bars) and animals with neonatal hippocampal lesions (Neo-Hibo; black bars). Note that animals in group Neo-C had no variance in the number of sessions for both phases.

Figure 5. Mean (± SEM) percent fear-potentiated startle, as expressed by log-transformed, for each cue in sham-operated controls (Neo-C; white bars) and animals with neonatal hippocampal lesions (Group Neo-Hibo; black bars). For both groups, aversive cues (A, AX) were

significantly different from safety cues (B, BX) (all $p < .05$), and the aversive cues were also significantly different from the transfer cue (AB) (all $ps < .05$).

Table 1*:* Extent of intended and unintended damage in Group Neo-Hibo

Cases	**Hippocampus**				**Amygdala**				**TH/TF**			
	L	R	Avg	W	L	R	Avg	W	L	R	Avg	W
Neo-Hibo-1	63.6	2.9	33.2	1.8	14	0	7	0	3.1	.5	1.8	0
Neo-Hibo-2	54.4	80.9	67.6	44	0	0	0	0	21.4	2.7	12.1	.6
Neo-Hibo-3	78.5	96.3	87.4	75.6	1.7	0	.8	0	6.1	5.5	5.8	.3
Neo-Hibo-4	20.3	67.3	43.8	13.6	0	4.7	2.4	0	15.3	0	7.6	0
Neo-Hibo-5	20.7	84.4	52.6	17.5	0	4.9	2.4	0	6.1	4	5.1	.2
Neo-Hibo-6	7.9	0	3.9	0	0	0	0	0	0	0	0	0
X	**40.9**	**66.4**	**48.1**	**25.4**	**2.6**	**4.6**	**2.1**	**0**	**8.7**	**2.1**	**5.4**	**.2**
Cases	**TE**				**ERh**				**PRh**			
	L	R	Avg	W	L	R	Avg	W	L	R	Avg	W
Neo-Hibo-1	0	0	0	0	2.6	0	1.3	0	0	0	0	0
Neo-Hibo-2	.6	0	.3	0	0	0	0	0	5.4	.5	2.9	0
Neo-Hibo-3	0	0	0	0	0	0	0	0	0	0	0	0
Neo-Hibo-4	1	0	.5	0	0	0	0	0	0	0	0	0
Neo-Hibo-5	0	0	0	0	0	1.5	.7	0	0	.5	.3	0
Neo-Hibo-6	0	0	0	0	0	0	0	0	0	0	0	0
X	**.3**	**0**	**.1**	**0**	**.4**	**.2**	**.3**	**0**	**.9**	**.2**	**.5**	**0**

Data are the estimated percentage of damage as assessed from MR (post-surgical FLAIR) images. L: percentage of damage to the left hemisphere; R: percentage of damage to the right hemisphere; Avg: average of L and R; W = (L x R)/100 [weighted index as defined by Hodos and Bobko (1984)]; X: group mean. Area Abbreviations: TH/TF (parahippocampal cortical area TH/TF), TE (temporal cortical area TE), ERh (entorhinal cortex), PRh (perirhinal cortex)

Table 2: Sessions per learning stage

	A+	A+B-	AX+BX-	Combined Safety Learning	Extinction
Group C					
Neo-C-1	2	2	2	4	5
Neo-C-2	Dropped				
Neo-C-3	2	2	2	4	5
Neo-C-4	2	2	2	4	2
Neo-C-5	2	2	2	4	2
Neo-C-6	Dropped				
X	**2**	**2**	**2**	**4**	**3.5**
Group A					
Neo-Hibo-1	2	2	2	4	2
Neo-Hibo-2	5	2	2	4	6
Neo-Hibo-3	2	2	2	4	2
Neo-Hibo-4	15	15	15	30	Failed
Neo-Hibo-5	2	15	15	30	Failed
Neo-Hibo-6	2	2	2	4	8
X	**4.7**	**6.3**	**6.3**	**12.7**	**4.5**

Data are the total number of sessions to reach criterion performance for the initial fear learning (Stage A+), the safety signal learning stages (A+B-, AX+BX-; Combined Safety Learning is the summed scores of the two safety signal learning stages), and the extinction stage. The X scores in bold are the group means per stage.

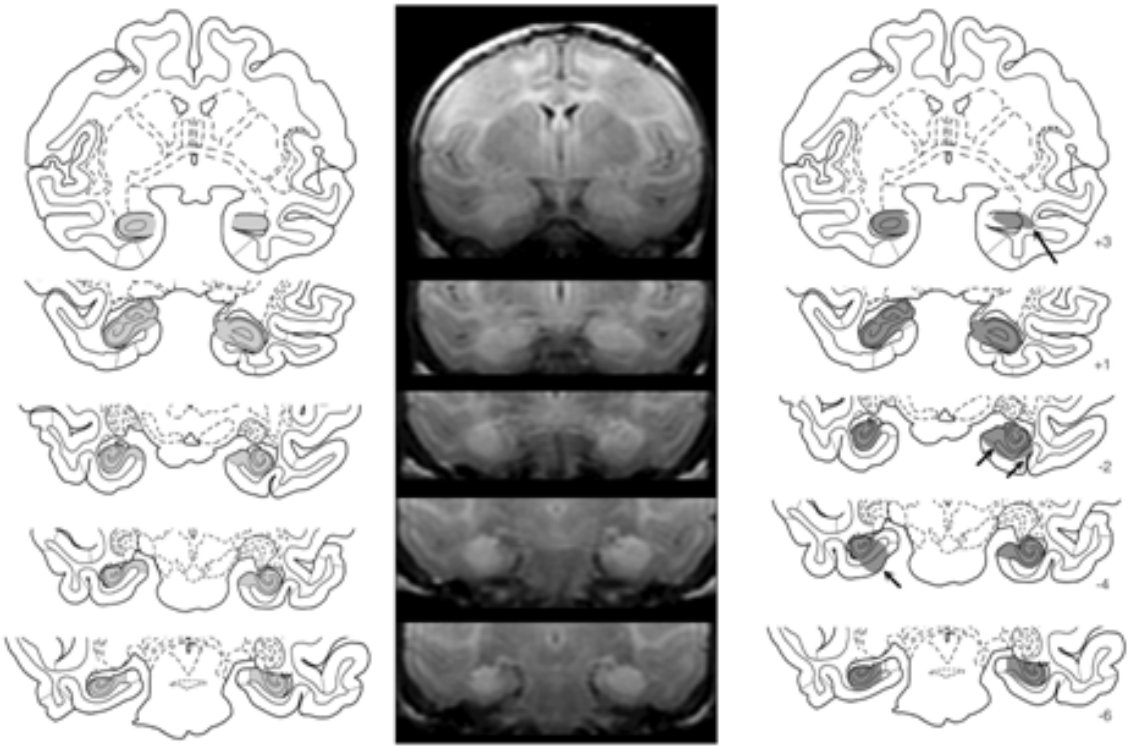
Table 3: Log-Transformed % Fear-Potentiated Startle

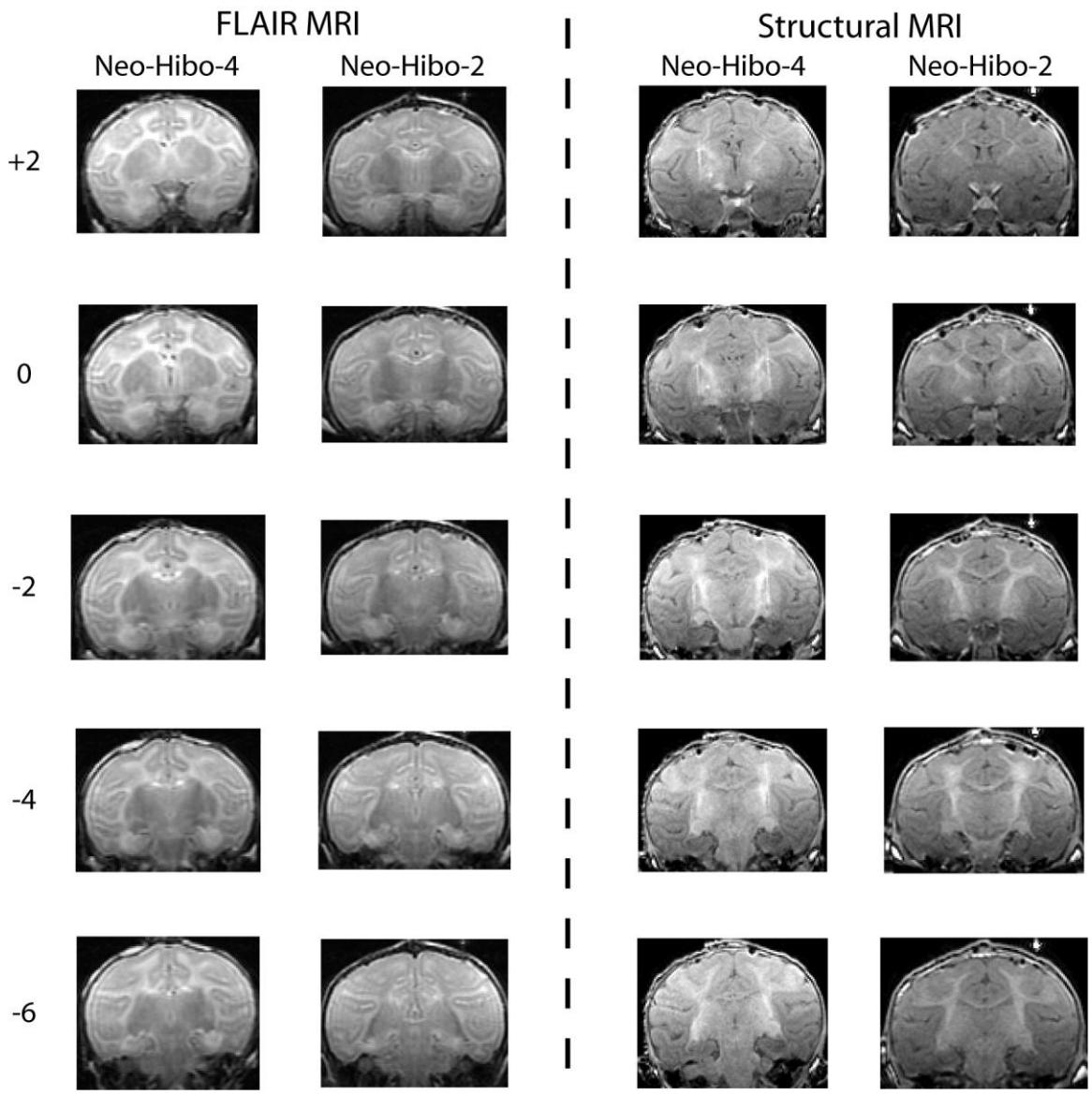
	A	B	AX	BX	AB
Group C					
Neo-C-1	3.35	2.07	3.57	2.35	1.90
Neo-C-2	Dropped				
Neo-C-3	2.00	1.48	1.77	1.27	1.85
Neo-C-4	3.58	2.46	3.80	2.51	3.54
Neo-C-5	2.56	1.64	1.36	1.23	2.04
Neo-C-6	Dropped				
X	**3.17**	**2.06**	**2.91**	**2.03**	**2.49**
Group A					
Neo-Aibo-1	2.74	2.20	2.96	2.42	2.54
Neo-Aibo-2	2.87	2.22	2.58	2.07	2.58
Neo-Aibo-3	2.79	1.58	2.53	1.52	2.69
Neo-Aibo-4	Failed				
Neo-Aibo-5	Failed				
Neo-Aibo-6	2.43	2.43	2.00	2.18	2.16
X	**2.71**	**2.11**	**2.52**	**2.05**	**2.49**

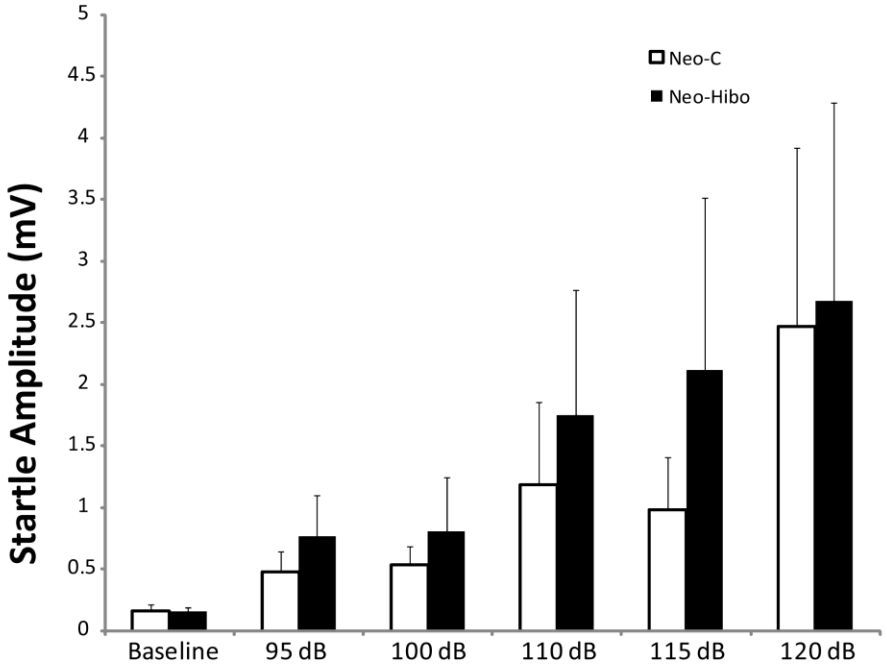
Data are the Log-Transformed %FPS amplitudes taken during the transfer test. Each individual score was obtained from the very first time the animal experienced that cue at the optimal decibel level (95dB or 120dB) for that particular animal. The X scores in bold are the group means per stage.

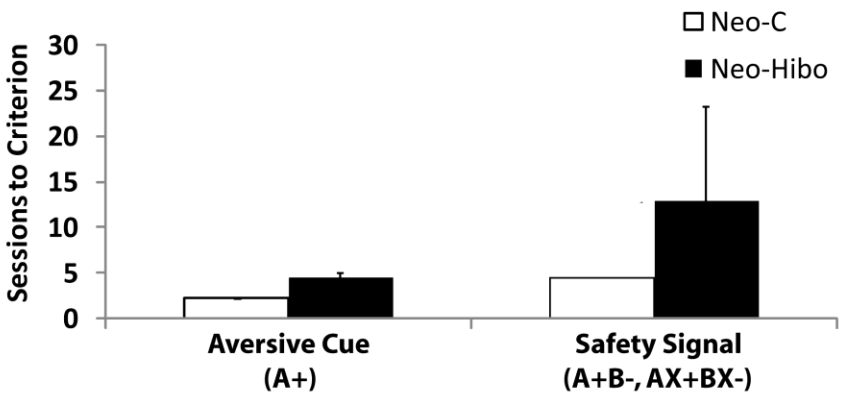
Neo-Hibo-3

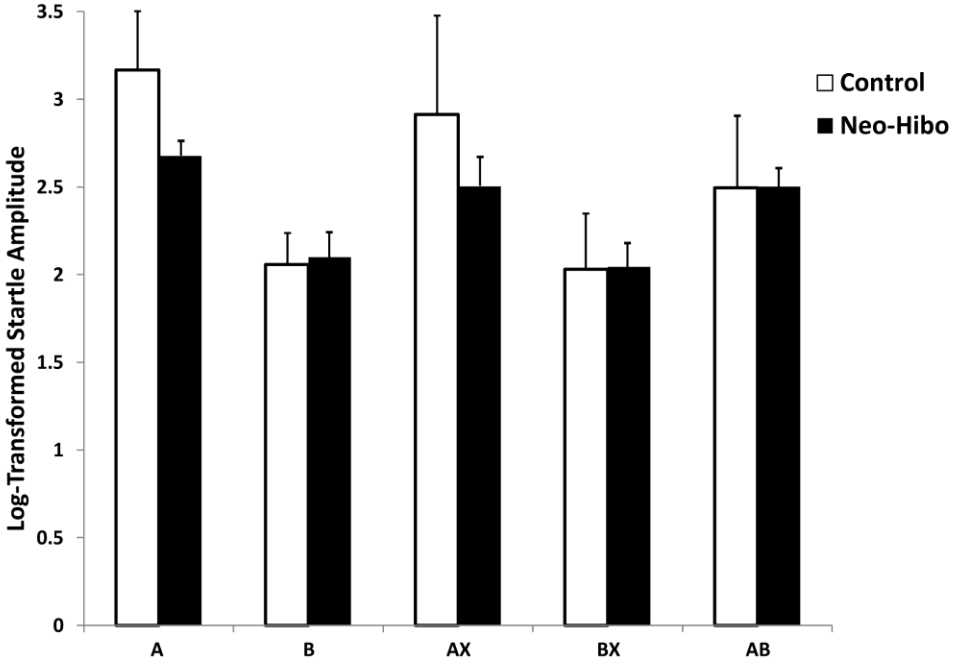
Intended Lesion Extent	1-Week Post Surgical FLAIR MRI	Reconstructed Lesion Extent




FLAIR MRI
Structural MRI
Neo-Hibo-4
Neo-Hibo-2
Neo-Hibo-4
Neo-Hibo-2
+2
0
-2
-4
-6


Startle Amplitude (mV)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Neo-C
Neo-Hibo
Baseline
95 dB
100 dB
110 dB
115 dB
120 dB


Neo-C
Neo-Hibo
Sessions to Criterion
30
25
20
15
10
5
0
Aversive Cue
(A+)
Safety Signal
(A+B-, AX+BX-)


Log-Transformed Startle Amplitude
3.5
3
2.5
2
1.5
1
0.5
0
A
B
AX
BX
AB
Control
Neo-Hibo

GENERAL DISCUSSION

The primary goal of this study was to investigate the neural basis of flexible fear learning and modulation using the non-human primate neonatal lesion model. Further understanding about the brain areas thought to be critical for fear learning, safety-signal learning, and the flexible inhibition of fear could greatly inform our understanding of anxiety disorders in humans. Currently, the amygdalocentric model of fear regulation is a predominant framework that has been used to study the neural basis of fear regulation and the consequences of malfunctioning of this brain system in many anxiety disorders, including PTSD (Rauch, Shin, & Phelps, 2006). In this model, the amygdala, which is known to have direct outputs to all physiological aspects of the fear response, is central for mounting a physiological response (Davis, 2000). It has then been proposed that structures outside the amygdala act upon it, via their direct and indirect connections, to suppress (or enhance) the excitation of the central nucleus of the amygdala when signals in the environment require modulation of the basic fear response (Rauch, Shin, & Phelps, 2006). Two structures, highlighted by recent research and thought to play this modulatory role, are the prefrontal cortex and the hippocampus. However, despite a growing number of human neuroimaging and clinical traumatic brain injury studies to support this view, no primate studies had selectively manipulated these structures to substantiate their critical contribution to safety-signal learning and modulation.

A primate model will clearly provide a significant translational model to study the brain structures involved in many anxiety disorders in humans for several reasons. First, lesion studies in humans suffer from selectivity in the brain areas damaged and fMRI studies are only correlational in nature. Second, while providing important findings, rodent studies suffer from lack of homology with brain structures in humans, especially when examining the involvement

of the prefrontal cortex in safety-signal learning and modulation. Finally, another important aspect for an adequate animal model to study the neurobiology of fear regulation is to consider the early ontogenetic origin of many anxiety disorders in humans, including PTSD. Thus, a more valid exploration of the amygdalocentric model of fear regulation in primates would be to use animals who received selective lesions in infancy. These were the main reasons that dictated the design of the experiments described in this thesis.

Using the AX+/BX- Fear-Potentiated Startle Paradigm, we predicted that, based on the current literature: a) animals with neonatal damage to the amygdala would be severely impaired during fear learning, and would not pass onto other discrimination problems, but, in the event that they eventually passed, that they would learn the safety signals normally. Additionally, they would have no difficulty either in down-regulating their fear in the presence of the safety-signal or in extinguishing fear responses when the aversive cue is not followed by the startle cue; b) animals with neonatal damage to the OFC would learn the initial fear and safety-signal cues, but will have difficulty using these signals to flexibly modulate their startle; and c) animals with neonatal hippocampal damage would have no difficulty in learning the initial fear, but would have difficulty during the conditioned inhibition phase, presumably due to deficits in contextual learning. As summarized in Table 1, the study provided several important findings, but not all consistent with our predictions.

First, adult monkeys given neonatal sham-operations learned all phases of the AX+/BX- paradigm in the minimum number of trials and thus provided additional support that performance on this paradigm is not affected by the nursery-rearing that these animals received as compared to the more naturalistic rearing that monkeys in the previous studies had received (Winslow et al., 2008; 2007; 2002). This could be important as there is preliminary data

suggesting that infant monkeys who were briefly separated and then reunited from their mothers develop abnormally, and have difficulty in learning to discriminate the fear/safety signal (Davis, personal communication).

Second, monkeys with neonatal lesions of the amygdala were retarded to fear the aversive cue, but given additional training sessions, they were able to learn it and to proceed with the other phases of the paradigm. Having learned to fear the aversive cue, they subsequently performed as well as control animals in learning the safety signal, in using the safety signal to modulate their fear of the aversive stimulus and in extinguishing their fear to the aversive stimuli when the startle cue stopped being presented with the aversive air-blast.

Third, unexpectedly, monkeys with neonatal lesions of areas 11 and 13 of the OFC performed normally in all phases of the paradigm, including fear/safety signal learning, modulation of fear or fear extinction, suggesting that these OFC areas may not be critical for down-regulating the amygdala during emotion regulation.

Finally, although two monkeys with neonatal hippocampal damage were severely impaired in their ability to learn either the fear or safety signals, four of the six animals with early hippocampal damage had no difficulty in fear/safety signal learning, modulation or extinction.

These results will be discussed in turn to point out how they can further inform the amygdalocentric model of fear regulation; how they may relate to the timing of the damage; and how they can enlighten the directions of future research.

Is the amygdala "central"

Complementing what has generally been found to be the case in the non-human primate literature, the data showed severe fear-learning impairments in adult monkeys that had received early amygdala damage. However, what would not have been predicted by the literature is the fact that all neonatally amygdalectomized animals were eventually able to learn to fear the aversive cue, despite receiving damage prior to fear training. Furthermore, the amygdalocentric model would not have predicted that safety signal processing would remain perfectly normal both in terms of learning and modulation; or that extinction would be unaffected. Rather, the amygdalocentric model would predict a general lack of fear, hence a lack of startle to the fearful cue. Although it could be argued that, in subjects with an intact amygdala, it is the amygdala that is central to the fear response, none of the earlier studies of fear learning in rodents and monkeys with amygdala lesions had trained the subjects more extensively to assess whether with additional training sessions the animals will eventually learn. Thus, although studies have revealed the electrophysiological and molecular components of fear acquisition in the amygdala and have shown that the amygdala is a key structure in fear acquisition, it is still possible that, in the absence of a functional amygdala, other structures may allow fear acquisition, though not as efficiently as the amygdala.

At the present time, it is difficult to conclude whether or not the age at which the amygdala damage occurred is a critical factor to allow the sparing of the ability to express fear-potentiated startle and further research will be needed to determine whether, as the neonatal amygdala lesions, amygdala lesions received in adulthood will result in a similar retardation in fear learning instead of a complete lack of it, and which other structures are able to carry this function. Currently, the bed nucleus of the stria terminalis is a strong candidate for taking over this function as it shares many connections with the amygdala, including reciprocal connections

with structures such as the hypothalamus and brainstem, and has been shown to play a role in the modulation of anxiety (for review see Davis, 2006).

Does the lateral OFC network down-regulate the fear response?

The data showed no evidence that the lateral network of the orbital frontal cortex (areas 11, 13, and insular cortex) plays a critical role in fear processing in this task. Thus, despite showing some subtle socioemotional behavioral abnormalities during dyadic interactions (Payne et al., 2007), animals with neonatal OFC lesions had no difficulty either learning the various cue associations, or using them in a flexible manner. In contrast, these animals displayed severe impairments in flexibly modulating their behaviors during appetitive association task (discussed below). Given the strong bi-directional communication between the lateral OFC network and the amygdala, these findings were surprising. However, they complement human neuroimaging data demonstrating that, during fear modulation, the strongest activation in the OFC occurs in the medial aspects of the prefrontal cortex rather than in the lateral OFC network (Quirk and Beer, 2006). In terms of the amygdalocentric model, we can now clarify the position that the lateral network is not the most critical component of the prefrontal cortex that is critical for flexibly modulating fear-learning, safety-signal processing, or extinction.

Does the hippocampus down-regulate the fear response?

With the exception of two animals that sustained inadvertent damage to both the dorsomedial and ventral striatum, the data showed that the hippocampus is not necessary for either fear/safety-signal learning, or the down-regulation of the fear response, either during conditioned inhibition or extinction. However, given the evidence from humans, rodents, and

monkeys that hippocampal dysfunction correlates with severe deficits in contextual learning abilities which may or may not be necessary for AX+/BX- learning, it is possible that the lack of impairment observed may relate to the timing of the lesions and will have to be further investigated.

The finding of severe learning deficits after combined damage to both the hippocampus as well as striatum, presents us with a question that cannot be answered within the scope of this study. As will be addressed below, because these animals seem to have normal hippocampal functions in terms of contextual recognition memory and modulation of emotional reactivity to threat signals, it is likely that the striatum may provide a primary mechanism for fear regulation.

Are these results due to the early lesion?

As with any damage, but particularly with brain damage occurring early in life, there is always the possibility for re-organization of brain circuitry leading to recovery of function. Thus, on the present results alone, it is still premature to conclude that the structures in question are normally involved in these processes. However, we do have extensive testing histories for all animals that do lend support to our determination of the likely-hood of compensatory mechanisms being set into motion. In addition, the varying developmental trajectories of each structure may help inform the most likely downstream effects of an early lesion.

For instance, of the three structures, the amygdala develops the earliest and is structurally well developed at birth, although protracted changes mostly in white matter have still been identified in primates (Payne et al., 2009for review see, Ulfig et al, 2003). Although there have been some noted metabolic consequences of early damage, such as hypo-activity in frontal cortices (Machado et al., 2008), as well as emergence of some stereotypies (Bauman et al.,

2008), in general, early lesions of the more adult-like amygdala might have similar consequences as compared to damage received in adulthood. Indeed, in the current study, we found that early amygdala damage resulted in fear-learning impairments, similar to those reported after adult amygdala damage (Antoniadis et al., 2007).

In contrast, the lateral network of the orbital frontal cortex is an extremely late developing structure and does not reach full functional maturity until approximately three years of age in monkeys (for review see Goldman-Rakic et al., 1997). Thus, one might predict a larger discrepancy between early versus late damage as compared to the amygdala. In fact, we have noted at least one instance of effect of timing of lesions in these animals on an appetitive task, which will be discussed in details below. In this particular case, early damage appears to have caused a developmental disruption, as animals with adult lesions to this same area have no learning impairments (Machado & Bachevalier, 2007). Thus, rather than functional compensation, early damage to the lateral network of the orbital frontal cortex results in a worsening of reward-association learning abilities. While we cannot rule out the possibility that this area normally plays an important role in the flexible modulation of fear, given that these animals were impaired to an even greater degree than animals with similar adult damage in reward-based learning and modulation, the sparing of fear regulation following the same lesions speaks rather in favor of the proposal that this orbital frontal network does not play an important role in the development of fear regulation.

Finally, the development of the hippocampus is somewhat protracted, although not to the extent of that of the orbital frontal cortex, showing functional maturity around 18 months (for review see, Seress, 2001; Payne et al., 2009; Lavenex et al., 2007). Early hippocampal damage may result in long-lasting deficits for certain tasks (incidental recognition memory, object and

spatial relational memory) both in human (Pascalis et al., 2004; 2009, Gadian et al., 2000), and non-human (Pascalis and Bachevalier, 1999; Killiany et al, 2005; Rehbein et al., 2005) primates, and rodents (Pereira et al., 2009; Marquis et al., 2008). However, in other instances, early damage to the hippocampus results in sparing, and even performance superior to controls (Mahut and Zola, 1973; Glavis-Bloom et al., 2008; Vargha-Khadem et al., 2003). Thus, it was unclear what the effects of early hippocampal damage might be for fear/safety-signal learning and the flexible modulation of fear. Animals with early hippocampal damage demonstrated normal fear/safety-signal learning, and normal modulation of emotion, which is consistent with other hippocampal-dependent tasks, such as those measuring contextual learning and memory abilities (Dore et al., 1998; Ridley et al., 2001). In conclusion, it is certainly plausible that the timing of the lesion may have resulted in sparing of function and that a different outcome will follow hippocampal damage received in adulthood.

Given that in the two hippocampal cases with unintended damage to the striatum fear learning and regulation was altered, it is relevant to discuss the potential effects of timing in relation to functional reorganization following striatal damage. Although very little is known about the ontogeny of the striatum, especially in primates, this structure appears to develop most closely to the amygdala. Both arise from the ganglionic eminence prenatally, and are fairly adult-like, at least structurally soon after birth (for review, see Jain et al., 2001). Thus, one might predict that similar to early amygdala damage, the effects of early striatal damage would not differ tremendously from striatal damage received in adulthood. While, it is difficult to make any strong conclusions with only two cases, the severity of the deficits seen in both cases does suggest little if any recovery of function, similar to damage received in adulthood (for review, see Da Cunha et al., 2008).

Differential effects of the amygdala and orbital frontal lesions on aversive versus appetitive learning and modulation.

We found that early damage to the amygdala resulted in severe fear learning impairments. In contrast, safety-signal and stimulus-reward association abilities remain perfectly intact as measured by either the Object Discrimination Reversal or Reinforcer Devaluation paradigm (Kazama, 2006; 2008). One interesting problem has yet to be solved regarding inconsistencies between the lesion model and neurophysiological studies. In some tasks (ex. reversal learning) lesions of the amygdala have no effect (Izquierdo & Murray, 2007; Kazama & Bachevalier, 2009), yet neurophysiological studies indicate that these same areas show differential neuronal activity (for review, see Salzeman, 2007) suggesting some kind of involvement. Thus, exactly how this non-critical neuronal activity is contributing to overall function during appetitive tasks is still unknown. Given that these same lesions affect some appetitive flexible decision-making, as measured by reinforcer devaluation (Izquierdo et al., 2004; Machado & Bachevalier, 2007), the amygdala, in conjunction with areas 11 & 13 of the OFC (discussed below) may be more important for higher-order appetitive processing based on changing internal states. Taken together, the primate amygdala appears to be highly central to basic fear learning, but not critical for simple appetitive learning.

In contrast to impairments in fear learning after early amygdala damage, we found that animals with damage to areas 11 & 13 of the OFC performed normally on the AX+/BX- Paradigm. This normal performance is somewhat unexpected, as flexible decision-making was severely impaired as measured by reinforcer devaluation, and thus, the sparing of function does not appear to be the result of early reorganization. Additionally, we found an effect of timing of

the lesion where early lesions disrupt abilities not affected by later lesions. While they show impaired flexible decision-making during the reinforcer devaluation task similar to the effects of adult lesions, animals with early damage were also severely impaired on learning large stimulus set (60-Pair) concurrent discrimination problems (Kazama et al., 2008).

The greater involvement of areas 11 and 13 in appetitive processing is consistent with neuroanatomical findings, indicating that lateral OFC receives more projections from the amygdala than it sends, whereas the ventromedial OFC send more projections to the amygdala than it receives (Barbas, 2007). Thus, ventromedial OFC may be in a better position to regulate amygdala activity under aversive conditions, and this information might then be sent to the lateral OFC for further higher-order processing. Taken together, it appears that other areas of the OFC may be more important for the flexible regulation of fear, while the lateral network may be more important for the flexible regulation of reward-seeking behaviors.

Where do we go from here?

As this is the first study to examine the neural basis of safety-signal learning and conditioned inhibition using non-human primates, it should certainly be considered just a starting point for further research. First, while we have established the effects of early damage to these three structures, there are several other areas of interest including the ventromedial prefrontal cortex, lateral prefrontal cortex, anterior cingulate cortex, and given the two strongly impaired hippocampal cases, the striatum. Second, although the early lesion model is arguably more clinically relevant for examining neurodevelopmental psychopathology, the use of temporary inactivation would be highly useful in answering questions raised by the current study regarding re-organization and recovery of function issues. Thus, the combined knowledge gained from both approaches would give us a more complete picture of the neural underpinnings of emotion

regulation. Third, understanding how safety signal learning and the flexible modulation of the fear response develop in normal animals could be highly relevant for clinical work, and could be used to identify critical windows within development where treatment/intervention would be most effective.

Currently, we are investigating other sub-regions of the orbital frontal cortex: ventromedial areas 14 and 25; lateral area 12; and middle areas 11, 13, and anterior insular cortex, using non-human primates with damage received in adulthood. Additionally, we will be investigating the normal development of these abilities using a modified version of the AX+/BX- Fear-Potentiated Startle Paradigm. Rather than using a light, tone, and fan as the various conditioned stimuli, the modified version relies on visual stimuli presented via a computer screen. Thus, we will be able to carry out repeated testing throughout the animal's development using many different visual stimuli.

Conclusions

In summary, the goal of the current study was to investigate the neural basis of fear/safety-signal learning and the flexible modulation using an early lesion model in non-human primates. Based on the results, we conclude that while the amygdala is important to fear learning, other areas are able to carry this function, albeit far less efficiently. Additionally, the amygdala does not appear to be critical for the flexible modulation of fear either in conditioned inhibition nor extinction. We also conclude that the lateral network of the orbital frontal cortex does not appear to be critical for fear processing, which suggests that the strong bi-directional connections between this area and the amygdala are used primarily for appetitive processes. Finally, results from the hippocampal study suggest that while early damage does not result in

fear processing impairments, it is possible that intact fear/safety signal learning could be due to re-organization. Thus, more studies need to be completed to determine if this effect would be different in animals with damage received in adulthood.

Table 1: Summary of performance

Group	Fear Learning	Safety-Signal Learning	Fear Modulation	Extinction
Neo-C	+	+	+	+
Neo-Aibo	Impaired	+	+	+
Neo-Oasp	+	+	+	+
Neo-Hibo (4)	+	+	+	+
Neo-Hibo/Striatal (2)	+/Impaired	Impaired	-	-

Summary of performance across the various phases of the AX+/BX- Paradigm. Plus signs (+) indicate normal performance and minus signs (-) indicate animals did not reach criterion on the previous phase and thus did not advance. For descriptions of Fear Learning (A+ Training), Safety-Signal Learning (A+B-, AX+BX- Training), Fear Modulation (AB Transfer Test), and Extinction (A-/AX- training) see the methods sections.

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