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Effects of Neonatal Amygdala Lesions on Fear Learning, Conditioned Inhibition, and Extinction in Adult Macaques

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Abstract

Fear conditioning studies have demonstrated the critical role played by the amygdala in emotion processing. Although all lesion studies until now investigated the effect of adult-onset damage on fear conditioning, the current study assessed fear-learning abilities, as measured by fear-potentiated startle, in adult monkeys that had received neonatal neurotoxic amygdala damage or sham-operations. After fear acquisition, their abilities to learn and use a safety cue to modulate their fear to the conditioned cue, and, finally, to extinguish their response to the fear conditioned cue were measured with the AX+/BX– Paradigm. Neonatal amygdala damage retarded, but did not completely abolish, the acquisition of a learned fear. After acquisition of the fear signal, four of the six animals with neonatal amygdala lesions discriminated between the fear and safety cues and were also able to use the safety signal to reduce the potentiated-startle response and to extinguish the fear response when the air-blast was absent. In conclusion, the present results support the critical contribution of the amygdala during the early phases of fear conditioning that leads to quick, robust responses to potentially threatening stimuli, a highly adaptive process across all species and likely to be present in early infancy. The neonatal amygdala lesions also indicated the presence of amygdala-independent alternate pathways that are capable to support fear learning in the absence of a functional amygdala. This parallel processing of fear responses within these alternate pathways was also sufficient to support the ability to flexibly modulate the magnitude of the fear responses.

Keywords

safety-signal learning; post-traumatic stress disorder; emotion regulation; rhesus monkey

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, Winslow, Davis, & Amaral, 2007) has used a fear-potentiated startle paradigm closely modeled after rodent studies (Winslow, Parr, & Davis, 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 continued to express high fear-potentiated

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startle. Thus, in primates, the amygdala plays a critical role in the acquisition of fear, but fear memory and its expression can be supported by other brain structures. 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 Sehlmeier, Schoning, Zwitterlood, Pfleiderer, Kircher, & Arolt, 2009). Robust amygdala activation was noted during the early stages of fear conditioning, but this activation decreased as conditioning proceeded longer. Interestingly, with longer time, the brain activation shifted from the amygdala to other structures, such as the anterior cingulate and insular cortices (Buchel, Morris, Dolan, & Friston, 1998; Everitt & Robbins, 2005; LaBar & Disterhoft, 1998), suggesting that the amygdala may be playing a key role only during early stages of fear acquisition.

The role of the amygdala in fear learning is well established across all species, but its contribution to the development of fear learning in early infancy is less understood. Although children typically have caregivers to help them guide their actions, they must learn to navigate emotional situations and to eventually make decisions on the relative safety or danger. Thus, the amygdala may be required early in life to support this learning. In addition, many neuropathological disorders in humans, such as anxiety, depression, and posttraumatic stress disorder, have a strong developmental component and are associated with dysfunction of the amygdala (for review, see Gillespie, Phifer, Bradley, Ressler, 2009; Machado & Bachevalier, 2003; Monk, 2008). Thus, information on the effects of early amygdala dysfunction in fear learning is clearly warranted.

Neonatal damage to the amygdala in rodents alter locomotor activity, social behaviors, stress-induced behaviors, prepulse inhibition, and acoustic startle response (Daenen, Van der Heyden, Kruse, Wolterink, & Van Ree, 2001; Daenen, Wolterink, Gerrits, & Van Ree, 2002a, 2002b; Daenen, Wolterink, Van Der Heyden, Kruse, & Van Ree, 2003; Wolterink et al., 2001). These changes were also associated with decreased cerebral glucose utilization later in life (Gerrits, Wolterink, van Ree, 2006). Yet, the effects of neonatal amygdala damage on simple fear learning have yet to be examined. Thus, in the present study, adult monkeys with neonatal amygdala damage and their age-matched controls were trained to associate a conditioned cue with an aversive, but painless, puff of air, using the fear-potentiated startle developed for nonhuman primates (Antoniadis et al., 2007; Winslow et al., 2002). Given that, similar to amygdala damage acquired in adulthood (Machado & Bachevalier, 2007, 2008), neonatal amygdala damage altered the ability to flexibly modulate behavioral responses in an appetitive task (Kazama, O'Malley, & Bachevalier, 2007) and blunted emotional reactivity to fearful stimuli (Raper, Kazama, & Bachevalier, 2009), we predicted that neonatal amygdala lesions would also impair 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, Noble, & Davis, 2008). Preliminary data of this study have already been published (Jovanovic, Kazama, Bachevalier, & Davis, 2010; Kazama, Heuer, Davis, & Bachevalier, 2010).

Method

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 surrogate-nursery-reared (see Goursaud & Bachevalier, 2007 for details)

with daily contact with a human caregiver and peers until young adulthood. They were then separated into single cages that allowed visual exploration but limited physical contact among individuals. Animals received brain surgeries between 8 and 12 days of age that included sham-operations (Group Neo-C, three males and three females) and neurotoxic lesions of the amygdala (Group Neo-Aibo, three males and three females). After 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 (Goursaud & Bachevalier, 2007; Nemanic, Alvarado, Price, Jackson, & Bachevalier, 2002) and will be briefly summarized below.

Pre- and Post-Surgical MRI Scans

Just before surgery, animals were anesthetized with isoflurane gas (1–2% to effect), intubated with an endotracheal canulae to maintain sedation, and secured in a stereotaxic nonferromagnetic 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 through-out 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. These neuroimaging procedures were repeated 7–10 days after surgery for the experimental animals only, and MR images were used to estimate lesion extent.

The T-1 images were used to precisely select and calculate coordinates of neurotoxin injection sites within the amygdala in animals of Group Neo-Aibo, using procedures described earlier (Málková, Lex, Mishkin, & Saunders, 2001; Nemanic et al., 2002; Saunders, Aigner, & Frank, 1990). The postsurgical MR images were used to visualize the location and extent of hypersignals resulting from edema.

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. Small bone openings were 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, four to six injection sites spaced 2 mm apart in the Medial/Lateral and Dorsal/Ventral directions were selected from the presurgical MR images and centered within the amygdala to include all amygdaloid nuclei while sparing the adjacent cortical areas. Injections of the neurotoxin ibotenic acid (Biosearch Technologies, Novato, CA) were made simultaneously through two 10- μ l 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 μ l of ibotenic acid (10 mg/ml in phosphate buffered saline, pH 7.4) was injected (0.2 μ l/minute) at each site. The needles were remained in place for an additional 3 minutes to allow diffusion of the drug before being retracted. For all animals, 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 postsurgical 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 (10 mg/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 presurgical MR images. Extent of hypersignals on FLAIR images (indicative of brain edema) were transposed onto drawings of coronal sections from a normal 2-week-old infant rhesus monkey atlas (J. Bachevalier, unpublished atlas) matched to the MR images. For each brain area, estimated volume of edema was measured on each drawn coronal section using Image J, and percent of estimated volume damage for each brain area was then calculated (see for details, Nemanic et al., 2002).

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 have previously been described (Antoniadis et al., 2007; 2009; Winslow, Noble, & Davis, 2008; Winslow, Parr, & Davis, 2002) and will be briefly summarized below. Table 1 lists the different stages of training in chronological order as well as the cues and startle noise intensity used at each stage.

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 conditional stimulus (CS) was a 4-s light produced by four overhead 20-W 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 pseudorandom and counterbalanced across groups. Thus, some animals received the light as the aversive CS, whereas others received the tone as aversive CS, and so on.

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, and 120 dB; 10 trials each). All trials were pseudorandomly intermixed throughout each session. Animals were then tested for prepulse inhibition before moving on to the AX+/BX– paradigm and data on prepulse inhibition will be published elsewhere.

Pretraining—Before 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 five trials each pseudorandomly ordered. Within each of the cue-startle trials, the startle stimulus was presented 4 sec after the onset of the CS. These pretraining sessions were repeated for each monkey until presentation of the safety signal (cue B) for that animal reduced the startle amplitude to less than 30% of startle amplitude obtained during 95 dB 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 pseudorandomly 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 four trial-types (that is, Noise Alone at 95 dB, Noise Alone at 120 dB, Cue A+ with 95 dB Noise, Cue A with 120 dB Noise) and were presented pseudorandomly six trials 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 pretraining startle to cue A+. The %FPS was defined as: $[\text{Mean startle amplitude on CS test trials} - \text{Mean startle amplitude on startle noise alone test trials}] / \text{Mean startle amplitude on startle noise alone test trials} \times 100$. For example, if during pretraining an animal had a mean startle amplitude of 5 mV in the presence of the A+ cue, and a baseline startle amplitude of 5 mV in the noise alone condition, then during the A+ Training Phase that animal would reach the criterion of 100% FPS when its startle exceeded 10 mV in the presence of the A+ Cue.

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 the following: 12 trials in which the safety cue (B) was presented with both startle noise intensities (95 dB and 120 dB, six 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), 12 trials in which cue A was paired with the startle noise (95 dB and 120 dB, six trials each), and 12 trials of startle noise alone (95 dB and 120 dB, six 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. For example, if the animal startled 0% FPS to the B– Cue, it would need to achieve a % FPS greater than 100 to reach criterion in the A+/B– Phase.

AX+/BX– training phase—Previous conditioned inhibition training in primates had shown 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 fear (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, six trials each), (b) 95 dB and 120 dB cue pairings (A, B, AX, BX, five trials each per noise intensity), and (c) 95 dB and 120 dB AB compound cue (five trials per noise intensity). All trials were pseudorandomly intermixed. Because the AB compound cue presentations were never paired with air-blasts and could be interpreted as a safety-signal by the animal on future presentations of that trial type, calculations of %FPS for each cue type were based only on the very first trial the animal experienced the AB compound cue.

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 (four trials of each type) to evaluate fear extinction. Training was completed when the animal returned to its pretraining 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 declined steadily 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, a Geisser-Greenhouse corrected repeated measures ANOVA compared 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 the control animals learned the task in the minimum of two sessions per phase, resulting in no variations in the group, non-parametric statistics were used to investigate group differences (Mann–Whitney *U*). Third, because previous reports (Winslow et al., 2008) indicated that startle values are not normally distributed, the transfer test data were transformed using a logarithmic base 10, and group comparisons were made with repeated measures ANOVAs.

Results

Lesion Extent

Assessment of extent damage via postsurgical MR images revealed extensive damage in all cases, averaging 53.2% in the left hemisphere and 71.8% in the right hemisphere (see Table 2, and Figure 1 for two representative cases). Although the extent of damage varied from case to case, it always included the central, medial, accessory basal, and dorsal areas of the basal nuclei but spared the ventral portion of the amygdala. Thus, in three cases (Neo-Aibo –1, –4, and –6), the damage was substantial and symmetrical, whereas in the remaining three cases (Neo-Aibo –2, –3, and –5), there was 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 was negligible for all cases, except for slight unilateral damage to the ventral aspect of the tail of the putamen in cases Neo-Aibo-1, –4, and –5 (see Figure 1).

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 neonatal 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 3. 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 with two 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 with 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 began at the fourth session, and animals of Group Neo-Aibo reached criterion by Session 6. Although sham-operated controls achieved a higher %FPS on their last day of training relative to animals with neonatal amygdala lesions, a group comparison of performance at their respective final training session did not reach significance ($t = 1.45$, $p > .05$).

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 3). Although animals with neonatal amygdala lesions required more sessions (average: 10.5) than controls (average: 4), this difference did not reach significance (Mann–Whitney U , $p > .05$). However, as shown in Table 3, 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+) for which Neo-Aibo-1 and Neo-Aibo-4 scored -72% and 7.1% FPS, respectively, despite their reinforcement, 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 4 and Figure 4, 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 with 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 3. 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.

Raw Baseline Startle Amplitude Across Stages

Finally, given that baseline startle amplitude measure was the basis of all prior analyses, a Group X Stage repeated measures ANOVA with a Greenhouse-Geisser correction was used to examine any potential changes in raw baseline startle across training stages. As seen in Table 5, the results revealed no significant change in baseline startle across either groups, $F(1, 6) = 1.60$, $p > .05$, or stages, $F(1.89, 11.33) = .146$, $p > .05$, and no interaction, $F(1.89, 11.33) = 2.067$, $p > .05$. Thus, although raw baseline startle amplitudes varied widely across individuals, there were no group differences in any of the stages of training.

Discussion

The 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, only the two animals with the most extended amygdala damage could not discriminate a fear signal from a safety one. The four remaining animals not only did discriminate between the fear and safety cues, but they were also able to use the safety signal to modulate and reduce the fear response and to extinguish the startle fear response when the air-blast was absent.

Baseline Acoustic Startle

Neonatal amygdala damage resulted in normal baseline acoustic startle, and, for both groups, baseline acoustic startles were stable across all stages of training. Thus, both groups showed increased startle in response to increased intensity, although the increase in startle responses in animals with neonatal amygdala lesions were slightly, but not significantly, greater in magnitude. This slight increase was mostly attributable to larger individual variability in startle amplitude after neonatal amygdala lesions. This variability, however, cannot be explained by extent of the amygdala lesions because 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 (PnC) (for review see Davis, 2006). The overall slight increase in acoustic startle after neonatal amygdala lesions in monkeys is reminiscent of that reported earlier after adult-onset amygdala lesions (Antoniadis et al., 2007). However, in this later study, the increase in acoustic startle after amygdala lesions reached significance; this finding was not replicated in a second study by the same authors (Antoniadis et al., 2009). Thus, altogether the findings suggest that, as in rodents, damage to the amygdala does not significantly alter acoustic startle.

Fear Learning

Neonatal damage to the amygdala did retard the acquisition of fear-potentiated startle but did not totally abolish this ability. Thus, only one animal in Group Neo-Aibo acquired the initial fear signal in two 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) because both studies demonstrated that fear conditioning is severely affected during the first phases of learning. However, the data further demonstrate that with additional pairing of the conditioned stimulus with the aversive stimulus, animals with neonatal amygdala lesions can eventually learn to fear the conditioned cue. Although the different outcomes of the two studies could be explained by several factors, such as timing of amygdala insult, which could have resulted in significant functional sparing after neonatal lesions, and lesion extent, which was larger in the Antoniadis and colleagues study (2007) as compared with the current one, the pattern of results in the current study rather suggests the existence of amygdala-independent alternate pathways for fear potentiated startle. This conclusion is supported by recent studies in rodents and humans.

Using a temporary inactivation of the basolateral nucleus of the amygdala (BLA), Ponnusamy and colleagues (Ponnusamy, Poulos, & Fanselow, 2007) showed that rats could acquire a context-specific long-term fear memory without the BLA provided that intensive overtraining was given, suggesting that alternate neural pathway could compensate for the absence of a functional amygdala. These researchers further demonstrated that the bed nucleus of the stria terminalis was a strong candidate for such alternate pathway (Poulos, Ponnusamy, Dong, & Fanselow, 2010). Similarly, 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 Sehlmeier 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; Knight, Smith, Cheng, Stein, & Helmstetter, 2004; LaBar & Disterhoft, 1998; 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. One interpretation of these data is that the acquisition of conditioned fear responses by the amygdala is time-sensitive and that fear response signals are then processed by other structures that support their long-term storage. Alternatively, the results could also suggest parallel, as opposed to, serial processing of the fear signals. Thus, amygdala-independent alternate pathways may be processing emotional valence in parallel but simply require longer time to form the stimulus-fear association as alluded to by others (Antoniadis et al., 2009; Ponnusamy et al., 2007; Poulos et al., 2010). 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; Everitt & Robbins, 2005; LaBar et al., 1998). 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 (Poulos et al., 2010). The orbital frontal cortex has also been proposed as a likely candidate structure (Antoniadis et al., 2009). Thus, these alternate routes could enable the animals to slowly acquire fear conditioning in the absence of a functional amygdala.

Finally, acoustic startle is only one component of the emotional response, and there may be other physiological components that could be altered after early amygdala damage. For example, in a recent study by Man and colleagues (2011), serotonergic lesions of the primate amygdala received late in life failed to disrupt appetitive Pavlovian learning but did retard the ability to generate changes in heart rate. Similar findings were also found in animals of the current study. Thus, measures of emotional reactivity and HPA axis regulation using the Human Intruder paradigm as well as basal diurnal cortisol rhythm were also collected in the animals of the current study. Consistent with the study of Man and colleagues (2011), despite their relative normal learning ability in the AX-/BX + paradigm, animals with neonatal amygdala damage emitted less freezing in the presence of the human intruder associated with blunted cortisol responses as compared with sham-operated controls; they also had a blunted basal diurnal cortisol rhythm (Raper et al., submitted).

Safety Signal Learning

The results showed that two animals with the largest amygdala lesions did not learn to discriminate between the aversive and safety cues. This lack of learning was mostly attributable to an extinction of the fear response to the aversive cue overtime, resulting in a low startle to both the aversive and safety cues. Thus, in these two animals, the addition of the safety cue in the training session blunted animals' reactivity to the aversive cue. One interpretation that cannot be discounted is that the smaller lesion size in the four animals that did learn the safety signal could have allowed for reorganization within the amygdala neural circuitry yielding sparing of function. However, there are two points that may discount this interpretation. First, based on the rodent literature, the central nucleus (Davis, 1992) or the intercalated cells (Likhtik et al., 2008) within the amygdala appear to be the key nuclei supporting fear expression and extinction. Yet all animals with neonatal amygdala lesions had extensive damage to these two areas. Second, even in the remaining four animals with neonatal amygdala lesions, which showed discrimination between the aversive and safety signals, there was a qualitative difference in how these animals learned this discrimination as compared with control animals. Typically, at the beginning of the safety learning phase, control animals reacted fearfully to the addition of the safety cue, but quickly learned that the safety cue was never followed by an air blast and thus extinguished their startle to the safety cue while maintaining a potentiated startle for the aversive cue. In contrast, amygdala-operated animals showed very little initial startle to the safety cue to begin with. Thus, even in these four animals, it is not yet clear whether they had learned anything about the safety signal because they did not respond to it 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 demonstrating an inherent and generalized lack of fear. This lack of fear in all amygdala cases is in fact a hallmark symptom of amygdala damage that has been noted in many species including humans (Adolphs, Tranel, & Damasio, 1998; Bechara et al., 1995; Tranel,

Gullickson, Koch, & Adolphs, 2006) and have been reported even when the amygdala damage occurs in infancy (Prather et al., 2001). Nevertheless, as discussed below, the transfer probe test demonstrated that indeed the four animals with neonatal amygdala lesions had learned the meaning of the safety cue because they were able to use it to modulate their fear reactivity to the aversive cue.

While there is much evidence suggesting that aversive associations are guided by the amygdala, basic appetitive associations may be striatal dependent. For instance, in a recent human neuroimaging study, Schiller and colleagues (Schiller, Levy, Niv, Le-Doux, & Phelps, 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, albeit unilateral, damage to ventral aspects of the striatum that could have affected the learning of the safety cue. Nevertheless, this additional striatal damage may not be the source of their lack of acquisition of the safety cue, because these two animals together with the four other animals of Group Neo-Aibo demonstrated normal performance in stimulus-reward associations tasks known to be mediated by the striatum, such as Object Discrimination task (Kazama & Bachevalier, 2012) and Concurrent Object Discrimination task (Kazama, Glavis-Bloom, & Bachevalier, 2008; Kazama, O'Malley, & Bachevalier, 2007).

Finally, a direct comparison of the effects of neonatal amygdala lesions with those of adult-onset lesions is impossible at the current time given that in both rodents and monkeys with adult-onset amygdala lesions, fear conditioning was totally abolished and animals could not be tested in safety signal learning or fear extinction. Thus, future studies assessing whether animals with adult-onset amygdala lesions could learn to fear with additional training are required to directly compare the effects of early onset versus adult-onset amygdala lesions.

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 reacquired fearful stimuli. Thus, conditioned inhibition may be a process independent of amygdala functioning. This conclusion questions a major, as yet 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). The amygdalocentric model holds that brain areas other than the amygdala, such as the hippocampus and prefrontal cortices, modulate fear responses stored in the amygdala. Although this view is supported by virtually all relevant human neuroimaging data reported to date (for reviews see Sehlmeier et al., 2009; Shin, Rauch, & Pitman, 2006), the lesion studies in monkeys provide a different interpretation. First, the amygdala is required for the acquisition of fear-potentiated startle (Antoniadis et al., 2007; present study), but not for the memory and expression of conditioned fear (Antoniadis et al., 2009). These data suggest that the amygdala has a time-limited role in fear learning and that memory of conditioning and the expression of the anticipatory fear leading to enhanced startle in the fear-potentiated startle paradigm can be mediated by other brain regions, such as the orbital frontal cortex (Kalin, Shelton, & Davidson, 2007; Laviolette, Lipski, & Grace, 2005; Machado, Kazama, & Bachevalier, 2009), insular cortex, and the bed nucleus of the stria terminalis (Ponnusamy et al., 2007; Poulos et al., 2010; Christianson et al., 2011). 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 on the memory of the fear responses via connections to other areas offering alternate routes to the primary amygdala-dependent startle pathway.

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 & Disterhoft, 1998; LaBar et al., 1998) and electrophysiological data in rodents (Quirk, Armony, & LeDoux, 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 relearning 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 during extinction is more likely representing an arousing effect of the aversive cue rather than a relearning 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; Kalisch, Korenfeld, Weiskopf, Seymour, & Dolan, 2006; Milad, Wright, Orr, Pitman, Quirk, & Rauch, 2007) and rodent models (Quirk, Likhtik, Pelletier, & Pare, 2003) have indicated that the medial prefrontal cortex and/or hippocampus may be critical for extinction. Thus, additional studies are required to more directly explore the critical brain areas involved in the extinction of learned fear.

Conclusions

In conclusion, the present results support the critical contribution of the amygdala during the early phases of fear conditioning that leads to quick, robust responses to potentially threatening stimuli, a highly adaptive process across all species and likely to be present in early infancy. The data also indicated the presence of amygdala-independent alternate pathways that are capable to support fear learning in the absence of a functional amygdala. This parallel processing of fear responses within these alternate pathways was sufficient to further support the ability to flexibly modulate the magnitude of the fear responses.

More generally, studies examining the neural circuitry subserving the expression and regulation of fear in nonhuman animals provide a foundation for understanding the neuroanatomical and neuropathological correlates of human mood and anxiety disorders such as PTSD. Findings of such experimental studies may lead to novel avenues to refine both cognitive and pharmacological treatments of anxiety. We have recently validated a repeated-measures version of the AX+/BX- paradigm (Kazama, Schauder, Davis, & Bachevalier, 2011) that will allow for within subjects design for pharmacological manipulations. Additionally, given that human studies suggest that PTSD may have a developmental component, this new version of the paradigm will permit longitudinal developmental study of fear conditioning and conditioned inhibition and of its neural substrate in nonhuman primates.

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Figure 1.

Intended lesion (left column) and extent of amygdala damage in two representative cases with the *least* amount of damage (Case Neo-Aibo-5, middle column) and the *greatest* amount of damage (Case Neo-Aibo-4, right column). Intended damage is shown in gray on coronal sections through the anterior-posterior extent of the amygdala of a normal infant macaque brain atlas (left column) and for the two representative cases. The positive numbers on the left side of each section indicate the distance (mm) from the interaural plan. Asterisks point to areas of slight unintended damage to the ventral striatum and the hippocampus on the left (see levels +3 to +5). Arrows indicate slight sparing of tissue within the ventral portion of the amygdala. Abbreviations: A – amygdala; ERh – entorhinal cortex; PRh – perirhinal cortex; TE, temporal cortical area – cytoarchitectonic fields of the temporal lobe as defined by von Bonin and Bailey (1947).

Figure 2.

Mean ($\pm$ 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, open circles) and for animals with neonatal amygdala lesions (Neo-Aibo; solid squares). The horizontal dotted line represents criterion of 100% FPS. Asterisk indicates $p = .05$ and # indicates $p = .08$.

Figure 4.

Mean ($\pm$ 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 $p < .05$).

Table 1**AX+/BX– Task Description**

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

Note. Chronological list of training stages as they occurred behavioral testing training (top to bottom). For each stage, the types of cues (light, tone, fan/A, B, X) presented and the Startle Noise (decibel of the 0.05-sec startle noise) used are given.

Table 2

Extent of Intended and Unintended Damage in Group A-Ibo

Cases	Anygdala			Hippocampal formation		
	L	R	Avg	L	R	Avg
Neo-Aibo-1	89	59.8	74.4	53.2	5.1	3.1
Neo-Aibo-2	42	77.6	59.8	32.6	0	0.8
Neo-Aibo-3	33	61.1	47.1	20.2	0	0
Neo-Aibo-4	62.1	90	76	55.9	1.9	3
Neo-Aibo-5	41.2	66.6	53.9	27.5	0	0
Neo-Aibo-6	52.1	75.6	63.8	39.3	5.6	10.3
X	53.2	71.8	62.5	38.1	2.1	2.9

Note. Scores 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 = $(L \times R)/100$ [weighted index as defined by Hodos and Bobko (1984)]; X = group mean.

Table 3

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

Note. Scores are 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. Neo-C = animals with sham operations; Neo-Aibo = animals with neonatal neurotoxic amygdala lesions. X designates group means per stage.

Table 4

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

Note. Scores are the log-transformed %FPS amplitudes taken during the probe transfer test. Although the animals experienced multiple trials of each cue type, each individual startle measure was calculated *only* using the startle of the very first time the animal experienced that cue. Additionally, results are calculated based on each individual animal's optimal decibel level (i.e. whether they had greater %FPS when startled at 95 dB or 120 dB). Conventions as in Table 2.

Table 5

Baseline Startle at 95 DB Noise Across Stages

Groups	A+	A+B-	AX+ BX-	AX+/BX- transfer test	Extinction
Neo-C-1	.26	.20	.31	.54	.43
Neo-C-3	.85	.84	1.06	1.52	1.96
Neo-C-4	7.6	.49	.38	.17	1.58
Neo-C-5	.59	1.08	.55	1.63	.69
X	2.33	.65	.58	.97	1.17
Neo-Aibo-1	.33	.62	—	—	—
Neo-Aibo-2	7.03	14.12	14.43	14.15	14.24
Neo-Aibo-3	1.74	4.71	2.43	.85	.62
Neo-Aibo-4	.54	—	—	—	—
Neo-Aibo-5	2.77	3.65	3.97	2.67	4.93
Neo-Aibo-6	.28	.44	.28	.27	.21
X	2.12	4.71	5.28	4.49	5

Note. Scores are median raw startle amplitudes in mV to the 95-dB for criterion session at each stage, except for the transfer test, for which the startle amplitude was taken from the first 95-dB Noise Alone trial.