

# Role of the Primate Amygdala in Fear-Potentiated Startle: Effects of Chronic Lesions in the Rhesus Monkey

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In experiment 1, we assessed the role of the primate amygdala and hippocampus in the acquisition of learned fear measured with fear-potentiated startle. Three groups of six rhesus monkeys were prepared with bilateral ibotenic acid lesions of the amygdaloid complex and the hippocampus or were sham operated. Selective ibotenic acid lesions of the amygdala, but not the hippocampus, blocked the acquisition of fear-potentiated startle. In experiment 2, we assessed the role of the primate amygdala in the expression of fear-potentiated startle. Surprisingly, animals that sustained amygdala damage after they successfully learned fear-potentiated startle expressed normal fear-potentiated startle, despite a complete amygdala lesion based on magnetic resonance imaging assessments. These results suggest that although the amygdala is necessary for the initial acquisition of fear-potentiated startle, it is not necessary for the retention and expression of fear-potentiated startle. These findings are discussed in relation to the role of the amygdala in emotional learning and in cross-species comparisons of emotional behavior.

**Key words:** primate; amygdala; fear-potentiated startle; fear conditioning; rhesus monkey; emotional learning; learning and memory

## Introduction

Fear-potentiated startle is a conditioning paradigm widely used in rodents to delineate neural substrates for the acquisition and expression of learned fear (Davis, 1992). The model capitalizes on the fact that there are a number of withdrawal or escape reflexes, such as the acoustically elicited eyeblink and whole-body startle reflex, which are increased in amplitude during a state of anticipatory fear. Substantial evidence indicates that the amygdala is pivotal in the acquisition and expression of conditioned fear (Kapp et al., 1992; Fanselow and LeDoux, 1999; LeDoux, 2000; Maren, 2001). Rats with bilateral damage to the amygdala fail to acquire fear-potentiated startle even if the damage is confined to the central (Kim and Davis, 1993) or basolateral (Sananes and Davis, 1992; Campeau and Davis, 1995) nuclei. Fear-potentiated startle performance that is reliably elicited in intact rats is abolished by an amygdala lesion, even if the lesion is restricted to the central (Hitchcock and Davis, 1986, 1987) or basolateral (Sananes and Davis, 1992; Campeau and Davis, 1995) nuclei.

As interesting as these studies in the rodent are, it is not clear

to what extent they can be extrapolated to human subjects. Studies on the neurobiology of primate emotion have also implicated the amygdala in the processing of emotional information and the mediation of fear responses (Aggleton and Passingham, 1981; Zola-Morgan et al., 1991; Amaral et al., 1992; Kalin et al., 2001; Prather et al., 2001; Mason et al., 2006). Although several studies implicate the macaque monkey amygdala in the processing of species-specific innate fears, they do not indicate whether the primate amygdala is also specialized for learning and expressing conditioned-fear behaviors. There is currently no published study that investigates the role of the primate amygdala in fear conditioning similar to the studies that have been performed in the rodent. Thus, the goal of the current study was to evaluate the role of the non-human primate amygdala in the acquisition and expression of fear-potentiated startle. We have chosen to conduct these studies in the rhesus monkey because of the wealth of information that is available on the neuroanatomy of the amygdaloid complex and on both the behavioral and electrophysiological analyses of medial temporal lobe structures. In two experiments, we made ibotenic acid lesions to selectively and permanently destroy cells in the primate amygdala. Based on data in rodents, we expected that pretraining lesions of the amygdala (experiment 1) would disrupt fear-potentiated startle acquisition and that post-training lesions of the amygdala (experiment 2) would disrupt expression of the fear-potentiated startle response.

## Materials and Methods

### Experiment 1: effects of amygdala or hippocampus lesions on the acquisition of fear-potentiated startle

The Institutional Animal Care and Use Committee of the University of California, Davis approved the protocol for the experimental procedures

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used in these studies. The protocol adheres to the *Guide for Care and Use of Laboratory Animals* adopted by the National Institutes of Health.

### Subjects and living arrangements

The 18 adult male rhesus monkeys (*Macaca mulatta*) used in this study were born and reared at the California National Primate Research Center (CNPRC) in outdoor half-acre enclosures and lived among a group of conspecifics in troops ranging from 70 to 120 monkeys. The subjects were all relocated from outdoor cages to indoor CNPRC housing at the same time and were habituated to the new conditions. Monkeys were housed individually in cages (28 × 22 × 46 inches). The rooms were automatically regulated on a 12 h light/dark cycle with lights on at 6:00 A.M. and off at 6:00 P.M., and room temperature maintained at 75–85°F. The subjects were fed a diet of monkey chow (Ralston Purina, St. Louis, MO) supplemented with fruit and vegetables and *ad libitum* water. They were randomly assigned to either receive bilateral ibotenic acid lesions of the amygdala (amygdala group,  $n = 6$ ) or of the hippocampus (hippocampus group,  $n = 6$ ) or to serve as the operated control group ( $n = 6$ ). The hippocampus lesion group was included as a medial temporal lobe lesion control group. Before the fear-potentiated startle experiment, these monkey cohorts had been tested on a set of socio-emotional tasks including emotional responsiveness, dyadic social interaction (Mason et al., 2006), and human intruder (Emery et al., 2001). After the fear-potentiated startle experiment, animals were tested on a spatial learning task (Banta-Lavenex et al., 2006).

### Surgical procedures

**Magnetic resonance imaging.** Animals were anesthetized individually with ketamine hydrochloride (10 mg/kg, i.m.) and medetomidine (25–50  $\mu$ g/kg, i.m.) and were then placed in a magnetic resonance imaging (MRI)-compatible stereotaxic apparatus (Crist Instrument, Hagerstown, MD). After scan completion, the medetomidine was reversed with atipamazole (0.15 mg/kg, i.m.). MRI scans served as brain atlases and were used to generate individualized injection coordinate matrices. T1 images were exported to Photoshop (version 5; Adobe Systems, San Jose, CA) and then Canvas (version 5; Deneba System, Miami, FL), to superimpose a calibrated grid that was used to calculate injection coordinates.

**Lesion surgery: ibotenic acid injections.** Anesthesia was induced with ketamine hydrochloride (10 mg/kg, i.m.), after which the animals were maintained on isoflurane (1.2–2%). After reaching a surgical anesthesia level, the animal was placed in the stereotaxic apparatus. Fentanyl (7–10 mg/kg/min, i.v.) was administered in combination with isoflurane to provide a stable level of anesthesia throughout the surgical procedure.

Using sterile procedures, the skull was exposed, and openings were made dorsal to the amygdala or to the hippocampal formation. The dorsoventral location of the amygdala or the hippocampus was verified electrophysiologically by lowering a tungsten microelectrode into the locations calculated initially by the MRI analysis. Adjustments were made according to salient electrophysiological features of the spontaneous neuronal activity of the amygdala and hippocampus. Two identical 10  $\mu$ l (26 gauge beveled needle) Hamilton syringes were used to simultaneously infuse ibotenic acid (10 mg/ml in 0.1 M PBS; Biosearch Technologies, Novato, CA) into each amygdala or each hippocampus. A unilateral amygdala lesion required three to four rostrocaudal injection planes, each with one to four mediolateral levels and one to three dorsoventral injection sites. A unilateral hippocampal lesion required seven to eight rostrocaudal injection planes, each with one to two mediolateral levels and one to two dorsoventral injection sites. One microliter was infused into each injection site at 0.2  $\mu$ l/min, for a total of 13–25  $\mu$ l per amygdala or 10–16  $\mu$ l per hippocampus. For all operated animals, the ibotenic acid injections were followed by (1) suturing of the dura, (2) filling the craniotomy with GelFoam (Amersham Biosciences, Peapack, NJ), and (3) suturing of the fascia and skin in three layers. The six sham-operated control animals had the same presurgical preparations, midline incision, and skull exposure. They were anesthetized for the average lesion surgery duration and had fascia and skin suturing in two layers. Postsurgical care for all experimental groups included vital sign monitoring as well as administration of antibiotics and analgesics when deemed necessary by veterinary staff.

**Postoperative T2-weighted scans: lesion verification.** Ibotenic acid-induced edema appears as a hyperintense signal in T2-weighted MR images and is used as a general indication of the injection locus (Saunders et al., 1990; Malkova et al., 2001). After a 10–14 d recovery period, animals underwent a second MRI procedure, and T2-weighted signals for each of the 12 lesion subjects were evaluated to confirm the location of the lesion.

### General experimental procedure

At the time of the experiment (~4.5 years after the lesions had been made), the mean age was  $11.4 \pm 0.4$  years in the control group,  $11 \pm 0.6$  years in the amygdala group, and  $11.7 \pm 0.6$  years in the hippocampus group. The mean weight was  $13.1 \pm 0.6$  kg in the control group,  $12.8 \pm 0.9$  kg in the amygdala group, and  $12.2 \pm 0.4$  kg in the hippocampus group. Each monkey was provided a primate collar (Primate Products, Miami, FL) and underwent daily pole and collar training for 60 d to permit habituation to the primate restraint chair. Aluminum transport cages (0.5 × 0.03 × 0.04 m) were used for transferring subjects from the colony home cage to the experimental room. For testing order, all subjects were randomly assigned to one of three, six-animal cohorts. Each cohort was composed of two subjects from the amygdala group, two from the hippocampus group, and two from the control group. Cohort 1 was tested on day 1, cohort 2 was tested on day 2, and cohort 3 was tested on day 3. Testing order was fixed across experimental phases, with each animal tested at the same time each day. Time of day was counterbalanced among the groups so that every experimental time slot was occupied by at least one animal from each experimental group.

### Apparatus

The rodent fear-potentiated startle apparatus (Cassella and Davis, 1986) modified for primate research is detailed and depicted in the study by Winslow et al. (2002). Briefly, a custom-built primate restraint chair within which the monkey was comfortably positioned for startle response recordings was enclosed within a ventilated, light- and sound-attenuated wooden chamber. The restraint chair was secured on the upper panel of a two-panel platform. Startle amplitude was measured with an accelerometer (model 7201-50; Endevco Corporation, San Juan Capistrano, CA) that was center mounted underneath the upper panel (60 × 40 × 1.91 cm). The two panels were bolted together and separated by heavy compression springs that maintained an interpanel distance of ~10 cm. A rubber stopper (6.57 cm diameter) was mounted on a 5.08 cm plastic block resting on the lower panel, located directly underneath the accelerometer. When the bolts connecting the panels were tightened, the accelerometer was pressed against the stopper, resulting in a highly dampened interface. Movement of the restraint box, resulting from a whole-body startle response, displaced the accelerometer and produced a signal that was integrated by the Endevco amplifier (model 104). The resulting voltage signal was proportional to the displacement velocity of the chair (Cassella and Davis, 1986). This signal was digitized and fed to a Macintosh computer and analyzed using custom software (Experimenter 3.0; Glass Beads, Newtown, CT). Startle response measurement was defined as the maximal peak accelerometer output during the first 600 ms after the startle-eliciting noise onset. Baseline activity was the maximal peak accelerometer output during a similar 600 ms time window but 30 s after the startle-eliciting noise offset (i.e., in the absence of any startle-eliciting noise).

### Stimuli

The startle stimulus was a computer-generated burst of white noise delivered through a wall-mounted speaker located 12 cm behind the animal's head. The conditioned stimulus (CS) consisted of light presentation for 4.2 s through four halogen lights (400 lux each) corner mounted to the ceiling. The noxious unconditioned stimulus (US) was the presentation of a 1.2 s, 100 pound per square inch compressed air burst with the nozzle located ~26 cm from the animal's face and neck.

### Specific behavioral procedures

**Phase I: baseline startle amplitude assessment.** The animal was transferred to the experimental chair and placed in the test chamber. For the first 10 min of the baseline testing session, there were no startle stimuli to let the

animal adjust to the darkness, isolation, and ambient noise (Cassella and Davis, 1986). At the end of the 10 min adjustment period, a 50 min test session began. During this 50 min period, blocks of startle stimuli consisting of white noise bursts (5–20 kHz) were presented at each of the following intensities: 80, 90, 100, 105, 110, 115, and 120 dB. There were seven blocks of the seven startle stimuli, so the animal was exposed to 49 randomly presented noise bursts at a 60 s intertrial interval (ITI).

**Phase II: light test to measure unconditioned effects of the light on startle amplitude.** The animal was placed in the test chamber and for the first 10 min was acclimated as described above. At the end of the 10 min period, a 20 min test session began. The 20 min session consisted of 20 startle stimuli at a 60 s ITI: 10 110 dB white noise bursts delivered alone (noise-alone trials), intermixed with 10 110 dB white noise bursts delivered 3.5 s after onset of a 4.2 s light. This test session was used to evaluate whether the light would have any unconditioned facilitatory or inhibitory effect on startle amplitude before its being paired with the aversive air blast.

**Phase III: fear-potentiated startle training and testing.** The animal was placed in the test chamber and for the first 10 min was acclimated as described above. After this, a 16 min session began that consisted of four training trials randomly intermixed with 12 testing trials each separated by a 60 s ITI. A training trial was used to produce the association between the light (CS) and the noxious air puff (US). Each training trial was initiated by light onset and followed by an air puff at one of the following delays: 1.5, 2.0, 2.5, or 2.7 s after the onset of the light. US onset time was varied in an effort to make the entire CS duration aversive (Davis et al., 1989). Testing trials were of two types. Either a startle stimulus was delivered alone or the startle stimulus was delivered 1.5 s after light onset. When training trials are intermixed with testing trials, relatively stable levels of fear-potentiated startle can be maintained across repeated training–testing sessions (Winslow et al., 2007). When the conditioned light came on, the animal did not know whether it would be followed by a startle stimulus, to measure fear, or an aversive air blast, to condition fear to the light. There was a total of three such mixed training–testing sessions at 3 d intervals. For each testing session, the raw startle scores were also expressed as percentage of fear-potentiated startle calculated as follows:

$$[(\text{light-noise} - \text{noise alone})/\text{noise alone}] \times 100.$$

Statistical analyses were performed on the raw data. The fear-potentiated startle scores were included for descriptive purposes.

Approximately 6 months after the experiment, amygdala- and hippocampus-lesioned animals were individually immobilized with ketamine hydrochloride (8 mg/kg), deeply anesthetized with Nembutal (50–100 mg/kg, i.v.), and prepared for intracardiac perfusion. Briefly, 1% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2 at 4°C) was infused at a rate of 250 ml/min for 2 min, followed by 4% paraformaldehyde infused at 250 ml/min for 10 min and at 100 ml/min for 50 min. The brain was removed from the skull, postfixed for 6 h in 4% paraformaldehyde, cryoprotected overnight in 10% glycerol in phosphate buffer and 2% dimethylsulfoxide, and finally cryoprotected for 3 d using 20% glycerol in phosphate buffer and 2% dimethylsulfoxide. Brains were frozen using the isopentane procedure (Rosene et al., 1986) and stored at  $-70^{\circ}\text{C}$ . A sliding microtome was used to cut coronal brain sections that were either 30 or 60  $\mu\text{m}$  thick. Tissue was preserved in cryoprotectant tissue-collecting solution (30% ethylene glycol and 20% glycerol in 0.005 M sodium phosphate buffer). For the next 2 weeks, the 60- $\mu\text{m}$ -thick sections were postfixed in a 10% formaldehyde solution at 4°C, rinsed, mounted onto gelatin-coated slides, and processed for Nissl staining. The sham-operated monkeys were returned to the colony.

### Experiment 2: effects of amygdala lesions on expression or retention of fear-potentiated startle

The goal of this second experiment was to evaluate the effects of bilateral amygdala lesions on the retention and expression of fear-potentiated startle.

In experiment 1, the light–response data (phase II) revealed that there are individual differences in the extent to which the presentation of the preconditioned light increases noise-induced startle. For experiment 2, we anticipated that some monkeys may fail to acclimate to the light. We made every effort, therefore, to screen out monkeys with an anxious



**Figure 1.** A photograph of the fear-potentiated startle apparatus (Med Associates). Arrows point to the speaker assembly (**a**), to one of the four ceiling lights (**b**), and to the floor of the chair (**c**), which has adjustable levels for different monkey heights. The load cell/strain gauge (**d**) is located under the upper panel of the platform. The animal is transferred in a custom-built primate restraint chair that is positioned on the platform and secured with side wing nuts.

disposition during the selection process. We evaluated monkey temperament by using a form of the human-intruder task (Kalin and Shelton, 2003). Animals with the lowest anxiety scores were selected from the available subject pool to have an initial pool of 10 animals. Our intent was to form two experimental groups with four monkeys in each group. With an initial 10-monkey cohort, two alternate subjects were available to go through the habituation process.

### Subjects

Two groups of subjects were used in experiment 2: a group that would receive bilateral amygdala lesions after fear conditioning ( $n = 4$ ) and a surgical control group ( $n = 4$ ). We started the experiment with a 10-monkey cohort for the reasons described above. The 10 adult male rhesus monkeys were similar to those described for experiment 1. They had all been relocated to indoor CNPRC housing facilities for at least 2 months and were habituated to indoor living conditions. The mean age was  $6.43 \pm 0.42$  years, and the mean weight was  $8.88 \pm 0.46$  kg. All animals were pair housed in the indoor facility, with the exception of one monkey housed alone because of the inability to identify a compatible cage mate.

### Apparatus

For this experiment, a newer version of the primate fear-potentiated startle apparatus was produced (Med Associates, St. Albans, VT). This version of the apparatus is illustrated in Figure 1. The restraint chair is enclosed within a ventilated light- and sound-attenuated cubicle and is securely fastened on the upper panel of a two-panel platform. A force transducer is suspended from the bottom part of the upper panel and converts the startle-induced panel compression into an electrical signal that is amplified, digitized, and stored for later analysis (version 2.1; Med

**Table 1. Volume (in mm<sup>3</sup>) of left and right amygdala in unlesioned monkeys and in the experimental subjects with bilateral ibotenic acid injections of the amygdala**

Group	Subject identification	Amygdala	
		Left	Right
Unlesioned monkeys	PM13-03	169.44	171.60
	PM15-03	161.76	159.36
	PM16-03	167.28	166.08
	PM17-03	178.56	174.96
	PM8-02	170.88	168.48
	M13-03	215.76	218.16
	Mean ± SE	177.28 ± 8.02	176.44 ± 8.62
Amygdala lesion	25729	63.26	47.85
	26672	30.03	11.64
	27317	13.91	32.66
	27581	22.93	56.13
	27695	3.79	5.68
	28022	12.95	18.74
Mean ± SE	16.72 ± 8.58	24.97 ± 8.28	

Associates). A ventilation fan mounted on the upper right corner of the back wall (30 cm behind the animal’s head) produced an ambient noise of ~65 dB. Startle response measurement was defined as the maximal signal recorded within 600 ms after startle-eliciting noise onset. Baseline activity was the maximal motion detected during a similar 600 ms time window 60 s after startle noise offset. The stimuli were identical to those in experiment 1. A programmable audio stimulator generated the startle stimuli that were delivered through a ceiling speaker located 15 cm above the animal’s head.

*Specific experimental procedures*

*Phase I: baseline startle amplitude assessment.* The animal was transferred to the experimental chair and placed in the test chamber. For the first 10 min, there were no startle stimuli, and the animal was acclimated as described in experiment 1. For the next 40 min, blocks of startle stimuli consisting of white noise bursts (5–20 kHz) were presented at each of the following intensities: 80, 90, 100, 110, and 115 dB. There were four blocks of the five startle stimuli at a 120 s ITI. Animals underwent 4 baseline days.

*Phase II: light test to measure unconditioned effects of the light on startle amplitude.* To establish whether the preconditioned light altered startle amplitude, startle stimuli were delivered in the dark (noise-alone trial) and during light (light-noise trial). The 40 min session consisted of 20 startle noise trials at a 120 s ITI: 10 100 dB white noise bursts presented in the dark, intermixed with 10 100 dB white noise bursts delivered 2.1 s after onset of a 4.2 s light. Animals were exposed to daily light-test sessions until light-induced startle enhancement was <10% on 2 successive days for all animals. All animals received therefore the same number of daily light-test habituation sessions.

*Phase III: prelesion fear-potentiated startle training and testing.* The animal was placed into the chamber and acclimated for 10 min. In the next 50 min session, 5 training trials were randomly intermixed with 20 testing trials and separated by a 120 s ITI. As in experiment 1, the training trial was used to produce the association between the light (CS) and the noxious air puff (US). Procedures for inducing fear-potentiated startle enhancement were identical to those used in experiment 1. The potentiated startle data were used to divide animals into the two experimental groups with equivalent fear-potentiated startle levels (Campeau and Davis, 1995). We randomly selected which of the two groups would receive amygdala lesions.

*Statistical analysis*

ANOVA procedures and Fisher’s least significant difference (LSD) *post hoc* comparisons were used for data analyses.

*Surgical procedures*

The surgeries were performed 14–45 d after acquisition of fear-potentiated startle. All imaging and surgical procedures were identical to those detailed in experiment 1. However, unlike experiment 1, the sur-

**Table 2. Percentage loss of the amygdala in the experimental monkeys relative to the mean value obtained from the unlesioned monkeys**

Subject identification	Amygdala	
	Left	Right
27695	98%	97%
28022	93%	89%
26672	83%	93%
27317	92%	81%
27581	88%	68%
25729	64%	73%
Mean	86%	84%

gical control animals did not receive a midline incision during their sham surgical procedure.

*Phase IV: postlesion retention test*

After a recovery period ranging from 26 to 36 d, retention testing was initiated. Each animal was introduced in the test chamber and had a 10 min acclimation period. The goal of this phase was to determine whether the animals retained their presurgically learned fear-potentiated startle. Thus, the 40 min session consisted of 20 startle test trials at a 120 s ITI: 10 100 dB white noise bursts delivered alone (noise-alone trial), intermixed with 10 100 dB white noise bursts delivered at one of the following delays: 0.5, 1.0, 1.8, or 2.8 s after the onset of the light (light-noise trial).

*Postsurgical T1-weighted scans: lesion verification*

The amygdala lesioned animals underwent an MRI procedure, 11–30 weeks following surgery. The imaging procedure was identical to that described in experiment 1. We acquired T1-weighted scans that were used to confirm the location of the lesion.

*Volumetric measurement of the amygdala*

Subjects used in these experiments are still participating in additional experimental procedures. Therefore, to demonstrate a lesion-induced decrease in the size of the amygdala, presurgical and postsurgical MR images were analyzed volumetrically using the Analyze 7.0 software package (BIR, Rochester, MN). A single trained neuroanatomist traced boundaries of the amygdala on the presurgical and postsurgical images.

**Results**

**Experiment 1**

*Histological analysis*

Individual amygdala volumes were estimated with the Cavalieri method (Gundersen and Jensen, 1987). Histological sections through the rostrocaudal extent of the amygdala were selected and microscopically evaluated for the extent of the lesion using an Aus Jena microfiche reader (Zeiss, Oberkochen, Germany). Using the nomenclature described by Price et al. (1987), the intact portions of the amygdala (with neurons apparent) were drawn for each section. Each line drawing was overlaid with a regular array of points, and the number of points within the boundary of the amygdala was recorded. The volume of the amygdala was estimated according to the following equation:

$$\text{Amygdala volume} = t \times (a/p) \times (P_1 + P_2 + P_3 + \dots + P_n),$$

where  $P_1, P_2, P_3, \dots, P_n$  are the number of points recorded for the number of consecutive sections,  $t$  is the sectioning interval, and  $a/p$  is the area associated with each point. The brains used for comparison come from a library of unlesioned rhesus monkeys. Table 1 lists the volumes of the amygdala in the experimental and in the unlesioned monkeys.

Table 2 lists the percentage loss of the amygdala in the experimental monkeys relative to the mean value obtained from the unlesioned monkeys.

We include photomicrographs of three levels (top to bottom)

through the full rostrocaudal extent of the amygdala in case MMU27695 (left) with the most complete and discrete amygdaloid lesion, PMI-17 03 (middle), and case MMU25729 (right) with the smallest lesion in the experimental group (Fig. 2). We also include higher-magnification photomicrographs at a mid-rostrocaudal level of the left (Fig. 3A) and right (Fig. 3B) amygdala of the PMI-17 03 and images at the same left (Fig. 3C) and right (Fig. 3D) level in case MMU27695 with the most complete and discrete amygdaloid lesion.

#### Control brains

The overall volume of the adult male rhesus monkey amygdala is  $\sim 180 \text{ mm}^3$  (Table 1).

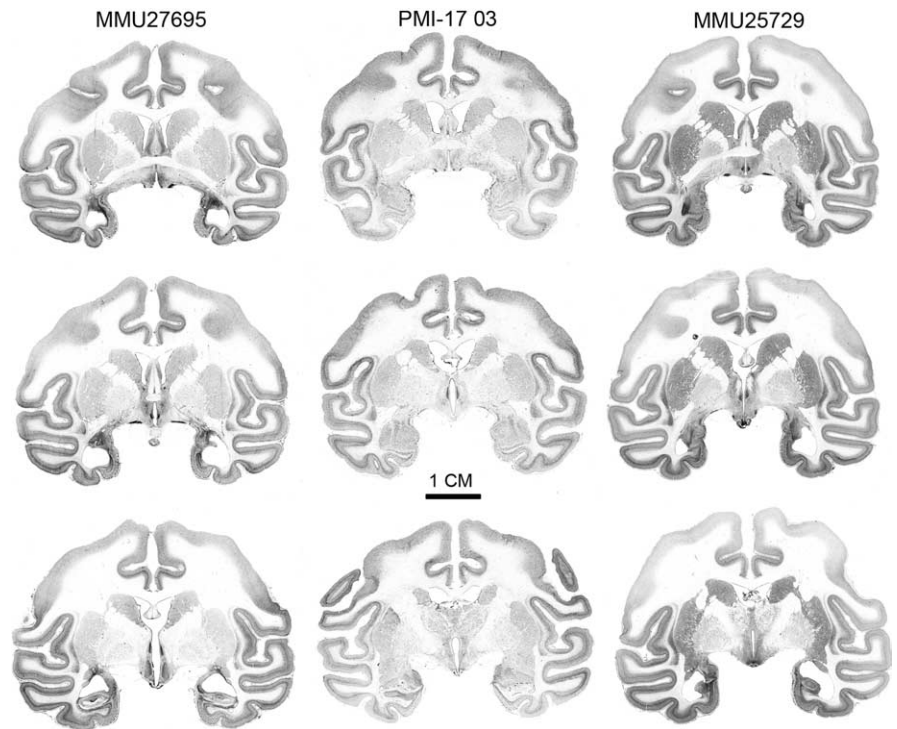
#### Amygdala lesioned brains

The ibotenic acid injections produced substantial cell loss in the amygdala in each of the experimental monkeys. The neurosurgery was designed to produce discrete damage to the entire amygdala. In general, the lateral, basal, accessory basal, and central nuclei were damaged but there was also some damage to the rostral hippocampus. Given that there was some variability in the amount of the amygdala damage, we include a qualitative description of the lesion in each case.

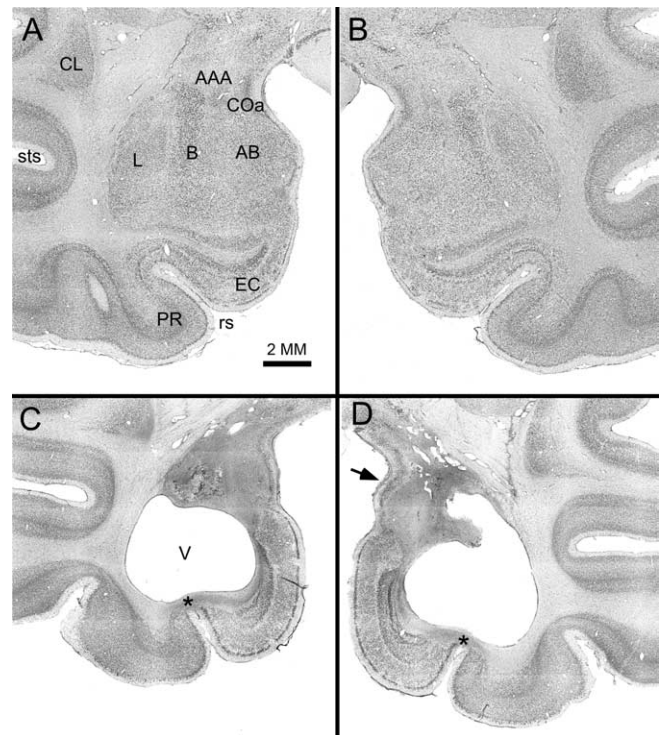
**Subject MMU27695.** This case represents the largest amygdala lesion with damage to 97% of the right and 98% of the left amygdala. The deep nuclei (lateral, basal, accessory basal, and paralaminar nuclei), the superficial nuclei (anterior cortical nucleus, nucleus of the lateral olfactory tract, medial nucleus, posterior cortical nucleus), the central nucleus, the anterior amygdaloid area, and the amygdalo-hippocampal area were eliminated bilaterally. Minimal sparing was detected only in the periamygdaloid cortex. Extraneous damage included the endopiriform nucleus, the ventral claustrum, and the cortices associated with the rhinal, anterior middle temporal, and superior temporal sulci through levels of the amygdala. There was also some damage to rostral levels of the hippocampus and dentate gyrus.

**Subject MMU28022.** The ibotenic acid injection eliminated 89% of the right and 93% of the left amygdala, producing the second largest lesion in the experimental group. On both sides, spared tissue was found only in the paralaminar nucleus, the accessory basal nucleus, and the amygdalo-hippocampal area. On the right side, there was slight sparing of the medial nucleus. Extraneous damage included the endopiriform nucleus, ventral claustrum, and cortices underlying the rhinal, the middle temporal, and the superior temporal sulci through levels of the amygdala. Minimal cell loss was detected in rostral levels of the hippocampus.

**Subject MMU26672.** The lesion eliminated 83% of the left and 93% of the right amygdala. Superficial areas were slightly spared on the right side (medial nucleus and periamygdaloid cortex) and on the left side (anterior cortical nucleus, nucleus of the lateral olfactory tract, periamygdaloid cortex), but the latter was also coupled with some minor sparing in the basal, accessory basal,



**Figure 2.** Photomicrographs of three levels [arranged from rostral to caudal (top to bottom)] through the amygdala in case MMU27695 (left) with the most complete and discrete amygdaloid lesion, an unlesioned monkey (center), and case MMU25729 (right) with the smallest lesion in the experimental group. Scale bar, 1 cm.



**Figure 3.** Higher-magnification photomicrographs of the left (A) and right (B) amygdala in the PMI-17 03 and images at the same left (C) and right (D) level in case MMU27695 with the most complete and discrete amygdaloid lesion. CL, Claustrum; AAA, anterior amygdaloid area; L, lateral nucleus; B, basal nucleus; AB, accessory basal nucleus; EC, entorhinal cortex; PR, perirhinal cortex; sts, superior temporal sulcus; rs, rhinal sulcus; COa, anterior cortical nucleus; V, ventricle. Asterisks indicate some extraneous damage in the perirhinal cortex in the fundus of the rhinal sulcus. The arrow indicates sparing of dorsally placed superficial nuclei. Scale bar, 2 mm.

and central nuclei. The amygdalo-hippocampal area was partially spared on both sides. Extraneous damage included the endopiriform nucleus, the ventral claustrum, and the cortices surrounding the rhinal and superior temporal sulci through levels of the amygdala.

**Subject MMU27317.** The ibotenic acid injection lesioned 81% of the right and 92% of the left amygdala. On both sides, spared tissue was detected only in superficial nuclei and in the amygdalo-hippocampal area. Extraneous damage included the endopiriform nucleus, the left ventral claustrum, and the cortex associated with the rhinal sulcus through levels of the amygdala. There was only slight damage to the CA1 and CA3 fields of the rostral hippocampal formation.

**Subject MMU27581.** In this case, 68% of the right and 88% of the left amygdala was damaged. In both hemispheres, the injections appeared to be placed more laterally than intended. Thus, the lateral nucleus was damaged extensively on both sides of the brain. On the right side, sparing occurred in the basal, accessory basal, and paralaminar nuclei as well as within the superficial nuclei, the central nucleus, and the amygdalo-hippocampal area. The left side was more extensively damaged with slight savings in the basal, the accessory basal, and the paralaminar nuclei, the periamygdaloid cortex, and the amygdalo-hippocampal area. There was some damage to the ventral portion of the claustrum and minimal damage to the rostral hippocampus.

**Subject MMU25729.** This case represents the smallest lesion in the experimental group with damage to 64% of the left and 73% of the right amygdala. The central nucleus appeared to be eliminated bilaterally.

#### Hippocampus lesions

A description of the lesion extents in the hippocampal lesion group has been presented by Banta-Lavenex et al. (2006). In general, the lesion extent was very consistent across all six experimental monkeys. The dentate gyrus, hippocampus, subiculum, presubiculum, and parasubiculum were heavily damaged. In some cases, the damage extended into the parahippocampal cortex. Consistently, across all animals, the entorhinal and perirhinal cortices were mostly intact, as was all of the amygdaloid complex.

#### Statistical analysis

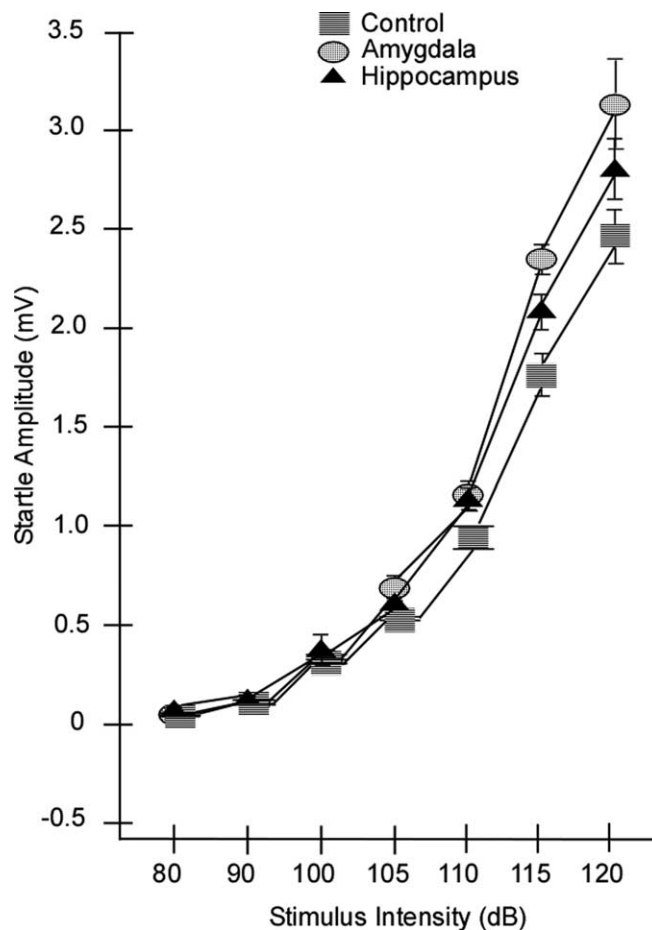
ANOVA procedures and Fisher's LSD *post hoc* comparisons were used for data analyses.

#### Phase I: baseline

An ANOVA using lesion as a between-subjects factor, noise intensity (80–120 dB), and block (1–7) as within-subjects factors indicated that increments in stimulus intensity were coupled with proportional increases in startle amplitude ( $F_{(6,12)} = 722.93; p < 0.001$ ) (Fig. 4). Startle amplitude did not vary across the different blocks. Interestingly, a significant group effect ( $F_{(2,15)} = 3.95; p < 0.05$ ) indicated that the amygdala group had higher startle amplitude than the control group ( $p < 0.02$ ). We had not anticipated a group difference in baseline startle (control vs amygdala). There is no precedent for this in the rodent literature, nor is there any evidence that lesions to the amygdala have an effect on baseline startle in the monkey. This effect will be evaluated further in future studies.

#### Phase II: light–response

The light did not increase noise-induced startle in any of the groups. This result comes from a repeated-measures ANOVA on startle responses with trial type (noise alone vs light–noise) as the



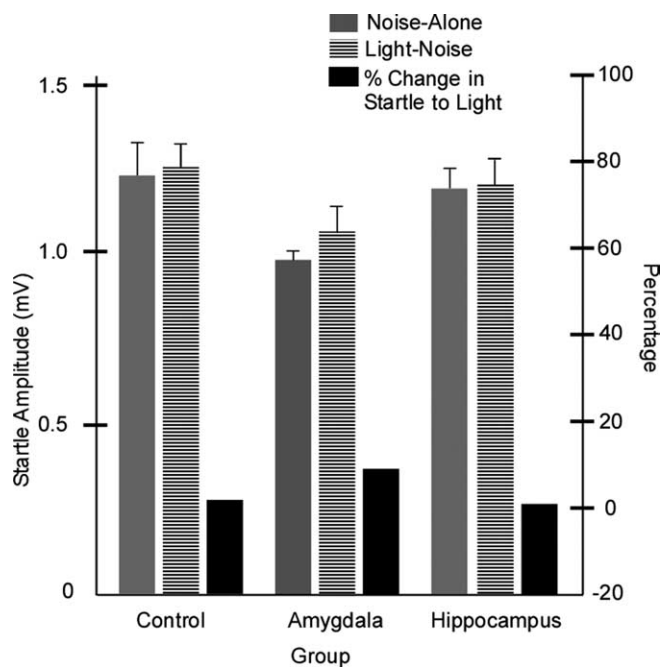
**Figure 4.** Mean startle amplitude (in mV) as a function of stimulus intensity. Acoustic stimuli varied from 80 to 120 dB. Responses are shown for animals in the amygdala ( $n = 6$ ) and hippocampus ( $n = 6$ ) lesion groups as well as the control group ( $n = 6$ ). Error bars at each data point denote SEM.

within-subjects factor and lesion condition as the between-subjects factor (Fig. 5). The analysis did not yield any significant main (trial-type or lesion condition) or interaction ( $p > 0.05$ ) effects. A closer look at the data indicates that the light increased startle by 9% in the amygdala group versus 2 and 1% in the control and hippocampus lesion groups, respectively.

The disproportionate unconditioned facilitation of startle in the amygdala lesion group prompted us to look more closely at the data from individual animals. The individual proportional scores are shown in Table 3. Two amygdala lesion animals (subjects 1 and 2) showed a 26% startle increase during the light-on condition. One control animal (subject 1) and one hippocampus-lesioned animal (subject 4) showed a 36 and a 15%, respectively, startle increase in the presence of the light.

Unfortunately, this analysis was done after all groups had already moved to the next training/testing phase. Our decision to proceed was based on the absence of statistical effects. However, in retrospect, it would have been better to continue habituation trials until the light no longer increased startle in any animal. This practice was performed in experiment 2 (see below). Only when light does not unconditionally facilitate startle can we conclude that light, after pairing with the noxious stimulus, leads to a fear-conditioned startle enhancement.

For the remaining analyses, we decided to exclude any animal showing preconditioned startle increases to the light that were



**Figure 5.** Mean startle amplitude (in mV) to 110 dB startle stimuli in the absence (noise alone) or presence (light–noise) of a 4.2 s light during the light–response phase. Error bars denote SEM. Black bars illustrate the percentage change in startle produced by light presentation (right axis).

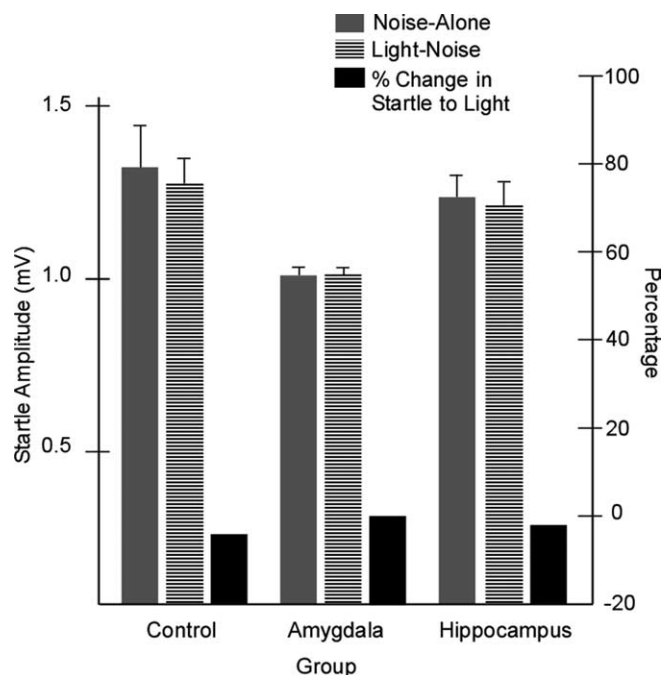
>10% relative to the noise-alone test trials. Four animals were excluded from the data analysis: two from the amygdala group, one from the control group, and one from the hippocampus group. The groups thus comprised four, five, and five animals in the amygdala lesion, hippocampal lesion, and control groups, respectively.

#### Phase II: light–response reanalysis

By removing the animals as described above, startle amplitude was not altered by the light in any of the groups (Fig. 6).

#### Phase III: fear-potentiated startle testing

Data transformation was used (square root) to comply with the theoretical assumptions of homogeneity of variance (Fig. 7). The graphs in Figure 7 (top) present the nontransformed data to better illustrate the animals' startle behavior. Transformed data were analyzed between the different groups ( $n = 3$ ), across the different test days (test days 1–3), and as a function of trial type (light–noise vs noise alone). The ANOVA revealed a significant fear-potentiated startle effect ( $F_{(1,33)} = 16.34$ ;  $p < 0.001$ ), indicating that startle was enhanced when the light was turned on, and a significant group effect ( $F_{(2,33)} = 4.12$ ;  $p < 0.03$ ). Only the control ( $p < 0.001$ ) and hippocampus lesion ( $p < 0.01$ ) groups acquired fear-potentiated startle; the amygdala lesion group did not. In the control group, light presentation increased startle in-



**Figure 6.** Mean startle amplitude (in mV) to 110 dB startle stimuli in the absence (noise alone) or presence (light–noise) of a 4.2 s light during the light–response phase. Animals with >10% light-induced startle enhancement have been eliminated from this analysis (for rationale, see Results, Experiment 1, Phase II: light–response). Error bars denote SEM. Black bars illustrate the percentage change in startle produced by light presentation (right axis).

tensity by 20% on test day 1, by 69% on test day 2, and by 102% on test day 3. In the hippocampus lesion group, light potentiated startle in a similar way, with a 20% increase on test day 1 that increased to 44% on test day 2 and 92% on test day 3. In the amygdala lesion group, in contrast, light led to no change in startle (0%) on test day 1, a 2% startle increase on test day 2, and a 17% startle increase on test day 3 (Fig. 7, bottom). With the three test-day scores collapsed, light increased startle by an average of 63% in the control group, by 49% in the hippocampus lesion group, but only by 6% in the amygdala group. This finding demonstrates that animals with bilateral damage to the amygdala fail to acquire fear-potentiated startle.

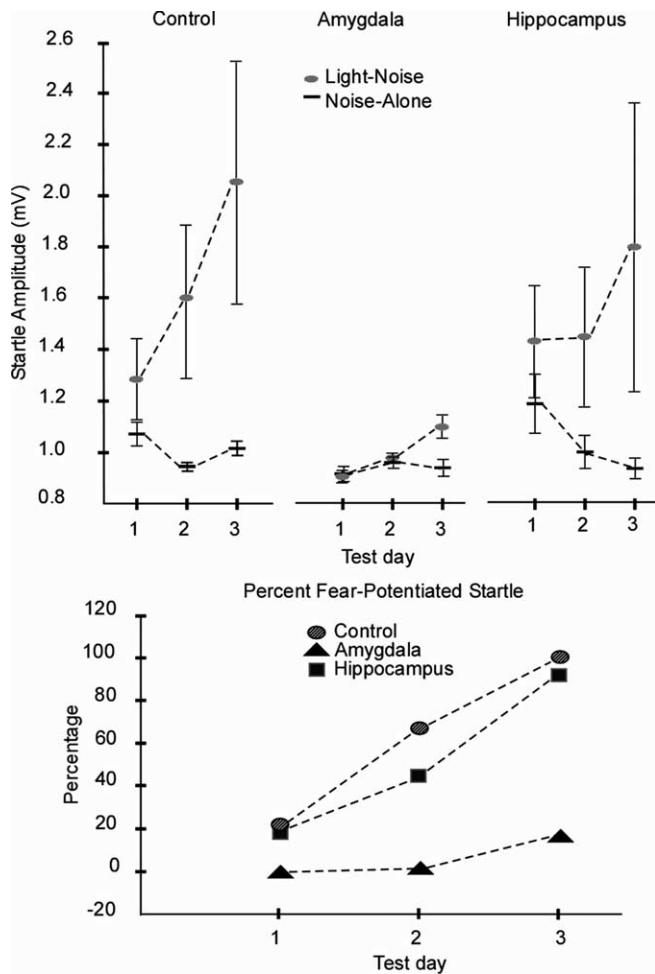
## Experiment 2

### MRI analysis of amygdala lesions

Whereas a decrease in the measured volume of the amygdala provides confidence that the lesion was placed properly, it does not allow a determination of whether remaining tissue is populated by neurons or not. Nor does the MRI provide evidence of the magnitude of extraneous damage. Given these caveats, there was a substantial decrease in amygdala volume in all experimental animals (Table 4). In three of the four monkeys (MMU32104, MMU32097, MMU30922), the ibotenic acid injections produced a 79–89% decrease in the volume of the amygdala. There was no evidence in these cases of any substantial sparing of amygdaloid nuclei. In subject MMU30642, volume was reduced by ~55%. It is likely that there was some sparing in this case, particularly of the most superficial regions of the amygdala. Presurgical and post-surgical MR images from case MMU32097 are shown in Figure 8. This case represents the most complete and discrete lesion of the amygdala. During the planning for these surgeries, particular care was paid to including the central nucleus within the lesion. It

**Table 3.** Percentage change in startle produced by light presentation during the light response phase

Subject	Control group	Amygdala group	Hippocampus group
1	36%	26%	1%
2	2%	26%	–6%
3	4%	–4%	3%
4	–14%	2%	15%
5	–7%	1%	0%
6	2%	0%	–9%



**Figure 7.** Top, Mean startle amplitude (in mV) to 110 dB startle stimuli in the presence (gray oval) or absence (line) of the 4.2 s light in the control ( $n = 5$ ), the amygdala ( $n = 4$ ), and the hippocampus ( $n = 5$ ) lesion groups during test days 1–3. Vertical bars at each data point denote SEM (bottom). The percentage change in startle produced by light presentation for the amygdala and hippocampus lesion groups as well as the control group during test days 1–3 is shown.

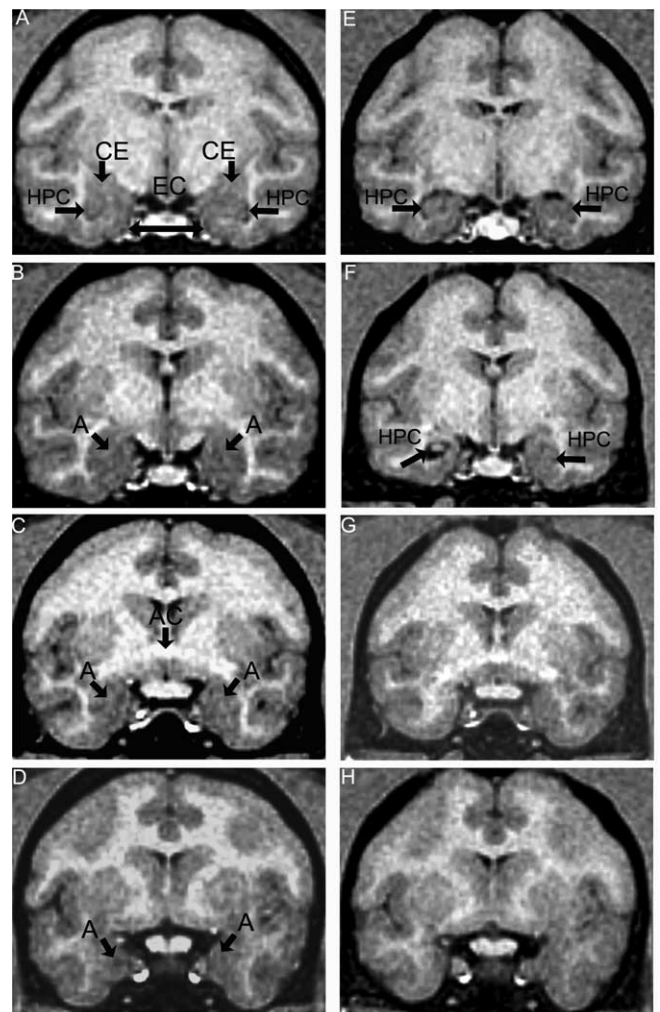
appears that the central nucleus was damaged extensively in all cases.

*Phase I: baseline startle amplitude assessment*

An ANOVA with noise intensity (80–115 dB), day (1–4), and block (1–4) as within-subject factors indicated that startle amplitude increased as a function of noise intensity ( $F_{(4,24)} = 6.96; p < 0.001$ ) (Fig. 9). Startle amplitude did not vary significantly across the different days, nor did it vary across the different blocks.

*Phase II: light-response*

In 5 of the 10 animals tested, the preconditioned light initially led to a >10% increase of startle (Fig. 10, left column). After nine habituation sessions, nine monkeys had reached a criterion of



**Figure 8.** Presurgical (A–D) and postsurgical (E–H) MR images in case MMU32097 with the most complete and discrete lesion to the amygdala. The sections are displayed in a caudal (top) to rostral (bottom) direction with a 2 mm intersection distance. The lesion produced a substantial distortion of the medial temporal lobe so other neuroanatomical landmarks were used to match the presurgical to the postsurgical section series. The presurgical sections cover the full 7–8 mm rostrocaudal length of the amygdala. A, Arrows point to the central nucleus of the amygdala (CE), the entorhinal cortex (EC), and the hippocampus (HPC). B–D, The amygdala (A) is displayed in B–D, and the anterior commissure (AC) is displayed in C. All postsurgical sections exhibit substantial shrinkage of the amygdala. E, F, The hippocampus (HPC) is prominent in the caudal areas where the central nucleus of the amygdala (CE) is normally found.

<10% light-induced startle increase on two successive days. These nine monkeys proceeded to fear-potentiated startle training. At the end of habituation, the group’s light-induced startle mean increase was –16%, indicating that light no longer enhanced startle amplitude.

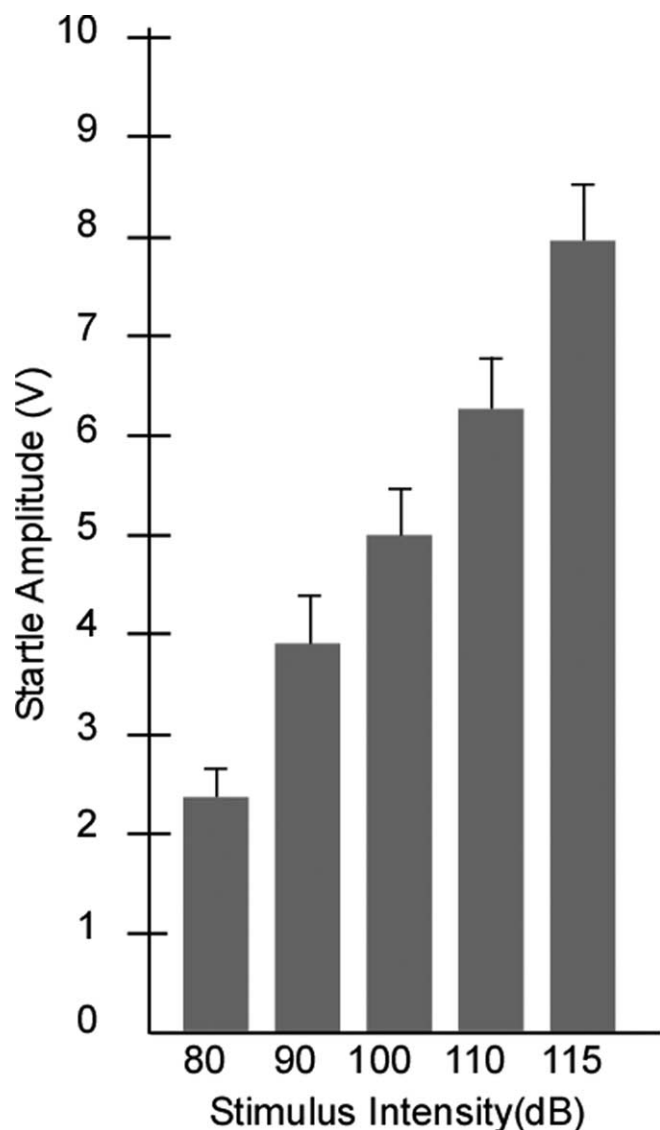
*Phase III: prelesion fear-potentiated startle testing*

Subjects had been assigned to either the amygdala lesion or control group based on their presurgery level of fear-potentiated

**Table 4.** Presurgical and postsurgical volume (in mm<sup>3</sup>) of left and right amygdala and percentage loss of the amygdala in the four experimental monkeys

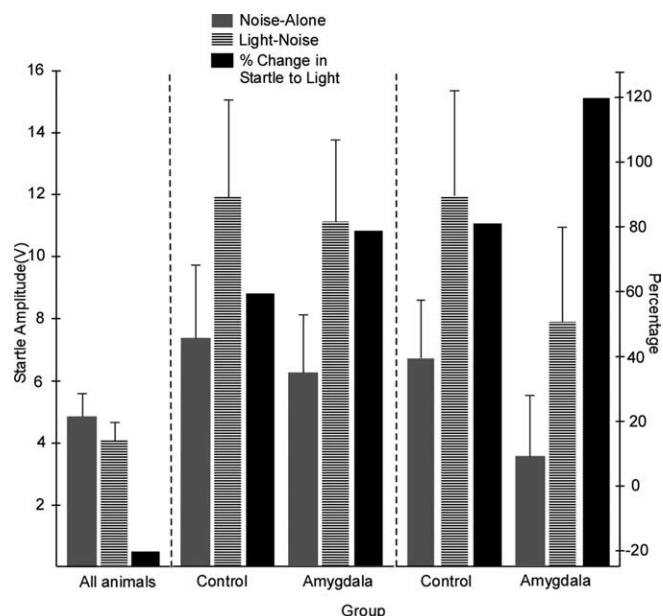
Subject	Preoperation		Postoperation		Percentage	
	Left amygdala	Right amygdala	Left amygdala	Right amygdala	Left amygdala	Right amygdala
30642	292.91	287.35	135.62	127.81	54%	56%
30922	319.64	291.99	67.32	51.57	79%	82%
32097	311.10	334.84	36.01	45.17	88%	87%
32104	281.13	272.71	30.68	30.03	87%	89%





**Figure 9.** Mean startle amplitude (in V) as a function of stimulus intensity in all animals. Acoustic stimuli vary from 80 to 115 dB. Data are averaged across four daily sessions. Error bars at each data point denote SEM.

startle. Prelesion fear-potentiated startle responses were analyzed by repeated-measures ANOVA with trial type (light–noise versus noise alone) as the within-subjects factor and lesion (group) as the between-subjects factor. The ANOVA revealed a significant fear-potentiated startle effect ( $F_{(1,6)} = 9.21; p < 0.03$ ), showing that light enhanced startle amplitude after being paired with the aversive air blast. There was no significant group effect, indicating that both groups showed the same degree of fear-potentiated startle. The light increased startle intensity by 60% in the control group and by 79% in the group that would undergo an amygdala lesion (Fig. 10, middle column). It is noteworthy that the exposure to unreinforced light presentations in the light–response phase could have retarded subsequent fear conditioning to that light (latent inhibition). However, the high fear-potentiated startle levels expressed during the prelesion test (60% in the control group and 79% in the group that would receive an amygdala lesion) indicate that latent inhibition was not a determining factor.



**Figure 10.** Mean startle amplitude (in V) to 100 dB startle stimuli in the absence (noise alone) or presence (light–noise) of a 4.2 s light during the light–response phase (left column) and the prelesion (middle column) and postlesion (right column) fear-potentiated startle testing phases. The gray and striped bars illustrate startle response magnitude (left axis). Error bars denote SEM. The black bars represent the percentage change in startle produced by light presentation (right axis) in all graphs.

#### Phase IV: postlesion fear-potentiated startle testing

Startle responses were analyzed by a repeated-measures ANOVA with trial type as the within-subjects factor and lesion as the between-subjects factor. The ANOVA revealed a significant fear-potentiated startle effect ( $F_{(1,6)} = 15.65; p < 0.01$ ), indicating that light enhanced startle amplitude. Somewhat unexpectedly, there was no significant group effect, indicating that the control and the amygdala lesion groups expressed similar levels of fear-potentiated startle. The light potentiated startle by 79% in the control group and by 121% in the amygdala lesion group (Fig. 10, right column).

#### Discussion

In the present experiments, the fear-potentiated startle test previously described by Winslow et al. (2007) was used with the rhesus monkey. In experiment 1, the goal was to examine whether in the non-human primate, as in the rat, the amygdala is essential for the acquisition of fear-potentiated startle. We found that the amygdala-lesioned monkeys were unable to acquire fear-potentiated startle. This is congruent with previous reports demonstrating that rats with lesions of the central (Kim and Davis, 1993) or the basolateral (Campeau and Davis, 1995) nuclei of the amygdala fail to acquire fear-potentiated startle. Moreover, as we had predicted from the literature on rat studies (Heldt et al., 2002), lesions to the primate hippocampus did not interfere with the acquisition of fear-potentiated startle (experiment 1).

In experiment 2, we sought to determine whether the non-human primate amygdala is involved in the retention and expression of the fear-potentiated startle response. A lesion of the entire amygdala was produced after animals had successfully acquired the fear-potentiated startle reflex. Surprisingly, these lesions did not block the retention and expression of fear-potentiated startle.

In a previous study, posttraining damage to large parts of the

amygdala, including the central nucleus and basolateral amygdala, blocked the expression of fear-potentiated startle (Kim and Davis, 1993). However, animals were able to reacquire the fear-potentiated startle when they were retrained (Kim and Davis, 1993). This finding indicates that a non-amygdala area is capable of mediating a fear-potentiated enhancement of the startle reflex in the rat (Kim and Davis, 1993). The current study provides evidence that a non-amygdala area can mediate the expression of a fear memory that originally depended on the amygdala for acquisition.

At the current time, it is not known what brain area(s) might mediate retention of fear-potentiated startle in the non-human primate in the absence of an intact amygdala. Although it is well established from research in the rat that the lateral nucleus of the amygdala is involved in the initial acquisition of learned fear associations (LeDoux, 2000), recent findings indicate that the medial prefrontal cortex also encodes and retains fear associations (Laviolette et al., 2005). Medial prefrontal cortex neurons show increased bursting activity to presentations of a fear-conditioned odor stimulus (CS+) relative to odors previously paired with the absence of footshock (CS-). Inactivating the basolateral amygdala prevented the development of differential neuronal responses in the medial prefrontal cortex. However, if animals had been trained with an intact amygdala, inactivating the amygdala did not diminish the differential response in the medial prefrontal cortex. The authors suggest that the initial fear association is established in the amygdala and is then transferred to the medial prefrontal cortex. This is certainly possible in the rhesus monkey as well because there are very substantial connections between the amygdala and prefrontal cortex in the non-human primate (Amaral et al., 1992).

Another brain region possibly involved in the mediation of fear conditioning is the bed nucleus of the stria terminalis (BNST). At least in the rat, the BNST sends direct projections to the same hypothalamic and brainstem nuclei as the central nucleus of the amygdala (Dong and Swanson, 2004). The BNST has been implicated in several measures of anxiety (cf. Walker et al., 2003), although under normal conditions it is not involved in fear-potentiated startle. However, it could well be recruited to mediate fear-potentiated startle under conditions in which the amygdala is destroyed. Using an overtaining design, Poulos and Fanselow (2005) found that pretraining lesions to the basolateral amygdala does not interfere with freezing behavior in the shock-associated context. However, if rats also receive a pretesting lesion to the BNST, they fail to show the conditioned fear response. This suggests that the BNST may have the potential of compensating for the absence of a functional amygdala (also see Ponnusamy and Fanselow, 2005).

Additional experiments in non-human primates are obviously needed. It would be of interest to determine, for example, whether transient inactivation of the BNST or perhaps the frontal cortex might reduce posttraining expression of fear-potentiated startle, either with or without an intact amygdala. Finally, it should be acknowledged that we have not yet conducted histological evaluation of the lesions in the animals in experiment 2. The completeness of those lesions was judged on the basis of postlesion MRIs. Because these MRIs look very similar to those in experiment 1, in which histological examination confirmed amygdala damage, we are quite confident that the animals in experiment 2 will be found to have comparable and extensive damage.

Experiment 2 had some procedural differences from experiment 1. Modifications were made to insure reliable elicitation of fear-potentiated startle and to eliminate potential confounds attributable to factors such as temperamental differences in the subject animals. For the following reasons, however, we do not believe that the changes have materially affected the outcome of the experiments. In experiment 1, baseline startle intensities were presented only once, and a 110 dB startle probe was selected. After experiment 1 was conducted, Lissek et al. (2005) suggested a more thorough analysis of baseline startle and selection of a startle stimulus that was of modest intensity. This information was used to enhance our strategy in experiment 2. To determine a consistent baseline startle response, we used fewer startle stimuli, over the same intensity range, but presented these stimuli over four daily sessions rather than just a single session. Based on the baseline response curves, we selected a 100 dB startle probe. Because the startle probes reliably elicited startle in both experiments, we can rule out the possibility that differences in baseline procedure contributed to the behavioral outcome. In fact, given the repeated baseline sessions in experiment 2, we might have predicted greater habituation to the startle stimulus. Yet, this was the experiment in which the amygdala lesion did not affect retention of the potentiated startle response. We also altered the amount of pretraining habituation to the light stimulus. In experiment 1, we determined that unhabituated light, before its pairing with the air puff, enhanced startle in certain animals. To eliminate the potential confound of this temperamental difference in prepotent animal responses, we explicitly habituated all animals to the light in experiment 2 before fear conditioning. Despite this additional training that, if anything, would have diminished fear-potentiated startle, high levels of fear-potentiated startle were nonetheless expressed in the prelesion period. Finally, the conditioning protocol in experiment 1 was different from that in experiment 2. In experiment 1, conditioning was performed in three sessions, and in each session, there were six light-noise and six noise-alone trials. In experiment 2, conditioning was done in a single session with 10 light-noise and 10 noise-alone trials. Relative to our major finding, it is important to note that the level of fear-potentiated startle demonstrated by the control animals was actually higher in experiment 1 than in experiment 2. Yet, there was no fear-potentiated startle expressed by the amygdala-lesioned animals in experiment 1, whereas there was equal or greater fear-potentiated startle expressed by the amygdala lesion group in experiment 2. We conclude, therefore, that although it would have been more elegant to have exactly the same design for each of these experiments, none of the experimental differences are likely to have led to the different outcomes for acquisition versus expression of fear-potentiated startle after the amygdala lesions.

In conclusion, our data in rhesus monkeys suggest that although the amygdala is necessary for the acquisition of fear-potentiated startle (experiment 1), memory for fear-potentiated startle may be stored in extra-amygdala areas that are sufficient for the expression of fear-potentiated startle (experiment 2). However, at the present time, the identity of these extra-amygdala areas is not known. Confirmation of these findings may have important implications for the treatment of pathological fear conditions such as acquired phobias.

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