

Role of the Amygdala in the Coordination of Behavioral, Neuroendocrine, and Prefrontal Cortical Monoamine Responses to Psychological Stress in the Rat

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Exposure to mild stress is known to activate dopamine (DA), serotonin (5-HT), and norepinephrine (NE) metabolism in the anteromedial prefrontal cortex (m-PFC). Neuroanatomical site(s) providing afferent control of the stress activation of the m-PFC monoaminergic systems is at present unknown. The present study used a conditioned stress model in which rats were trained to fear a substartle-threshold tone paired previously with footshock and assessed for behavioral, neuroendocrine, and neurochemical stress responses. Bilateral NMDA-induced excitotoxic lesioning of the basolateral and central nuclei of the amygdala was performed before or after training. Pretraining amygdala lesions blocked stress-induced freezing behavior, ultrasonic vocalizations, adrenocortical activation, and dopaminergic metabolic activation in the m-PFC. Post-training amygdala lesions blocked stress-induced m-PFC DA, 5-HT, and NE

metabolic activation. Post-training amygdala lesions also blocked stress-induced freezing and defecation, and greatly attenuated adrenocortical activation. These data provide evidence of amygdalar control of stress-induced metabolic activation of the monoaminergic systems in the m-PFC, as well as amygdalar integration of behavioral and neuroendocrine components of the rat stress response. These results are discussed in terms of possible relevance to stress-induced exacerbation of schizophrenic symptoms and the pathophysiology of post-traumatic stress disorder.

Key words: dopamine; norepinephrine; serotonin; prefrontal cortex; nucleus accumbens; corticosterone; freezing; ultrasonic vocalization; amygdala; post-traumatic stress disorder; schizophrenia

Animals, including humans, respond to perceived threat with a coordinated set of psychophysiological reactions known as the stress response. In many animals, the stress response is characterized by cessation of ongoing behavior, increased startle reactivity, alterations in cardiovascular functioning, changes in autonomic tone, and activation of neuroendocrine axes. Within the brain, the dopamine (DA), norepinephrine (NE), and serotonin (5-HT) biogenic amine systems that innervate the cortex, and in particular the medial prefrontal cortex (m-PFC), also are activated in response to threatening stimuli (Bliss et al., 1968; Thierry et al., 1976; Weiss et al., 1981; Deutch and Roth, 1990; Tanaka et al., 1990; Inoue et al., 1993). Early investigations (Bliss, 1968) indicated that exposure of rats to an uncontrollable stressor such as footshock resulted in decreased cortical levels of tissue NE and concomitant increases in the levels of noradrenergic metabolites, whereas the same stressor resulted in accelerated metabolism of DA and 5-HT without changing the absolute level of these amines.

Metabolic activation of the DA system in the rat m-PFC is one of the most intensively studied central neurochemical correlates of the stress response (Deutch and Roth, 1990). Exposure to mild stress preferentially activates this m-PFC DA system while not

affecting DA metabolism in other terminal regions, such as the caudatoputamen. The DA cell bodies that are activated by mild stress are located in the ventral tegmental area (VTA) of Tsai in the A10 catecholamine cell group (Lindvall and Bjorklund, 1984) and project to the cortex where they terminate on dendritic shafts of spiny pyramidal cells and/or GABAergic interneurons (Sequela et al., 1988; Goldman-Rakic et al., 1989; Gellman and Aghajanian, 1993; Vincent et al., 1993).

Neuroanatomical tract tracing studies using various tracing techniques have demonstrated that the medial aspect of the central nucleus of the amygdala projects to the VTA, as well as to the locus coeruleus and raphe nuclei (Wallace et al., 1989; Gonzales and Chesselet, 1990; Wallace et al., 1992). Electrophysiological studies have demonstrated that a large majority of presumptive DA cells in the VTA respond to electrical stimulation of the amygdala over both mono- and polysynaptic pathways (Maeda and Mogenson, 1981).

The current study was initiated to investigate the effects of excitotoxic lesion of the amygdala on the behavioral, neuroendocrine, and central neurochemical components of the stress response to determine whether some or all of these responses are coordinated concurrently within the amygdala (LeDoux, 1992). A battery of tests was developed to measure various components of the rat stress reaction elicited in response to a tone paired previously with footshock. Parameters monitored in the present work include freezing behavior, locomotor activity, ultrasonic vocalization, defecation, and serum corticosterone levels. The effects of amygdectomy on stress-induced alterations in DA, NE, and 5-HT utilization in the m-PFC, as well as on the mesolimbic DA

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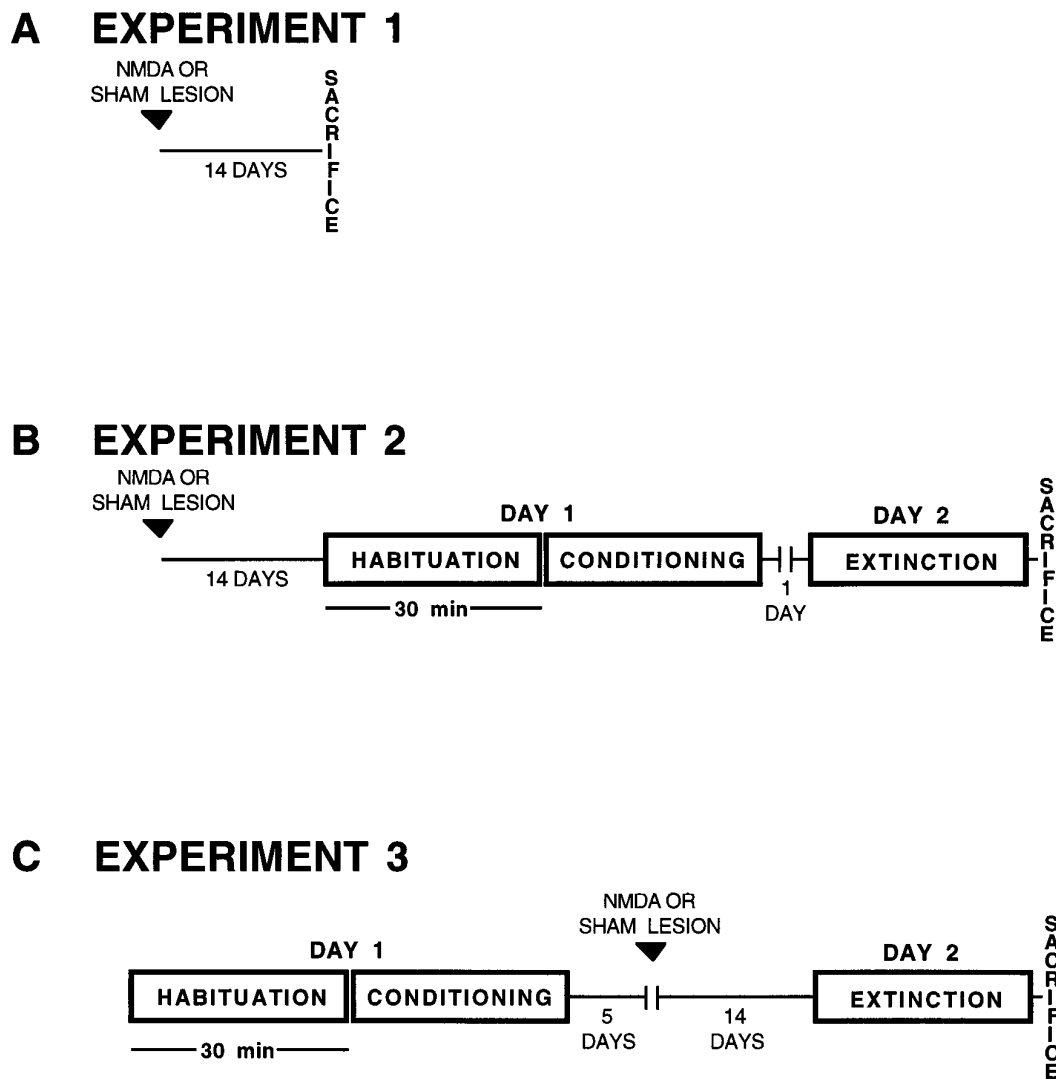


Figure 1. Experimental design for testing the effect of amygdala lesions on the behavioral, neuroendocrine, and neurochemical components of the stress response. *A* shows design of experiment 1 for testing effects of lesions on basal serum corticosterone and basal medial prefrontal cortical biogenic amine metabolism. *B* shows design of experiment 2 for testing the effect of *pretraining* amygdala lesions on stress responses. *C* shows design of experiment 3 for testing the effect of *post-training* amygdala lesions on stress responses. Note that in the post-training design, all animals were surgically naive at the time of conditioning.

and 5-HT systems innervating the nucleus accumbens septi (NAS), also were assessed.

MATERIALS AND METHODS

Animals and housing conditions. Male albino Sprague–Dawley rats (Camm, Wayne, NJ) weighing 300–400 gm were used. All rats were caged individually and acclimated for 2 weeks before experimentation. Rats were maintained on a 12:12 hr light/dark schedule with lights off at 1500 hr. Food and water were available *ad libitum*. All studies were conducted in the dark during the active phase of the rat diurnal activity cycle, beginning 1–6 hr after dark onset. The rationale for testing in the dark was to approximate the psychophysiological conditions when these nocturnal animals are most likely to encounter threatening stimuli under natural conditions.

Apparatus. Testing was conducted in a modified standard conditioning chamber isolated in a sound attenuation cubicle. All surfaces of the chamber were washed with 70% ethanol after each animal exposure. An infrared television camera (Sanyo Electronics, Japan) and infrared illuminator were positioned over the test cage and connected to a video recording system located in an adjoining room. Ultrasonic vocalizations were detected using a miniature narrow-bandwidth microphone (Panasonic, Tokyo, Japan) with a center frequency of 21 kHz. The microphone

signal was amplified, filtered, digitized, and analyzed by computer. Behavioral events (freezing, grooming, crossings, and rears) were monitored remotely on a television screen and coded by an observer using an electronic push-button/toggle-switch assembly connected to a computer.

Surgical procedures. A delivery cannula was constructed from a 5 cm length of 30 gauge stainless steel tubing and attached to the needle of a 2 μ l Hamilton syringe positioned in a manual micrometer. Rats were anesthetized with sodium pentobarbital (65 mg/kg) and atropine bromide (0.3 mg/kg), intraperitoneally, and placed in a stereotaxic frame using blunt ear bars to protect the tympanic membranes. NMDA (Sigma, St. Louis, MO) was dissolved in PBS, final pH 7.4, at a concentration of 20 μ g/ μ l and delivered at an infusion rate of 0.08 μ l/min via an injection cannula. An infusion volume of 0.20 μ l NMDA was delivered at depths of 8.3 mm and 8.1 mm below the skull surface at 2.5 mm posterior to bregma and 4.5 mm lateral to the midline (Paxinos and Watson, 1986). Sham lesions were conducted using PBS infusions at the same coordinates and delivery volumes. All lesions were bilateral.

Conditioning and testing procedures. All studies were conducted in the dark during the active phase of the rat diurnal activity cycle. Assignment to treatment group was random. Animals were run at the same time each evening throughout these studies. The experimental design used in this study is described below and presented schematically in Figure 1.

Experiment 1. For determination of the effects of amygdectomy on basal m-PFC biogenic amine metabolism and basal serum corticosterone, animals received sham or excitotoxic lesions of the amygdala as described above. After a 2 week recovery period, animals were removed from their home cages and killed. These animals were not exposed to the test apparatus.

Experiment 2. To test the effect of *pretraining* amygdectomy on stress responses, animals were prepared with sham or excitotoxic amygdala lesions. These groups were subdivided further into sham lesion/nonstressed (Sh/NS), sham lesion/stressed (Sh/S), amygdectomy/nonstressed (A-/NS), and amygdectomy/stressed (A-/S) groups. After a 2 week postlesion recovery period, each animal was introduced into the test chamber and allowed to explore the cage freely for 30 min. In the last 5 min of this habituation period, three 5 sec, 56 dB, substartle-threshold white noise tones were presented randomly by computer. A 30 min conditioning period then was initiated. In the stress groups, animals were presented with ten 5 sec, 56 dB white noise tones that coterminated with a 0.5 sec, 0.4 mA footshock (measured according to the method of Sananes and Davis, 1992). One tone-footshock pair was presented randomly within each of 10 consecutive 3 min intervals. In the nonstressed control groups, animals were treated as above except that they did not receive footshock. At the conclusion of the conditioning period, test subjects were returned to their home cages. The next day, animals were reintroduced into the testing chamber for 30 min. During this extinction trial, all animals were presented randomly with ten 56 dB white noise tones *without* footshock. At the conclusion of the extinction trial, animals were decapitated rapidly and trunk blood was collected for determination of serum corticosterone and brain tissue obtained for neurochemical analyses.

Experiment 3. To test the effect of *post-training* amygdectomy on stress responses, stressed and nonstressed control groups were habituated and conditioned in the testing chamber as described above. The animals were divided into three groups: Sh/NS, Sh/S, and A-/S. Animals then were subjected to either sham or excitotoxic lesions of the amygdala 5 d after conditioning. After lesioning, the animals were allowed to recover for 2 weeks before extinction testing was conducted as detailed above. Note that these post-training lesioned animals were surgically naive at the time of conditioning.

Behavioral measurements. Ultrasonic vocalizations had to meet the following criteria: (1) Remote behavioral observation of the animal confirmed deep, prolonged respiration at ~0.5 Hz preceded by short, shallow rapid panting at ~3–4 Hz (Fryszak and Neafsey, 1991); (2) Call duration was >20 msec as visualized on a digital oscilloscope; and (3) Call tracings appeared on both the oscilloscope and computer monitor screen. Freezing was defined strictly as absence of visible movement of the animal, including vibrissae, except that related to respiration. This behavior has been used as an index of fear in the rat (Blanchard and Blanchard, 1969; Fanselow, 1980; Conti et al., 1990). Crossings were coded each time the base of the tail crossed gridlines drawn on the video monitoring screen. Rears were counted when an animal raised both forepaws off the cage bars and was not otherwise engaged in grooming behavior. Defecation was quantitated by counting the number of fecal boli present in the test chamber at the end of the conditioning or extinction trial. Animals were coded initially by an observer not blind to the experimental conditions. Video records of 11 randomly selected animals were recoded by an observer blind to the experimental conditions. Statistical analysis showed a very high correlation between results generated by unblind and blind behavioral observations (crossings and rearings, $r^2 = 0.98$; freezing, $r^2 = 0.99$). Therefore, the initial unblinded behavioral results were used for all statistical analyses.

Neurochemical and neuroendocrine analyses. Neurochemical analyses were performed on *ex vivo* tissue samples harvested from the same animals used for behavioral and neuroendocrine measurements. Schematic depictions of the regions dissected for neurochemical analysis are presented in Figure 2. Tissue DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-HT and its metabolite 5-hydroxy-indoleacetic acid (5-HIAA) were isolated according to a modification of the procedure of Reinhard et al. (1982). These compounds were assayed using reversed-phase HPLC coupled with amperometric electrochemical detection. Regional levels of DA and 5-HT system metabolic activity were assessed by calculating the ratio of the acid metabolite to the parent neurotransmitter in postmortem tissue. We did not observe any significant changes in the absolute tissue level values of DA or 5-HT in response to stress, a finding consistent with a large body of previous work dating back to the 1960s (Bliss et al., 1968) and with previous work from this laboratory (Goldstein et al., 1994). Therefore, as in our previous work,

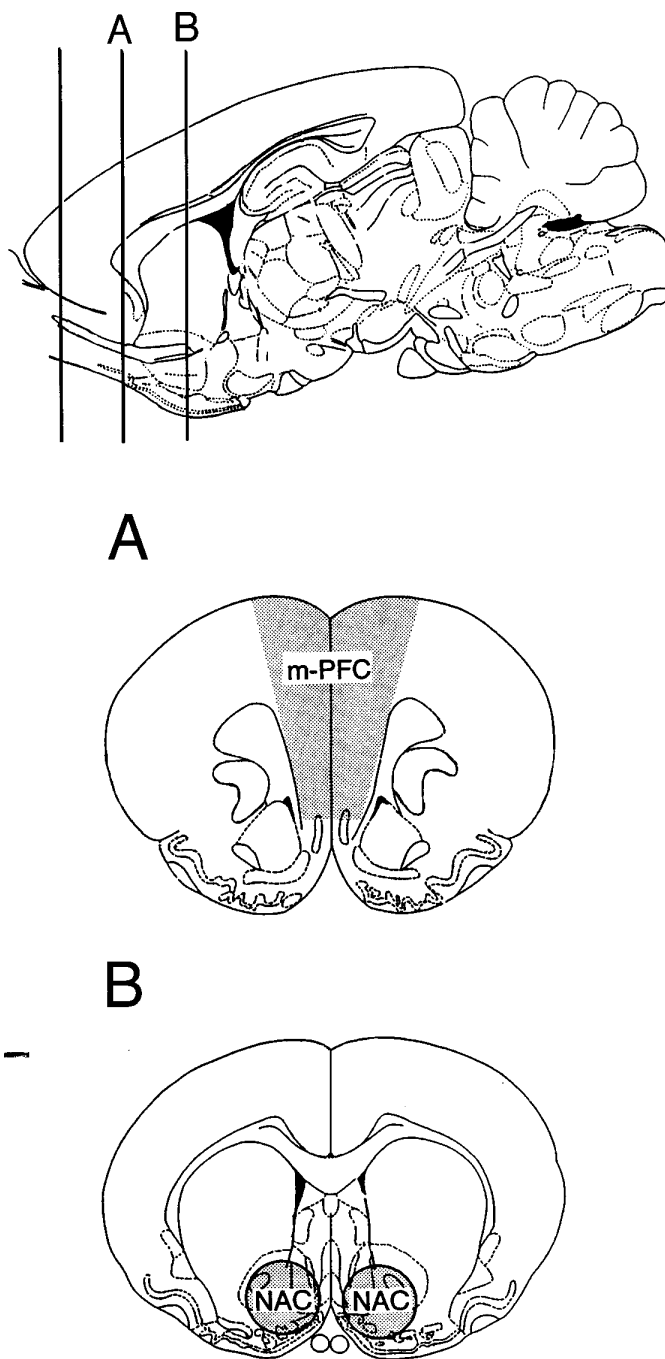


Figure 2. Atlas diagram detailing tissue regions harvested for *postmortem* monoamine neurochemical analyses. Tissue sections (2 mm) were obtained after killing and dissected as indicated. *m-PFC*, Medial prefrontal cortex; *NAC*, nucleus accumbens.

we present DA and 5-HT metabolic activity as the ratio of metabolite to parent neurotransmitter. Measurement of cortical NE metabolism was determined in a separate assay from tissue measurements of the major rat noradrenergic metabolite 3-methoxy-4-hydroxyphenylglycol sulfate (MHPG), by a modification of the method of Elsworth et al. (1983). Results are expressed as total MHPG (i.e., free plus sulfate conjugated MHPG). We did not measure endogenous levels of NE in the current study. Serum corticosterone was measured by a magnetic radioimmunoassay developed in this laboratory as detailed in a previous report (Goldstein et al., 1994). Tissue samples from some animals were excluded from analysis because of technical difficulties with individual dissections or sample preparation.

Histological procedures. Formalin-fixed brains were sectioned (40 μ m),

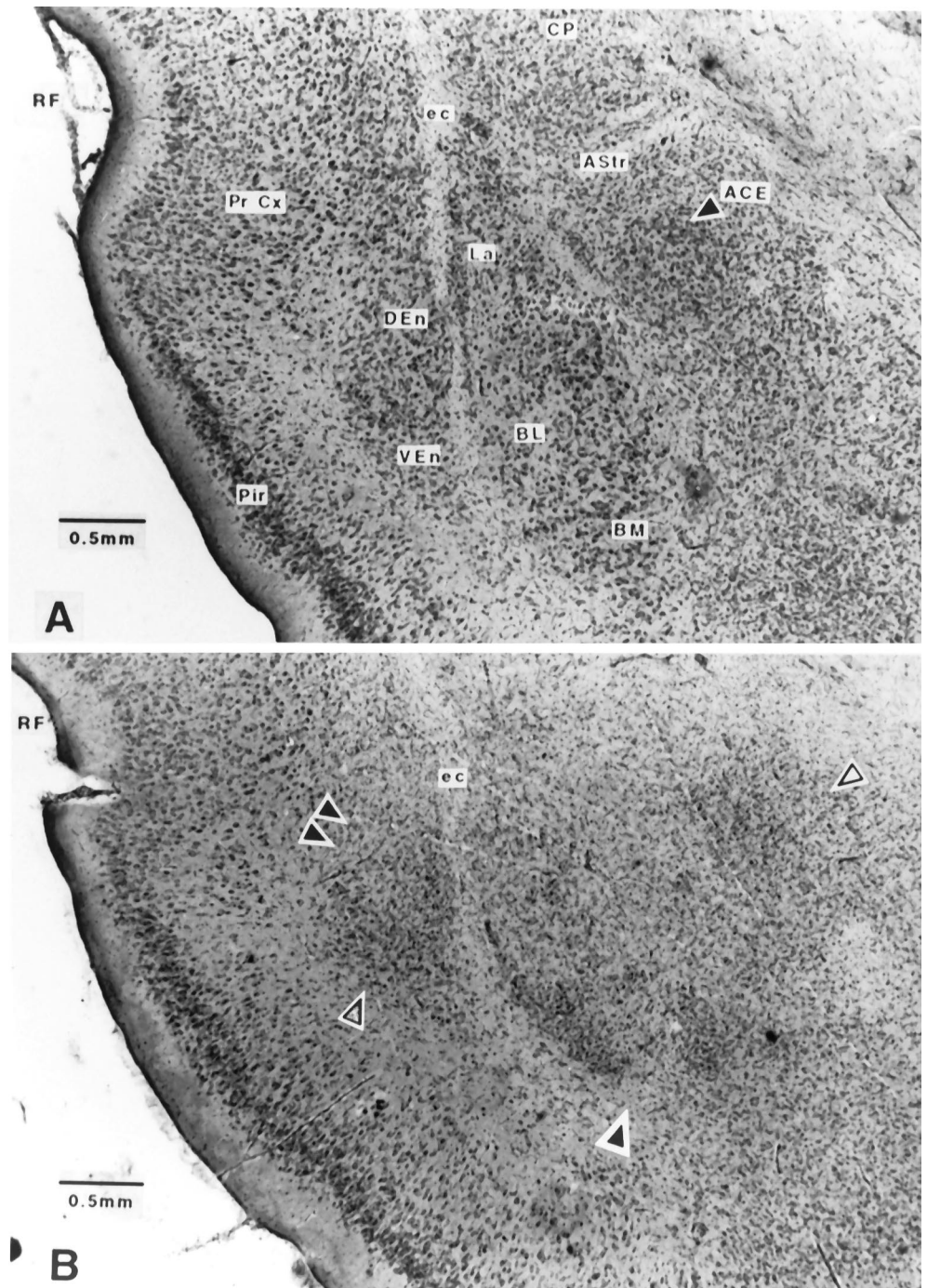


Figure 3. Sham and excitotoxic lesions of the amygdala. *A*, Sham lesions of the amygdala. *ACE*, Central nucleus of the amygdala; *AStr*, amygdalostratial transition zone; *BL*, basolateral nucleus of the amygdala; *BM*, basomedial nucleus of the amygdala; *CP*, caudatoputamen; *DEn*, dorsal endopiriform nucleus; *ec*, external capsule; *La*, lateral nucleus of the amygdala; *Pir*, piriform cortex; *Pr Cx*, perirhinal cortex; *VEn*, ventral endopiriform nucleus. *B*, Excitotoxic lesions of the amygdala. *RF*, Rhinal fissure; *ec*, external capsule. Note heavy gliosis and neuronal dropout in the lateral, basolateral, and central nuclei of the amygdala. *Double black arrows* and *single shaded arrow* indicate gliosis and neuronal dropout in dorsal and ventral endopiriform nuclear regions, respectively. *Single black arrow* points to neuronal dropout in the ventral basolateral and basomedial nuclei of the amygdala. *Single open arrow* points to heavy gliosis and neuronal dropout in the central nucleus of the amygdala. Photomicrographs at $4.5\times$ after formalin fix and cresyl violet stain. Scale bars are as noted in photomicrographs.

mounted, and stained with cresyl violet. The extent of neuronal cell loss and reactive gliosis was assessed microscopically and transcribed onto atlas sections from Paxinos and Watson (1986). Histological criteria for inclusion in the amygdalotomy group required evidence of bilateral damage (i.e., heavy gliosis, neuronal degeneration and dropout, and/or cavitation) within the rostrocaudal extent of the basolateral and central amygdala nuclei.

Statistics. Data from experiments 1 and 3 were analyzed by one-way ANOVA for significant effect of treatment. A two-way ANOVA was used in experiment 2 to test for significant effects of lesion, treatment, and lesion-by-treatment interaction. The Student–Newman–Keuls post hoc multiple comparison procedure was used for comparison among multiple means when appropriate. Temporal analyses of behavioral data were made using repeated-measures ANOVA. All analyses were conducted using the SuperANOVA computer program (Abacus Concepts, Berkeley,

CA). Values are expressed as means \pm SEM. Statistical significance was set at $p < 0.05$.

RESULTS

Verification of lesions

Representative photomicrographs of amygdala lesions are shown in Figure 3. A schematic depiction of the range of amygdala lesions is shown in Figure 4. The saline-injected sham control animals did not sustain damage to any part of the amygdala, adjacent perirhinal cortex, or caudatoputamen. Examination of brain sections from animals sustaining excitotoxic lesion of the amygdala revealed marked bilateral gliosis and bilateral neuronal

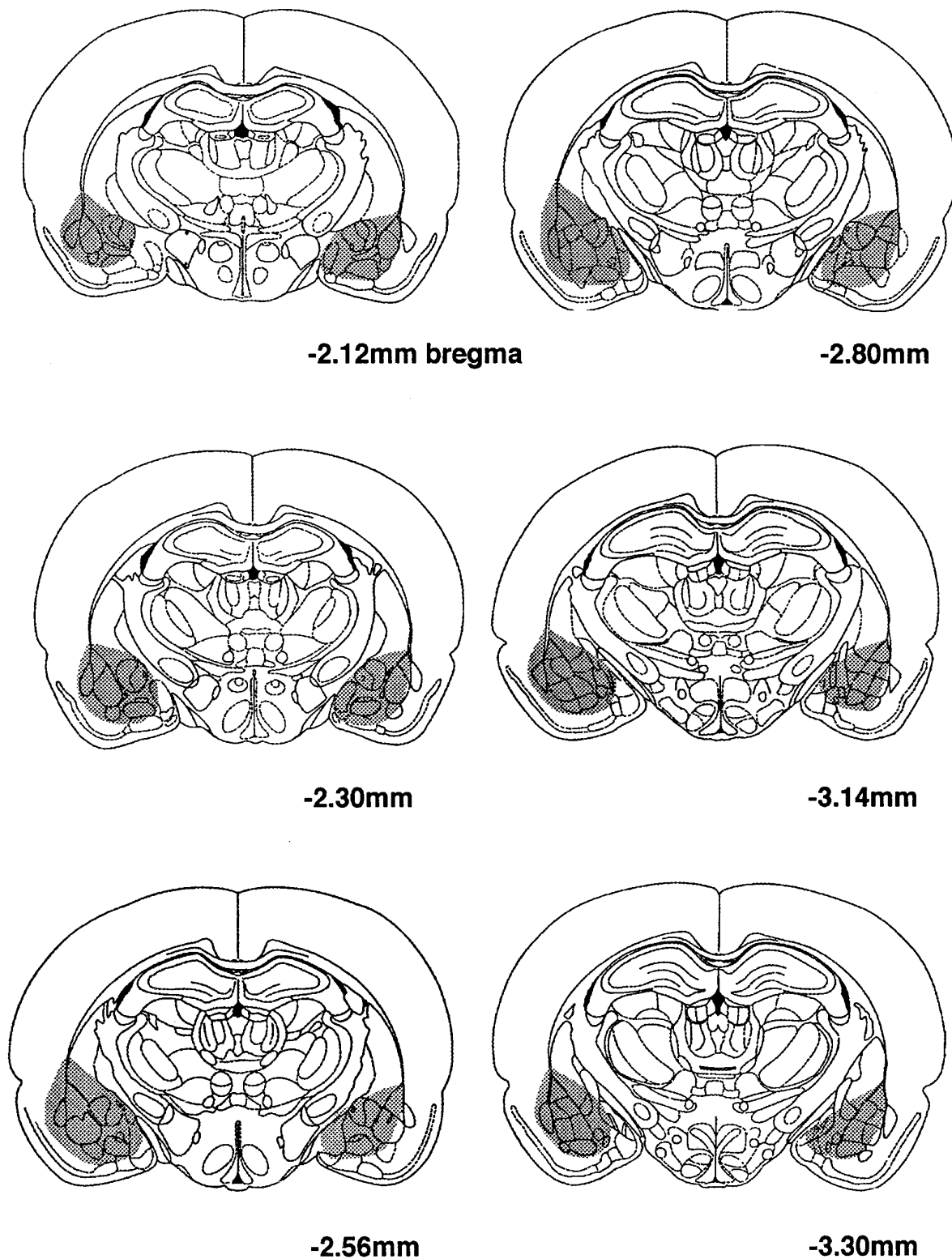


Figure 4. Depiction of the range of amygdala lesions in this study (superimposed on schematic diagram reproduced from Paxinos and Watson, 1986).

degeneration throughout the rostral–caudal extent of this structure, typically extending between 2.00 mm and 3.30 mm posterior to bregma (Paxinos and Watson, 1986). The lesion area included the lateral and basolateral nuclei as well as the central nucleus. The largest lesions spread medially to include the basomedial and

medial nuclear complexes. Some animals in the pretraining and post-training experiments also sustained damage unilaterally or bilaterally in the dorsal and ventral endopiriform nuclei. These histological findings are consistent with other descriptions of large NMDA lesions in these areas (Cahill and McGaugh, 1990; Gal-

Table 1. Effect of amygdala lesion on basal neurochemistry and serum corticosterone

m-PFC		SHAM <i>n</i> = 8	AMYG- <i>n</i> = 9	
DA	(ng/mg protein)	1.80 ± 0.22	1.93 ± 0.22	ND
DOPAC	(ng/mg protein)	0.25 ± 0.03	0.26 ± 0.03	ND
DOPAC/DA		0.14 ± 0.01	0.13 ± 0.01	ND
5-HT	(ng/mg protein)	10.8 ± 0.87	11.52 ± 0.83	ND
5-HIAA	(ng/mg protein)	2.32 ± 0.18	2.49 ± 0.19	ND
5-HIAA/5-HT		0.21 ± 0.01	0.22 ± 0.02	ND
Corticosterone	(ng/ml serum)	98.8 ± 20.4	67.7 ± 13.8	ND

Effect of amygdala lesion on basal cortical DA and 5-HT utilization in the m-PFC and basal serum corticosterone. Animals were subjected to bilateral sham or excitotoxic lesioning of the amygdala 2 weeks before being killed. Animals were never exposed to the conditioning chamber and were simply removed from the home cage before being killed. ND, No statistically significant effect of lesion, $p > 0.05$.

lagher et al., 1990; Sananes and Davis, 1992). In 8 of the original 26 lesioned animals in the pretraining experiment, the lesions were judged incomplete by the criteria above and were excluded from the data analyses. Only 1 of 10 lesioned animals in the post-training experiment failed to meet lesion criteria.

Experiment 1: effect of amygdala lesions on basal corticosterone, DA, and 5-HT metabolism in the m-PFC

To examine the effect of amygdectomy on basal serum corticosterone levels and m-PFC biogenic amine utilization, animals were prepared with either sham or NMDA-induced amygdala lesions. Two weeks after lesioning, animals were killed. Note that these animals were not exposed to the conditioning apparatus. Mean m-PFC DOPAC/DA and 5-HIAA/5-HT levels for sham- and amygdala-lesioned animals were statistically indistinguishable as shown in Table 1. Thus, excitotoxic lesions of the amygdala do not alter basal m-PFC DA or 5-HT metabolism when compared with sham lesions. Likewise, no significant difference was noted in serum corticosterone levels between sham- and amygdala-lesioned animals, 99 ± 20 and 68 ± 14 ng/ml, respectively; note the trend toward lower serum corticosterone levels in the lesioned group. Measurement of MHPG was not made in this set of animals.

Experiment 2a: effect of pretraining amygdala and sham lesions on conditioned stress-induced behavioral, neuroendocrine, and m-PFC DA responses

In experiment 2, the effects of *pretraining* NMDA lesions of the amygdala on behavioral, neuroendocrine, and neurochemical indices of aversive conditioning were assessed. Two weeks after sham or excitotoxic lesion of the amygdala, animals were subjected to aversive conditioning with extinction trial testing on the following day. Note that data presented in this section are derived from responses during the extinction trial on day 2 when animals are presented only with the tone (i.e., without footshock).

Behavioral results are presented in Figure 5A. Significant effects on freezing behavior were observed for lesion, $F_{(1,32)} = 59$, $p < 0.0001$; treatment, $F_{(1,32)} = 58$, $p < 0.0001$; and lesion by treatment interaction, $F_{(1,32)} = 54$, $p < 0.0001$. Both the Sh/NS and A-/NS groups demonstrated low levels of freezing, with mean percent of time freezing: 2 ± 2 and $0.3 \pm 0.3\%$, respectively. These two groups were statistically indistinguishable. In contrast, the Sh/S group exhibited marked freezing behavior, $64 \pm 8\%$, which was statistically different from the Sh/NS and A-/NS groups, $p < 0.0001$. The A-/S group showed block of this stress-induced

freezing response with a mean freezing response of $1 \pm 1\%$, $p < 0.0001$ versus the Sh/S group. The A-/S freezing response was statistically indistinguishable from either of the two nonstressed groups.

The effects of pretraining amygdectomy on serum corticosterone are depicted in Figure 5B. Significant effects on adrenocortical activation were detected for lesion, $F_{(1,33)} = 14$, $p < 0.001$; treatment, $F_{(1,33)} = 5$, $p < 0.05$; and lesion by treatment interaction, $F_{(1,33)} = 4$, $p < 0.05$. Both the Sh/NS and A-/NS groups demonstrated low, statistically indistinguishable corticosterone levels: 137 ± 30 and 87 ± 14 ng/ml, respectively. In contrast, the Sh/S group exhibited a marked stress-induced elevation in serum corticosterone to 277 ± 46 ng/ml, a 102% increase over the Sh/NS group, $p < 0.01$. The A-/S group showed complete block of this stress-induced serum corticosterone elevation with a mean level of 83 ± 25 ng/ml; this was significantly different from the Sh/S group, $p < 0.001$, and statistically indistinguishable from either the Sh/NS or A-/NS groups. This result cannot be attributed to amygdala lesion-induced suppression of baseline serum corticosterone levels (see above, Experiment 1).

The effect of pretraining amygdectomy on m-PFC DA utilization is shown in Figure 5C. Significant effects on m-PFC DA activation were detected for lesion, $F_{(1,33)} = 9$, $p < 0.005$; treatment, $F_{(1,33)} = 7$, $p < 0.02$; and lesion by treatment interaction, $F_{(1,33)} = 15$, $p < 0.001$. Both the Sh/NS and A-/NS groups demonstrated low DOPAC/DA ratios, 0.16 ± 0.2 and 0.18 ± 0.02 , respectively. In contrast, the Sh/S group exhibited a marked stress-induced elevation in m-PFC DA utilization to 0.28 ± 0.02 , a 71% increase in the DOPAC/DA ratio versus the Sh/NS group, $p < 0.001$. This effect was blocked in the A-/S group, which exhibited a mean DOPAC/DA ratio of 0.16 ± 0.01 ; this was significantly different from the Sh/S group, $p < 0.001$, and statistically indistinguishable from the Sh/NS and A-/NS groups. This result cannot be attributed to amygdala lesion-induced suppression of the baseline m-PFC DOPAC/DA ratio (see above, Experiment 1).

Experiment 2c: effect of pretraining amygdala lesions on behavioral indices of unconditioned stress

Behavior during the conditioning period on day 1 also was altered by amygdectomy (Fig. 5D). Note that the data to be presented in this section refer to behavior observed during the conditioning period (second half-hour on the training day, day 1) when animals were receiving either tone-shock pairs or tones alone without shock. Animals that were subjected to the tone alone during conditioning did not freeze, vocalize, or defecate during the second half-hour of training on day 1.

A significant effect of amygdectomy was noted on freezing behavior *during conditioning*, $F_{(1,17)} = 68$, $p < 0.0001$. The mean percentages of time engaged in freezing during the conditioning session for the Sh/S and A-/S groups were 53 ± 6 and $3 \pm 1\%$, respectively. A significant effect of lesion also was noted for ultrasonic vocalizations *during conditioning*, $F_{(1,18)} = 11$, $p < 0.005$. The mean number of vocalizations during the conditioning session for the Sh/S and A-/S groups were 239 ± 80 and 0 ± 0 calls, respectively. A significant effect of lesion also was noted for stress-induced defecation *during conditioning*, $F_{(1,18)} = 24$, $p < 0.0001$. The mean number of fecal boli produced during the second half-hour of the conditioning session for the Sh/S and A-/S groups were 14 ± 1 and 4 ± 1 fecal boli, respectively. Thus, the amygdectomy group showed blockade of freezing, ultrasonic vocalization, and defecation during presentation of tone-shock pairs when compared with the sham-lesioned animals.

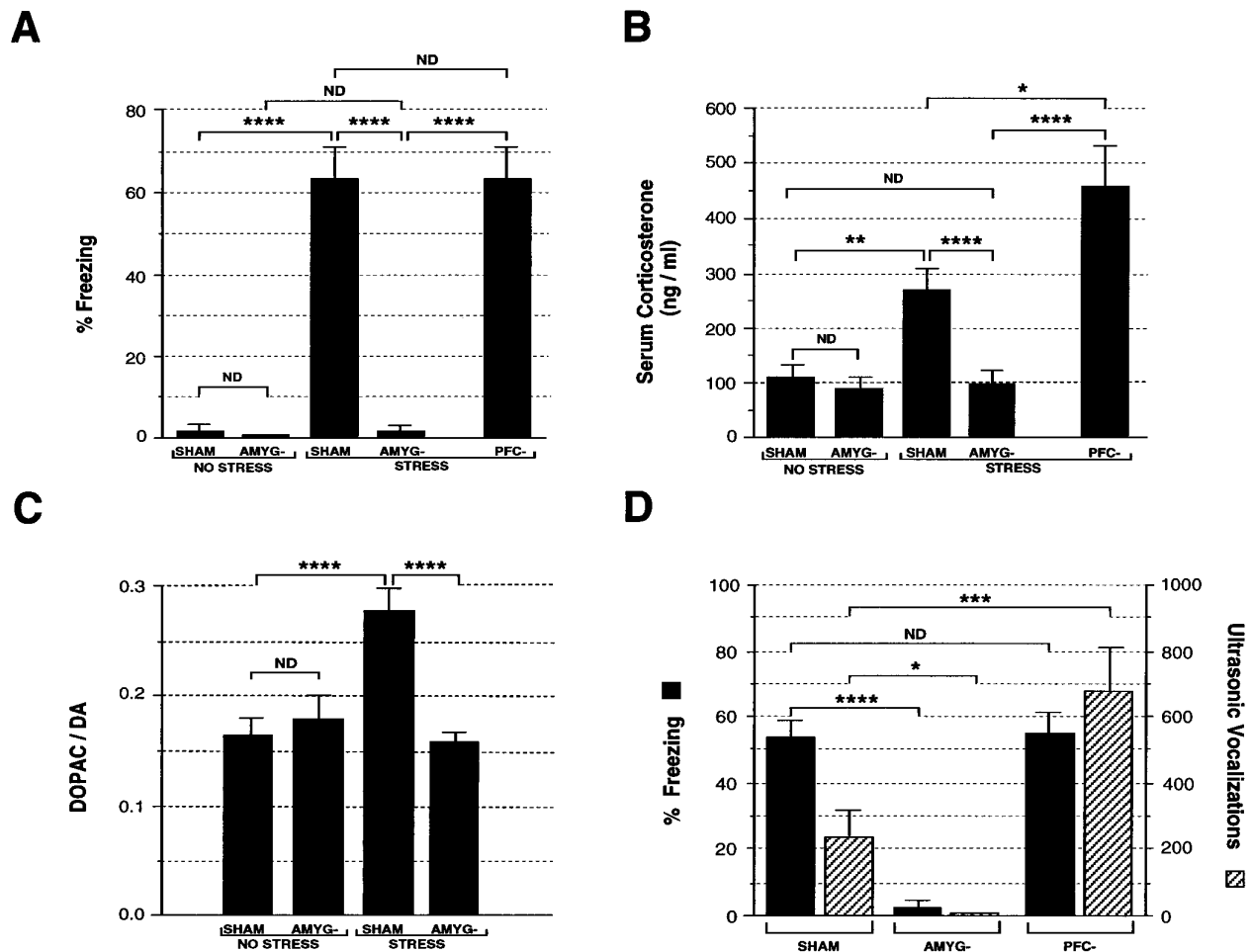


Figure 5. Effect of pretraining amygdala lesions on behavioral, neuroendocrine, and neurochemical indices of the stress response. *A* shows effect of pretraining amygdala lesions on stress-induced freezing behavior. *B* shows effect of pretraining amygdala lesions on stress-induced serum corticosterone levels. *C* shows effect of pretraining amygdala lesions on stress-induced DA metabolism (expressed as the DOPAC/DA ratio) in the m-PFC. *D* shows effect of amygdala lesions on stress-induced freezing and ultrasonic vocalization during the conditioning period. Animals were subjected to bilateral sham (SHAM) or excitotoxic lesions of the amygdala (AMYG-) 14 d before conditioning. STRESS and NO STRESS indicate experimental conditions in which animals were or were not exposed to shock during conditioning, respectively. Number of animals per group are (*A–C*) SHAM/NO STRESS, 10; AMYG-/NO STRESS, 7; SHAM/STRESS, 9; AMYG-/STRESS, 10 (*A*) and 11 (*B, C*); and (*D*) SHAM, 9; AMYG-, 10. ****, Statistically significant difference, $p < 0.0001$; ***, statistically significant difference, $p < 0.001$; **, statistically significant difference, $p < 0.01$; *, statistically significant difference, $p < 0.05$; ND, no statistically significant difference.

Experiment 3: effect of post-training amygdala lesions on behavioral and neuroendocrine indices of conditioned stress

Because the pretraining amygdala lesions appeared to alter several indices of the behavioral stress response during aversive conditioning, additional studies were carried out to assess the effect of post-training amygdala lesions on the behavioral, neuroendocrine, and neurochemical conditioned stress responses. In this series of experiments, aversive conditioning was conducted before lesioning (Fig. 1), thus avoiding the potentially confounding effect of the lesion on learning. Note that nearly 3 weeks (19 d) intervened between conditioning and testing.

ANOVA revealed a significant effect of treatment on freezing behavior, $F_{(2,19)} = 29$, $p < 0.0001$ (Fig. 6A). The mean percentages of time engaged in freezing during the extinction period were 0.5 ± 0.5 , 48 ± 9 , and $0.3 \pm 0.2\%$ for the Sh/NS, Sh/S, and A-/S groups, respectively. Post-training amygdala lesions thus resulted in blockade of the stress-induced freezing response.

Changes in locomotor behavior, measured as the combined score for crossing and rears, also were noted in the post-training amygdala lesions group. ANOVA revealed a significant effect of treatment on total locomotor activity, $F_{(2,18)} = 46.10$, $p < 0.0001$. The mean locomotor scores during the extinction period were 282 ± 32 , 61 ± 18 , and 317 ± 17 for the Sh/NS, Sh/S, and A-/S groups, respectively. Note that within the sham lesion groups, the stress condition resulted in a 81% decrease in locomotor activity; post-training amygdala lesions blocked this stress-induced suppression of locomotion. The temporal course of this effect is presented in Figure 6B.

ANOVA revealed a significant effect of treatment on the number of fecal boli produced during the extinction trials, $F_{(2,18)} = 16$, $p < 0.02$. Mean number of fecal boli in the Sh/NS, Sh/S, and A-/S groups were 2 ± 1 , 6 ± 1 , and 1 ± 1 , respectively.

Adrenocortical activation also was affected by post-training amygdala lesions (Fig. 6A). ANOVA revealed a significant effect of treatment on serum corticosterone levels, $F_{(2,18)} = 17$, $p < 0.0001$. The mean serum corticosterone levels after the extinction period

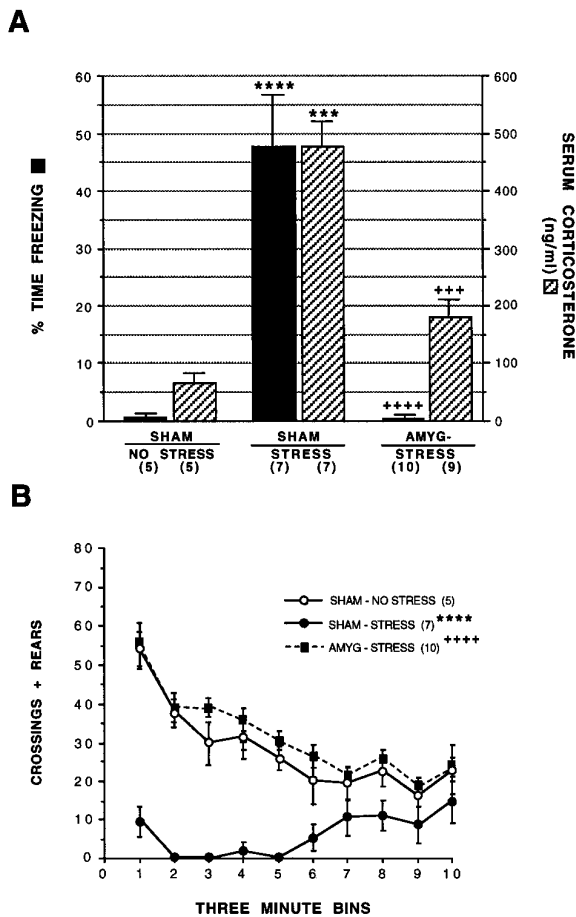


Figure 6. Effect of post-training amygdala lesions on behavioral and neuroendocrine indices of the stress response. *A* shows effect of post-training amygdala lesions on stress-induced freezing behavior and serum corticosterone levels. *B* shows effect of post-training amygdala lesions on the temporal course of locomotor activity (*Crossings + Rears*) during the 10 successive 3 min time bins that make up the 30 min extinction period. Note that the amygdala lesion group exposed to stress behaves similarly to the sham lesion control group, which was not exposed to stress. Animals were subjected to bilateral sham (*SHAM*) or excitotoxic lesions of the amygdala (*AMYG-*) 5 d after conditioning. *STRESS* and *NO STRESS* indicate experimental conditions in which animals were or were not exposed to shock during conditioning, respectively. ****, Statistically significant difference versus the sham/no stress group, $p < 0.0001$; ***, statistically significant difference versus the sham/no stress group, $p < 0.001$; +++++, statistically significant difference versus the sham/stress group, $p < 0.0001$; +++, statistically significant difference versus the sham/stress group, $p < 0.001$. Numbers in *parenthesis* indicate number of animals in each group.

were 65 ± 17 , 465 ± 76 , and 157 ± 25 ng/ml for the Sh/NS, Sh/S, and A/S groups, respectively. Post-training amygdalaectomy thus resulted in a 66% attenuation of the stress-induced adrenocortical response.

Experiment 3: effect of post-training amygdala lesions on neurochemical indices of stress

The results of neurochemical analysis of m-PFC DA utilization are shown in Figure 7*A*. ANOVA revealed a significant effect of treatment on m-PFC DA utilization, $F_{(2,18)} = 21$, $p < 0.0001$. The mean DOPAC/DA ratios after the extinction period were 0.12 ± 0.01 , 0.25 ± 0.03 , and 0.13 ± 0.01 for the Sh/S, Sh/NS, and A/S groups, respectively. Note that within the sham lesion groups, conditioned stress resulted in a 124% increase in m-PFC DA

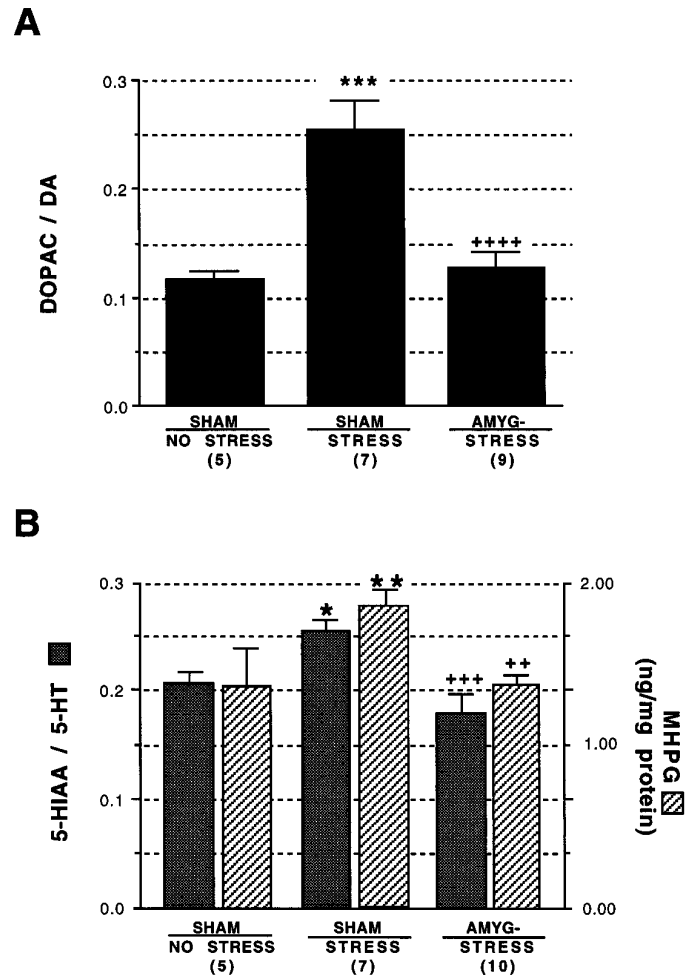


Figure 7. Effect of post-training amygdala lesions on neurochemical indices of the stress response in the m-PFC. *A* shows effect of post-training amygdala lesions on stress-induced DA utilization in the m-PFC. ***, Statistically significant difference versus the sham/no stress group, $p < 0.001$; +++++, statistically significant difference versus the sham/stress group, $p < 0.0001$. *B* shows effect of post-training amygdala lesions on stress-induced 5-HT utilization and NE metabolism in the m-PFC. Animals were subjected to bilateral sham (*SHAM*) or excitotoxic lesions of the amygdala (*AMYG-*) 5 d after training. *STRESS* and *NO STRESS* indicate experimental conditions in which animals were or were not exposed to shock during conditioning, respectively. **, Statistically significant difference versus the sham/no stress group, $p < 0.01$; *, statistically significant difference versus the sham/no stress group, $p < 0.05$; +++, statistically significant difference versus the sham/stress group, $p < 0.001$; ++, statistically significant difference versus the sham/stress group, $p < 0.01$. Numbers in *parenthesis* indicate number of animals in each group.

utilization, compared with the 71% increase when analyses were done on tissue obtained immediately after aversive conditioning. Post-training amygdalaectomy resulted in blockade of this stress-induced elevation in m-PFC DA utilization. Also note that the mean m-PFC DOPAC/DA ratio for the A/S group was statistically indistinguishable from the Sh/NS control group, paralleling the results from the pretraining lesion study.

To answer the question of whether the effect of post-training amygdalaectomy on DA utilization is specific for this neurotransmitter system, neurochemical measurements of 5-HT and NE utilization were performed on the same m-PFC tissue samples on which the DA analyses were conducted. These data are shown in Figure 7*B*. ANOVA revealed a significant effect of treatment on

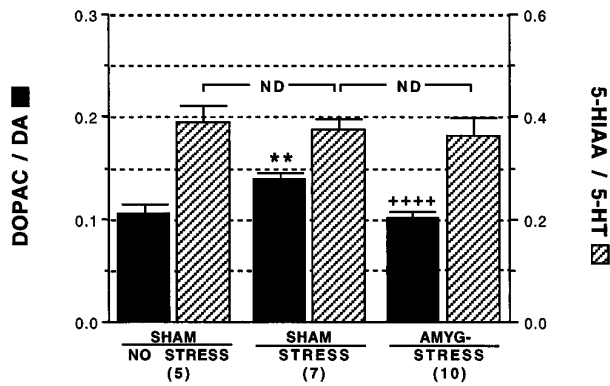


Figure 8. Effect of *post-training* amygdala lesions on stress-induced DA and 5-HT utilization in the NAS. Animals were subjected to bilateral sham (SHAM) or excitotoxic lesions of the amygdala (AMYG-) 5 d after conditioning. STRESS and NO STRESS indicate experimental conditions in which animals were or were not exposed to shock during conditioning, respectively. **, Statistically significant difference versus the sham/no stress group, $p < 0.01$; +++++, statistically significant difference versus the sham/stress group, $p < 0.0001$; ND, no statistically significant difference between groups, $p > 0.05$. Numbers in parenthesis indicate number of animals in each group.

m-PFC 5-HT utilization, $F_{(2,19)} = 11$, $p < 0.001$. The mean 5-HIAA/5-HT ratios after the extinction period were 0.21 ± 0.01 , 0.26 ± 0.01 , and 0.18 ± 0.001 for the Sh/NS, Sh/S, and A-/S groups, respectively. Note that within the sham lesion groups, the stress condition resulted in a statistically significant 22% increase in m-PFC 5-HT utilization, $p < 0.05$, whereas post-training amygdectomy resulted in blockade of this stress-induced elevation, $p < 0.001$. Also note that the mean m-PFC 5-HIAA/5-HT ratio for the A-/S group was statistically indistinguishable from the Sh/NS control group. ANOVA also revealed a significant effect of treatment on m-PFC MHPG concentration, $F_{(1,19)} = 7$, $p < 0.01$. The mean m-PFC MHPG concentrations after the extinction period were 1.4 ± 0.2 , 1.8 ± 0.1 , and 1.4 ± 0.04 ng/mg protein for the Sh/S, Sh/NS, and A-/S groups, respectively. Note that within the sham lesion groups, the conditioned stress resulted in a statistically significant 27% increase in m-PFC MHPG concentration, whereas post-training amygdectomy resulted in blockade of this stress-induced elevation of MHPG. Also note that the mean m-PFC MHPG concentration for the A-/S group was statistically indistinguishable from that of the Sh/NS group.

The regional specificity of the effect of amygdectomy on m-PFC DA utilization was assessed by examining the DOPAC/DA ratio in the NAS. These data are shown in Figure 8. ANOVA revealed a significant effect of treatment on NAS DA utilization, $F_{(2,19)} = 22$, $p < 0.0001$. The mean DOPAC/DA ratios after the extinction period were 0.11 ± 0.01 , 0.14 ± 0.005 , and 0.11 ± 0.002 for the Sh/NS, Sh/S, and A-/S groups, respectively. Note that within the sham lesion groups, the conditioned stress resulted in a statistically significant 23% increase in NAS DA utilization, whereas post-training amygdectomy resulted in blockade of this stress-induced elevation, $p < 0.0001$. Also note that the mean NAS DOPAC/DA ratio for the A-/S group was statistically indistinguishable from the Sh/NS control group. Note that the 5-HT system in the NAS was not activated by stress (Fig. 8). Mean 5-HT utilization values for the Sh/NS, Sh/S, and A-/S groups were 0.39 ± 0.04 , 0.38 ± 0.02 , and 0.37 ± 0.04 , respectively. Noradrenergic metabolism in the NAS was not assessed.

DISCUSSION

The principal finding of this study is that bilateral excitotoxic amygdala lesions result in blockade of the mesocortical monoaminergic responses to stress induced by reexposure to stimuli paired previously with an unconditioned stressor. The amygdala lesions also attenuated associated adrenocortical activation, freezing, ultrasonic vocalization, and defecation. These findings suggest that the amygdala is important in linking aversive stimuli to the normally contingent behavioral, neuroendocrine, and cortical monoamine responses to psychological stress.

Pretraining versus post-training lesions of the amygdala

Pretraining amygdectomy blocked behavioral responses to footshock and to stress induced by reexposure to stimuli paired previously with footshock. It is noteworthy that amygdala lesions have not been found to alter flinch amplitude or the flinch threshold to footshock (Cahill and McGaugh, 1990; Sananes and Davis, 1992). However, the finding that pretraining amygdectomy blocked ultrasonic vocalization, freezing, and defecation during conditioning suggests that this lesion may have interfered with the perception and/or expression of fear during conditioning and, thus, may have altered aversive memory formation.

This possibility prompted a study using post-training amygdectomy; i.e., subjects were conditioned *before* lesioning, thus obviating the potentially confounding effect of the lesion on efficacy of conditioning. As with the pretraining lesions, post-training lesions greatly attenuated adrenocortical activation and blocked freezing, defecation, and conditioned stress-induced activation of m-PFC monoamine metabolism.

The amygdala and afferent control of the m-PFC DA, NE, and 5-HT systems

Increased DA utilization in the m-PFC is produced by a variety of unconditioned aversive stimuli (Thierry et al., 1976; Fadda et al., 1978; Reinhard et al., 1982; Claustre et al., 1986; Roth et al., 1988; Pei et al., 1990), unconditioned psychological stress (Kaneyuki et al., 1991), and conditioned stress (Herman et al., 1982; Deutch et al., 1985; Goldstein et al., 1994). Exposure to more substantial or prolonged stress results in recruitment of the mesolimbic DA system innervating the NAS (Roth et al., 1988). Consistent with these studies, we demonstrated a robust increase in m-PFC DA utilization in response to stimuli paired previously with footshock; a more modest increase was noted in the NAS.

The finding that both pretraining and post-training amygdectomy block the increase in m-PFC DA utilization implicates the amygdala in afferent control of the mesocortical DA system response to psychological stress. This extends work by Davis et al. (1994) that demonstrated that amygdala lesions block activation of the m-PFC DA system in response to footshock and novelty. The central nucleus of the amygdala projects to the VTA (Gonzales and Chesselet, 1990), site of the DA perikarya that innervate the m-PFC. In addition, the central nucleus communicates indirectly with the VTA through projections to the bed nucleus of the stria terminalis, periaqueductal gray (PAG), lateral hypothalamus, and nucleus parabrachialis (Phillipson, 1979).

Alternatively, amygdala influence on m-PFC DA metabolism could involve control of DA release and/or metabolism locally within the m-PFC. This hypothesis is based on the presence of reciprocating connections between the cortex-like basolateral amygdala nucleus and m-PFC (Krettek and Price, 1977; Sesack et al., 1989; McDonald, 1991). Electrophysiological studies have

demonstrated that stimulation of the basolateral nucleus alters neuronal firing in the m-PFC with latencies consistent with mono- and polysynaptic pathways (Perez-Jaranay and Vives, 1991). The m-PFC in turn sends efferent projections that synapse on DA neurons in the VTA (Sesack and Pickel, 1992).

Exposure to stress also results in regional increases in NE metabolism in rodents, (Dunn, 1988; Tanaka et al., 1990) (for review, see Glavin, 1985), primates (Mason et al., 1968; Redmond, 1987), and humans (Rubin et al., 1970; Maas et al., 1971; Sweeney et al., 1978). Recent studies using *in vivo* microdialysis have shown that m-PFC NE release is enhanced by unconditioned stress (Cenci et al., 1992; Nakane et al., 1994; Finlay et al., 1995). These findings are consistent with observed stress-induced increases in electrophysiological activity in locus coeruleus neurons (Jacobs et al., 1991).

Consistent with these observations, we have demonstrated that NE metabolism in the m-PFC is activated by the stress of reexposure to stimuli paired previously with footshock. We did not measure endogenous levels of NE in the current study. It is very likely, however, that the tissue levels of NE decline after exposure to our conditioned stress model, similar to findings documented in a wealth of previous work from many other laboratories investigating the effect of stress on the noradrenergic system (Tsuda et al., 1986). The noradrenergic metabolic activation noted in the present study was blocked by post-training amygdectomy, suggesting that the amygdala may provide functional afferent regulatory input to the NE system during psychological stress. The central nucleus of the amygdala is known to send indirect (Aston-Jones et al., 1986) and direct (Cedarbaum and Aghajanian, 1978; Wallace et al., 1989) projections to the locus coeruleus, site of the NE cell bodies that innervate the cortex (Lindvall and Bjorklund, 1984). Immunohistochemical studies also have demonstrated that afferents from the central nucleus of the amygdala impinge on tyrosine hydroxylase-positive neurons in the rostral portion of the locus coeruleus (Wallace et al., 1989, 1992).

Stress-induced increases in brain 5-HT metabolism also have been observed previously (Thierry et al., 1968; Dunn, 1988), although not in all studies (Kaneyuki et al., 1991). Inoue et al. (1993) demonstrated an increase in m-PFC 5-HIAA levels in rats reexposed to a context paired previously with footshock, a finding consistent with the present as well as a previous study from our laboratory (Goldstein et al., 1994).

The demonstration that postconditioning amygdectomy blocks activation of the m-PFC 5-HT system resulting from reexposure to stimuli paired previously with footshock suggests that the amygdala also provides afferent regulatory input to the 5-HT system during psychological stress. The central nucleus of the amygdala sends projections to the dorsal raphe nucleus (Wallace et al., 1992); serotonergic neurons from both the dorsal and median raphe nuclei in turn project to the m-PFC (Azmitia and Segal, 1978; Moore et al., 1978).

The amygdala and the adrenocortical response

Studies in nonprimate mammals (Redgate, 1970; Redgate and Fahringer, 1973) have confirmed Mason's early pioneering work in awake primates demonstrating activation of the hypothalamic–pituitary–adrenocortical axis by electrical stimulation of the amygdala (Mason, 1959). Recent work in rodents indicates that the amygdala, and more specifically, the central nucleus of the amygdala, is involved in adrenocortical stress activation (Beaulieu et al., 1986; Roozendaal et al., 1991; Van de Kar et al., 1991). The present findings corroborate these studies and are consistent with known neuroanatomical connections of the central nucleus to

distal structures controlling the adrenocortical response (Swanson et al., 1983; van der Kooy et al., 1984; Gray et al., 1989).

Effects of amygdectomy on ultrasonic vocalization, freezing, and defecation

Ultrasonic vocalizations in the 22 kHz range are elicited under a variety of situations that evoke defense responses in laboratory rats and are thought to be antipredator signals to conspecifics (Blanchard et al., 1991; Miczek et al., 1991). The present study demonstrates that amygdectomy blocks stress-induced ultrasonic vocalization. It is known that both auditory and somatosensory information reach the lateral and basolateral amygdala directly from the thalamus, as well as via indirect connections through the cortex (LeDoux, 1992). The lateral and basolateral amygdala send projections to the central nucleus, which projects to the ventral aspect of the PAG (Hopkins and Holstege, 1978), an area that in turn projects to laryngeal, pharyngeal, respiratory, and perioral motoneurons in the nucleus ambiguus and retroambiguus (Jurgens and Pratt, 1979; Yajima et al., 1982; Holstege, 1989). Electrical stimulation within the PAG elicits ultrasonic vocalization in anesthetized rats (Yajima et al., 1980). Thus, amygdectomy may disrupt the relay of incoming sensory information to output neurons activating ultrasonic vocalization.

Both pretraining and post-training amygdectomy also blocked stress-induced freezing, findings consistent with other reports (Blanchard and Blanchard, 1972; Roozendaal, 1991). The PAG appears to be an important effector nucleus for organizing stress-induced freezing, because lesioning of either the PAG or the central nucleus of the amygdala results in complete abolition of this response (LeDoux et al., 1988). Stress-induced defecation also was blocked by amygdectomy, possibly by disrupting the connection of the central nucleus to the dorsal motor nucleus of the vagus (Schwaber et al., 1982; Veening et al., 1984).

General considerations

The present study extends the growing literature implicating the amygdala in the coordination of behavioral, neurohumoral, and central neurochemical responses to psychological stress. However, our data do not allow us to make claims concerning the precise mechanism(s) responsible for this effect. It is possible that the lesioned animals can no longer integrate incoming sensory information. It is also possible that the effector nuclei for the various arms of the stress response have been disconnected so that the stress responses simply cannot be expressed. A third possibility is that the lesioned animals are expressing certain stress responses, but not those measured in this study. Finally, amygdectomy may have a direct effect on one or a limited number of stress responsive systems, which interact secondarily with other systems to give rise to the observed results. Future research will be necessary to clarify these issues.

Nevertheless, the findings do suggest a role for the amygdala in afferent control of the m-PFC DA system response to stress. This may be relevant clinically, because cortical DA dysfunction is proposed to play a role in schizophrenia (Weinberger et al., 1986; Davis et al., 1991; Goldstein and Deutch, 1992; Reynolds, 1992) and symptoms of schizophrenia are thought to be stress responsive (Rabkin, 1980; Bebbington et al., 1993). Amygdala input to the VTA, thus, may be an important neurobiological substrate for the mediation of symptom exacerbation in this disorder. Psychological stress, dysregulation of central monoamine systems, and the amygdala also have been proposed to play a role in the development of post-traumatic stress disorder (PTSD) (Charney

et al., 1993; Southwick et al., 1993; Goldstein et al., 1994). The present findings integrate these factors and, thus, may contribute to our understanding of PTSD, as well as other anxiety and affective disorders (Chrousos and Gold, 1992; Post, 1992).

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