The Basolateral Nucleus of the Amygdala Is Necessary to Induce the Opposing Effects of Stressful Experience on Learning in Males and Females

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The basolateral nucleus of the amygdala (BLA) has been implicated in the modulation of learning after stress. Acute inescapable stress enhances classical eyelink conditioning in male rats, whereas the same stressor impairs eyelink conditioning in female rats. The experiments here directly assessed whether inactivation of the BLA during stress exposure would block both the stress-induced facilitation in males and the retardation of eyelink conditioning in females. To this end, the BLA was temporarily inactivated by infusion of the GABA agonist muscimol before acute stressor exposure. All rats were trained in a different context 24 h later. Males infused with muscimol before the stressful event did not exhibit facilitated eyelink conditioning, whereas those infused with the vehicle emitted more conditioned responses than unstressed males. Females infused with muscimol before stress did not express a deficit in conditioning, whereas those infused with vehicle and stressed emitted fewer conditioned responses than unstressed vehicle controls. These data demonstrate that neuronal activity within the BLA during stress exposure is necessary to modulate learning 24 h later in a new context. Thus, the BLA is necessary to induce the long-term effect of stressful experience on conditioning regardless of sex and direction of modulation.

Key words: stress; eyelink conditioning; basolateral amygdala; muscimol; sex differences; pavlovian conditioning

Introduction

Acute stress facilitates acquisition of many associative learning tasks, such as fear conditioning (Maier, 1990; Cordero et al., 2003; Rodrı́guez Manzanares et al., 2005) and eyelink conditioning (Shors et al., 1992; Beylin and Shors, 1998; Neufeld and Mintz, 2001; Shors, 2001). Based on its established role in emotions and emotional learning, the basolateral nucleus of the amygdala (BLA) is a structure likely to modulate learning after stress. The BLA is critically involved in the stress-induced modulation of classical conditioning (Neufeld and Mintz, 2001; Rodrı́guez Manzanares et al., 2005). Antagonism of NMDA receptors within the BLA at the time of stress exposure blocked the facilitation of eyelink conditioning (Shors and Mathew, 1998). Similarly, a stress-induced enhancement of contextual fear conditioning was abolished by intra-amygdala administration of the GABA agonist midazolam before stress exposure (Rodrı́guez Manzanares et al., 2005). In tasks such as passive avoidance, the BLA positively modulates memory consolidation (McGaugh and Roozendaal, 2002; Roozendaal et al., 2002) and also modulates activity in the hippocampus during learning and stress (Vazdarjanova and McGaugh, 1999; Kim et al., 2001; Huff and Rudy, 2004; Blankenship et al., 2005; Korz and Frey, 2005). In general, an intact amygdala is necessary for stress-induced facilitation of classical fear and eyelink conditioning (Shors and Mathew, 1998; Neufeld and Mintz, 2001; Rodrı́guez Manzanares et al., 2005). It is important to note that these reports were conducted exclusively in male animals (Shors and Mathew, 1998; Neufeld and Mintz, 2001; Rodrı́guez Manzanares et al., 2005).

In contrast to males, female rats express a profound deficit in classical eyelink conditioning after a stressful experience, especially when they are stressed in diestrus and trained in proestrus, when estrogen levels are high (Shors et al., 1998; Wood et al., 2001). The presence of glucocorticoids is necessary to express the facilitation of eyelink conditioning in males (Beylin and Shors, 2003) but is not necessary for the retarded acquisition expressed by females (Wood et al., 2001). These sex differences may ultimately relate to the circuitry used to modulate learning after stress. Numerous studies find that glucocorticoids injected directly into the BLA enhance aversive learning and memory (Roozendaal et al., 2002). Because the BLA is generally implicated in the facilitation of learning, we asked whether the nucleus might also be necessary for decremented responses after stress. Here we used reversible inactivation of the BLA at the time of the stressor to assess its role in the modulation of associative learning in both male and female rats.

Materials and Methods

Subjects. Male and cycling female Sprague Dawley rats between 90 and 120 d of age (obtained from a breeding facility at Rutgers University)
were used. Rats were housed in groups of three until surgery. After sur-
gery, rats were housed alone in standard plastic “shoebox” cages (44.5 cm
g long, 21.59 cm wide, and 23.32 cm high). Rats had ad libitum access to rat
chow and water and were maintained on a 12 h light/dark cycle. All experi-
ments were conducted with full compliance to the rules and regu-
lations specified by the Public Health Service Policy on Humane Care and
Use of Laboratory Animals and the National Institutes of Health Guide for
the Care and Use of Laboratory Animals.

Surgery. Rats were anesthetized with sodium pentobarbital (50 mg/kg
for males and 40 mg/kg for females). After being placed in the stereotaxic
instrument, the scalp was cleaned with Betadine, and an incision was made.
Guide cannulas (23 gauge; Plastics One, Roanoke, VA) were im-
planted bilaterally aimed at the basolateral nucleus of the amygdala (an-
terior-posterior, −3.6; mediolateral, ±2.4; dorsoventral, −7.6 from surface
of skull). Cannulas and headstages were fixed with dental cement and
four jeweler screws. In preparation for eyeblink conditioning, four eyelid
muscles (insulated stainless-steel wire, 0.005 inch) were implanted
through the upper eyelid (orbicularis oculi muscle). Rats were allowed a
minimum of 1 week recovery before eyeblink conditioning began.

Vaginal cytology. Stages of estrus were monitored daily beginning the
day after surgery and a minimum of 5–7 d before training. Sterile cotton
swabs were dipped in physiological saline and gently inserted into the
vaginal canal, to collect loose epithelial cells. These cells were applied
to slides, and stained with a 1% Toluidine Blue solution. Cells were rinsed
and dehydrated with 95% EtOH. Each phase of the estrous cycle was
identified using a microscope. Females were stressed in diestrus and
trained in proestrus, when estrogen levels are increasing (Shors et al.,
1998; Wood et al., 2001). Animals that failed to exhibit a normal estrous
cycle were eliminated from the study.

Infusions. During infusions, stylets were replaced with infusion cannu-
as protruding 1 mm past the guide cannula. Infusion cannulas were
attached to a microinfusion pump via polyethylene tubes attached to 10
µl Hamilton syringes. The syringe and tubes were filled with water, and a
small air bubble separated the water from the artificial CSF (ACSF) or
muscimol solution. Rats were infused with 0.25 g of muscimol into
the upper eyelid (orbicularis oculi muscle). Rats were allowed a
minimum of 1 week recovery before eyeblink conditioning began.

Conditioning chamber acclimation followed by stressor exposure in a
novel context. Rats were placed in the conditioning boxes for an acclima-
tion period. During this time, spontaneous eyeblinks were recorded. Rats
were then transported to a separate room and infused with either ACSF
or muscimol. After infusions, rats were placed in a different context that
consisted of a white wooden soundproof box with no illumination. Rats
were placed in restraint tubes, and electrodes were attached to the tail.
Thirty 1 s, 1 mA stimulations were delivered at 1 min intervals for 30 min,
for a total of 30 tail shocks. Rats in the no-stress condition were infused
with ACSF and returned to the home cage.

Trace eyeblink conditioning. Twenty-four hours after stressor expos-
sure, rats were placed in the eyeblink conditioning chambers and trained
with the trace eyeblink conditioning procedure for 150 trials/d for 4
consecutive days (600 trials total). The conditioned stimulus (CS) was
an 83 dB, 250 ms white noise. The unconditional stimulus (US) was a 100
ms periorbital shock (0.65 mA). The CS and US were separated by a 500
ms trace interval, in which no stimuli were delivered. Trials were pre-
sented in blocks of 10 in the following order: one CS-alone trial, four
paired trials, one US-alone trial, and four paired trials, with an intertrial
interval of 25 ± 5 s. Conditioned responses (CRs) were eyeblinks that
occurred during the 500 ms trace interval on paired trials and 750 ms
after the CS offset on CS-alone trials. The percentage of CRs emitted
across blocks of trials was analyzed using repeated-measures ANOVAs.

Results

Histology

After training, rats were deeply anesthetized and transcardially
perfused with saline followed by 10% formalin. Brains were ex-
tacted and then postfixed in 10% formalin for 3–4 d and then
transferred to storage in 30% sucrose in formalin. Coronal sec-
tions (40 µm) were taken throughout the BLA using a cryostat.
Cannula placements within the BLA were between −2.30 and
−3.30 mm relative to bregma. Reconstruction of cannula place-
ments for those rats retained in analysis is depicted in Figure 1.
Cannulas were judged to be within the BLA if the tips were bilat-
erally on the dorsal boundary of the lateral nucleus of the amygd-
ala. Site of drug infusion was assessed by track markings of the
infusion cannula, which protruded 1 mm beyond the guide can-
numa. Rats were excluded from analysis if cannula placements
were not within the BLA or if the BLA was damaged by the can-
nula or the infusion. Each condition was comprised of the follow-
ing males: ACSF-no stress (n = 8); ACSF-stress (n = 6); musci-
mol (mus)-stress (n = 6); females: ACSF-no stress (n = 8);
ACSF-stress (n = 6); mus-stress (n = 8).

Behavioral results

Behavioral results are presented in Figure 2. In Figure 2, A and C,
eyeblink conditioning trials are presented in 10-trial blocks for the
first 50 trials to depict any possible differences between groups in early trials. Thereafter, conditioning trials are pre-
sented in 50-trial blocks. ANOVA revealed no significant group
differences in spontaneous blinks emitted during the acclimation
session in males (F<sub>2,17</sub> < 1). Repeated-measures ANOVA on the
first 5 blocks of 10 trials failed to find a significant effect of trial
(F<sub>6,88</sub> = 1.22; p > 0.05) or a trial × condition interaction (F<sub>6,88</sub>
< 1). The main effect of condition did not reach significance
(F<sub>2,17</sub> = 3.42; p = 0.057). Bonferroni’s post hoc tests found no
significant differences between groups ( p > 0.05). A repeated-
measures ANOVA across the 16 blocks of eyeblink conditioning
revealed a significant effect of trial (F<sub>15,245</sub> = 19.41; p = 0.0001),
as males expressed an increase in conditioned responding across
trial blocks. The trial × condition interaction did not reach sig-
ificance (F<sub>15,245</sub> < 1). The main effect of condition was significant
(F<sub>2,17</sub> = 5.69; p = 0.013). Bonferroni’s post hoc tests con-
firmed that males treated with ACSF before stress exposure
expressed more conditioned responses than unstressed rats ( p =
0.019) or rats treated with muscimol before stress exposure ( p =
0.038). Rats treated with muscimol before stress exposure did not
differ from unstressed, ACSF-treated controls ( p > 0.05). Thus,
rats treated with muscimol before stress exposure did not express
facilitated learning relative to ACSF-treated rats exposed to stress,
and performed similarly to unstressed rats.

Analysis of spontaneous blink rates of females during the ac-
climation session and before conditioning failed to find any
Group differences (F<sub>2,19</sub> < 1). Analysis of the first 5 blocks of 10
trials found no effect of trial (F<sub>6,76</sub> < 1), nor a trial × condition
interaction (F<sub>6,76</sub> = 1.19; p > 0.05). The main effect of condition
was not significant (F<sub>2,19</sub> = 2.76; p > 0.05). Thus, stress or
drug treatment did not influence early responding. A repeated-
measures ANOVA across the 16 blocks of eyeblink conditioning
depicted in Figure 2C revealed a significant effect of trial (F<sub>15,245</sub>
= 12.13; p < 0.0001), as females expressed an increase in condi-
tioned responding as training progressed. The trial × condition
interaction did not reach significance (F<sub>15,245</sub> = 1.35; p > 0.05).
The main effect of condition was significant (F<sub>2,19</sub> = 5.43; p =
0.014). Bonferroni’s post hoc analyses confirmed that female rats
treated with ACSF before stress exposure did not express as many
conditioned responses as unstressed females ( p = 0.042) or fe-
nales treated with muscimol before stress exposure ( p = 0.019).
Rats treated with muscimol before stress exposure did not differ
from unstressed female rats ( p > 0.05). Thus, muscimol before
stress exposure abolished the deleterious effect of stress on sub-
sequent eyeblink conditioning.
Figure 2, B and D, depicts the percentage of rats to reach 60% conditioned responding on any block of 50 trials. The majority of unstressed females attained this level of conditioned responding (87.5%), whereas stress reduced the number of females reaching this criterion (33.3%). Importantly, a higher percentage of female rats treated with muscimol in the BLA (75%) during the stressor reached criterion relative to stressed females. Male rats exhibited the opposite pattern of results. Only 50% of unstressed males reached a criterion of 60% conditioned responding, whereas all male rats exposed to stress reached criterion. When the BLA was inactive during the stressor, only one-half of the males reached criterion, similar to unstressed males. These results indicate that exposure to the acute stressor is affecting, not only acquisition, but also how many animals attain a learning criterion of 60% conditioned responses.

Discussion

In both males and females, BLA inactivation with muscimol during the stressor prevented the modification of classical eyeblink conditioning. These data were somewhat anticipated given the established role of the amygdala in aversive conditioning (LeDoux, 2000; Roozendaal et al., 2002). However, involvement of the BLA in decremented learning after stress in females has not been previously demonstrated. Although the BLA is not necessary for eyeblink conditioning, lesions of the BLA profoundly retard acquisition (Neufeld and Mintz, 2001; Lee and Kim, 2004; Lindquist and Brown, 2004), suggesting that the BLA is involved in the modulation of learning during arousing and fearful conditions (Packard and Cahill, 2001; McGaugh, 2004). Inescapable stress elicits fear that generalizes beyond the context in which inescapable stress was conducted, and this fear is evident before US delivery (Maier, 1990). This generalized fear facilitates aversive conditioning in a novel context in males (Maier, 1990). The present results suggest that activity within the BLA elicited during the stressor may potentiate contextual fear expressed during eyeblink conditioning and thereby enhance acquisition in male rats. Similarly, enhancement of GABAergic inhibition within the BLA during acute restraint stress blocks the stress-induced enhancement of contextual fear conditioning in male rats (Rodríguez Manzanares et al., 2005). These relationships have not been demonstrated in females.

If the BLA is mediating the emotional components of stress exposure and eyeblink conditioning, it follows that fear disrupts eyeblink conditioning in females. Whether fear expressed by stressed females during eyeblink conditioning is different or more persistent than that expressed by unstressed females or males is unknown. The influence of stress on eyeblink conditioning is dependent on different hormones, which may modulate the expression of fear differently in males relative to females. The effect of stress on eyeblink conditioning in females is dependent on the presence of estrogen (Wood and Shors, 1998), and acute stress elicits a prolonged elevation in estrogen levels (Shors et al., 1999). Estrogen receptor agonists can disrupt the conditional inhibition of fear in females (Toufexis et al., 2007), and estrogen modulates many types of learning (e.g., Zurkovsky et al., 2007). The presence of estrogen during training in females exposed to acute stress may be enhancing the expression of fear to a degree that disrupts their ability to acquire the CS–US association or emit adaptively timed CRs.
Whether the opposing effect of stress on eyeblink conditioning relies on intrinsic amygdala changes is unknown. Exposure to the stressor used here dramatically and persistently impairs multiple-unit activity within the BLA (Shors, 1999), and chronic stress enhances synaptic connectivity in the BLA (Vyas et al., 2006). Furthermore, glucocorticoids within the BLA enhance consolidation of aversive learning (McGaugh and Roozendaal, 2002; Roozendaal et al., 2002) and increase excitability of principal neurons within the BLA (Duvarci and Pare, 2007). To our knowledge, these results are restricted to male rats. Direct examination of sex differences in response to stress within the BLA found that although serotonin and dopamine levels are lower in the amygdala of female than in that of male rats, stress exposure elevates these transmitter levels above those of males (Mitsushima et al., 2006). Females also expressed a larger increase in systemic corticosterone after stress exposure relative to males (Mitsushima et al., 2006), despite the fact that glucocorticoids are not necessary for the effect of stress on eyeblink conditioning in females (Wood et al., 2001). Thus, the female response to stress may be attributable to changes in neural structures sensitive to fluctuation of estrogens, because the deleterious influence of stress on learning occurs when estrogen levels are highest (Wood and Shors, 1998). Whether this possible sensitivity to estrogens is within the BLA or afferent/efferent structures remains unknown.

The hippocampus is also necessary in males and females for the influence of stress on acquisition of classical eyeblink conditioning (Bangasser and Shors, 2007). Thus, the hippocampus is critical for the modulatory role of stress in both sexes beyond its putative role in learning (Bangasser and Shors, 2007). The BLA may interact directly with the hippocampus during the stressor to modify subsequent learning in both males and females. The hippocampus exhibits morphological and excitability changes in response to stress (Galea et al., 1997; Kim et al., 2001; Shors et al., 2001) as well as eyeblink conditioning (Geinisman et al., 2001; Moyer et al., 1996). The BLA modulates neuronal activity within the hippocampus in response to stress exposure (Kim et al., 2001; Korz and Frey, 2005) and many learning tasks, including eyeblink conditioning (Pare, 2003; Huff and Rudy, 2004; Blankenship et al., 2005; McIntyre et al., 2005). The hippocampus is sensitive to fluctuating estrogen levels (Woolley et al., 1990; Yankova et al., 2001); whether estrogen levels directly influence BLA—hippocampal interactions during stress exposure and eyeblink conditioning is unknown. It is possible that inactivation of the BLA at the time of stress exposure may disrupt amygdala-dependent modulation of the hippocampus and, in turn, abolish the influence of stress on learning in both males and females.

The current results indicate that the BLA is critically engaged during a stressful event and can modify learning at least 24 h later in male and female rats. A direct comparison of neural substrates used by males versus females to respond to stress may provide insight into sex differences in emotional memory, as well as in the prevalence of posttraumatic stress disorder in women (Cahill et al., 2001; Canli et al., 2002; Tolin and Foa, 2006). The results presented here suggest that the amygdala is not only engaged by stress but is necessary to initiate the opposing effect of stressful experience on classical conditioning.

**References**


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