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# INACTIVATION OF THE BED NUCLEUS OF THE STRIA TERMINALIS SUPPRESSES THE INNATE FEAR RESPONSES OF RATS INDUCED BY THE ODOR OF CAT URINE

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**Abstract—In this study, we investigated whether two brain regions, the bed nucleus of the stria terminalis (BNST) and the basolateral amygdala (BLA), affected male rats' (*Rattus norvigicus*) ability to innately discriminate between a predator odor (cat urine) and female rat urine. Muscimol, a GABAa receptor agonist, was bilaterally microinjected into either the BNST or BLA of rats through implanted stainless-steel guide cannulas to temporarily inactivate these brain nuclei. The behavioral responses of the treated rats to female rat urine and cat urine were then tested in an experimental arena. Compared to a saline infusion control, the injection of muscimol into the BNST strongly reversed the innate aversion of rats to cat urine but the injection of muscimol into the BLA had no effect. Furthermore, intra-BNST infusion of muscimol caused rats to be equally attracted to urine from cats and female rats but intra-BLA infusion did not stop rats manifesting fear on exposure to cat urine and exploratory behavior on exposure to female rat urine. We conclude that the BNST plays a more crucial role in modulating innate fear responses in rats than the BLA.** 

**Key words: basolateral amygdala, bed nucleus of the stria terminalis, muscimol, predator odor, behavior, brain anatomy.**

## INTRODUCTION

Innate fear of predator odor is beneficial to prey animals as this can allow them to modify their behavior before predators are encountered (Edut and Eilam, 2003).

The fundamental brain structures involved in the formation, consolidation, and retrieval of fear memories have now been identified (Fendt and Fanselow, 1999; LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001; Maren and Quirk, 2004). For example, the amygdala is a candidate region for the part of the brain where fear memories are originated and stored. The basolateral complex of the amygdala (BLA; consisting of the lateral, basolateral, and basomedial nuclei) is where CS (conditional stimulus) and US (unconditional stimulus) information converge and associate (LeDoux, 1998; Davis and Whalen, 2001; Maren, 2001; Schafe et al., 2001; Fanselow and Gale, 2003). However, some studies have produced contradictory results; for example, Fendt et al. (2003) found that the amygdala was unrelated to innate freezing, whereas another study found that inactivation of the medial nuclei of the amygdale of rats blocked freezing to predator odor (Muller and Fendt, 2006).

The bed nucleus of the stria terminalis (BNST), known as the extended amygdala, is adjacent to the anterior commissure in the basal forebrain and forms the rostral part of the continuum. The BNST has been strongly implicated in mediating responses to stimuli that contain an affective salience. Several studies have suggested that the BNST mediates behavioral responses to acute and chronic aversive stimuli (Casada and Dafny, 1991; Walker and Davis, 1997). This is supported by evidence that the BNST is activated in response to stress and modulates anxiety-related behaviors in several animal models. More specifically, BNST inactivation has been found to decrease the neuroendocrine and behavioral responses to stress (Gray et al., 1993), and to block some forms of unconditioned fear (Walker and Davis, 1997; Davis et al., 1997).

In the laboratory, the odors most frequently employed to elicit fear responses in rodents include cat odor and trimethylthiazoline (TMT), a synthetic compound isolated from fox feces (Maury et al., 1984). Rats exposed to either cat odor or TMT often display a variety of behavioral and physiological responses indicative of fear, including standing still, avoidance, and increased secretion of stress hormones. However, rodents appear to engage in risk assessment more frequently in tests involving cat odor (Holmes and Galea, 2002; Hebb et al., 2004) than TMT (Rosen, 2004). Cat urine has been widely used in stress experiments on rodents. Different studies have, however, reported contradictory results; for example, Bramley et al. (2000) found that the odor of cat urine strongly blocked the motor activity of Kapiti rats, while

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*Abbreviations:* BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CS, conditional stimulus; MeA, medial amygdala; SD, Sprague Dawley; TMT, trimethylthiazoline; US, unconditional stimulus.

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Blanchard et al. (2003) found that the cat urine odor could not induce rats to stand still. In the present study, we took female rat urine odor and cat urine odor as the two quite opposite social odor stimuli; the paired odors may provide a higher sensitivity for social recognition. We investigated whether inactivation of the BLA or BNST in rat blocks the fear responses induced by the cat urine stimulus.

## EXPERIMENTAL PROCEDURES

### Subjects and housing

Thirty-two male Sprague Dawley (SD) rats (Vital River Laboratory Animal Technology Co. Ltd.) weighing 290–330 g at the time of surgery were used. These were maintained in a vivarium for 1 week before surgery to acclimatize. Rats were housed individually in plastic cages (37 × 26 × 17 cm) in a room maintained on a 14:10-h light/dark cycle (lights on at 19:00) and at 23 ± 2 °C. Food and water were available *ad libitum*. All animal procedures were performed in accordance with current Chinese legislation and approved by the Institute of Zoology's Animal and Medical Ethics Committee.

### Odor probes

Urine probes from female rats were collected using a metabolic cage at the animal facility of the Institute of Zoology. Urine probes from healthy male cats were obtained by a local veterinarian by means of bladder catheterization. All urine samples were collected fresh and stored in a refrigerator at −20 °C until used. Samples were presented in odor exposure experiments as follows; 1 ml of cat or female SD rat urine was placed in a 6-cm diameter Petri dish which was then put in a corner of the experimental arena 5 cm from the walls.

### Surgery

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed into a stereotaxic frame (RWD Life Science Co., Ltd) with blunt ear bars. Two stainless-steel guide cannulas (diameter, 0.68 mm) (RWD Life Science Co., Ltd) were implanted bilaterally into the brain targeting either the BNST (0.2 mm caudal, ±1.7 mm lateral, 4.5 mm ventral to bregma) or the BLA (2.8 mm caudal, ±5.0 mm lateral, 7.0 mm ventral to bregma) (Paxinos and Watson, 1997). The cannulas were fixed to the skull with dental cement and three anchoring screws. Rats were injected intraperitoneally with a prophylactic (0.4 ml) dose of antibiotics comprising 300 mg/kg penicillin and 300 mg/kg streptomycin immediately after surgery. After surgery and between tests, stylets (diameter, 0.4 mm) were inserted into the cannulas to maintain patency. Rats were given 5–7 days to recover from surgery before testing.

### Apparatus for odor exposure and behavioral testing

All behavioral tests were conducted in an arena [75 cm (L) × 45 cm (W) × 35 cm (H)] with a cover made from transparent Plexiglas and 15 equal area subfields (15 × 15 $cm^2$) on smooth floor with a grid lines marked on it (Liu et al., 2010). Rat behaviors were monitored by video and are defined as follows: (1) Freezing; the rat is completely immobile except for slight vibrissae movement associated with respiration. This behavior was used as a measure of fear. (2) Locomotion; this parameter was measured by the total number of floor grids crossed by rats in each trial. (3) Grooming; the rat licks, scratches, or ''cleans'' any part of its body with its head or forelimbs. (4) Contact; the rat makes direct tactile contact with the source of the stimuli, including chewing or touching with its vibrissae.

### Procedure for odor exposure

To acclimatize rats to the experimenter they were handled daily (10−15 s per rat) for 5 consecutive days following surgery. To familiarize the rats with the arena, each animal was placed in it together with an odorless saline solution in a petri dish (1 ml saline) for 10 min each day during the same consecutive 5 days. On the day after this acclimatization period, each rat received (in a pseudo-randomized manner) bilateral injections of either 1.1 nmol of muscimol (Sigma Aldrich, America) [dissolved in 100 nl of saline] or saline alone (100 nl) into the BNST ($n$ = 15) and 2.2 nmol of muscimol [dissolved in 200 nl of saline] or saline alone (200 nl) into the BLA ($n$ = 17) at a rate of 100 nl/min using a 10 µl syringe and Hamilton mini-pump connected to a 33-gauge needle (outer diameter: 0.4 mm; extending 2 mm beyond the guide cannula) via polyethylene tubing. The needle was kept in place for another 2 min after injections were completed to ensure complete diffusion of the drug after which the dummy stylet was replaced. Previous research has shown that drugs injected in this way diffuse into an area of approximately 1-mm diameter from the injection site (Fendt et al., 2003). Testing began 5 min after infusion. The cat or female rat urine samples were put in one corner of the arena, and the rat was put in the opposite corner. The rat's behavior was monitored for the next 10 min by a video-recorder as described above. All behavioral tests were carried out between 9:00 and 17:00 h. Samples were presented in a pseudo-randomized order and the corner in which urine samples and rats were placed was also changed in a pseudo-randomized fashion. The experimental arena was cleaned with boiled water and 70% ethanol after each test and ventilated with clean air for 30 min (ca. 15 l/min) before the next test commenced. Videotapes from all experiments were analyzed by two observers who were blind to the condition of the animals. The percentages of time spent freezing or in contact with odor stimuli were calculated for each rat for 10 min of each experimental session. Locomotion, measured as the number of floor lines crossed by each rat during the course of each trial, was also recorded.

### Verification of injection sites

At the end of each experiment, rats were humanely killed with an overdose of sodium pentobarbital and 20 nl of 4% Evens Blue solution was infused into each cannula to verify the placement of the injection needle. The rats' brains were then rapidly removed and fixed in 4% paraformaldehyde for 48 h before being sectioned on a cryostat (Leica, Germany). Thirty-micrometer sections were taken. The locations of the injection sites were identified with reference to Paxinos and Watson (1997). Only results obtained from animals that were confirmed to have had injections into the BNST and BLA were used in statistical analyses.

### Statistical analysis

All values of data are given as means ± SE. Statistical analysis of the data was accomplished with a two-way analysis of variance (ANOVA), using drug (muscimol/saline treatment) as one factor and odor (female rat urine/cat urine stimuli) as the second factor, and then the post hoc Tukey's tests for multiple comparisons were conducted between groups when a significant F ratio was obtained. All statistical assessments were two-sided and considered significant when $p < 0.05$. Statistical analyses were performed using SPSS statistical software 16.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Histological verification of injection sites

Fig. 1 shows that the BLA and BNST were accurately targeted for injection in the rats (Fig. 1A and B) with the filled circles indicating the injection sites in BLA and BNST (Fig. 1C and D).

### The duration of contact with odor stimuli

Table 1 shows that the duration rats spent in contact with cat urine was markedly shorter than that with female rat urine after the rats were injected with saline into either BNST or BLA ($p < 0.05$ and $p < 0.01$, respectively, for BNST and BLA groups), while inactivation of the BNST with muscimol not only increased the duration of rats' contact with cat urine (interaction of drug and odor: $F_{(1,26)} = 4.28$; $p < 0.05$) although the increase in duration of rats exposed to female rat urine was not significant ($p > 0.05$), but also reduced or even inverted the difference in rats' contact duration between female rat urine and cat urine ($p > 0.05$). However, Injection of muscimol into the BLA had no effect on avoidance of rat to cat urine (interaction of drug and odor: $F_{(1,30)} = 1.08$; $p > 0.05$). Inactivation of BLA with muscimol could not, as the BNST inactivation could, invert the decreased duration of rats' contact with cat urine compared to female rat urine ($p < 0.01$).

### Freezing in response to cat urine

Fig. 2 indicates that the freezing rate of rats injected with saline into BNST or BLA induced by exposure to cat urine was obviously higher than that to female rat urine ($p < 0.01$, for both BNST and BLA saline groups), which indicates that the rats displayed a strong freezing response to the cat urine, while a significant difference was not observed in freezing response of rats injected with muscimol into the BNST between exposed to female rat urine and cat urine odor ($p > 0.05$), which indicates that inactivation of BNST with muscimol blocked the freezing behavior (interaction of drug and odor: $F_{(1,26)} = 4.66$; $p < 0.05$). In contrast, while blocking the BLA with muscimol did not affect the freezing response elicited by cat urine odor (interaction of drug and odor: $F_{(1,30)} = 1.34$; $p > 0.05$); the freezing rate when rats exposed to cat urine is much higher than that to female rat urine after the BLA inactivation ($p < 0.01$).

### Grooming induced by cat urine

Table 2 shows that the grooming duration of rats injected with saline into BNST or BLA exposed to cat urine was shorter than that to female rat urine ($p < 0.05$ for both the two groups), while muscimol injection to BNST not only increased the grooming duration of rats exposed to cat urine (interaction of drug and odor: $F_{(1,26)} = 4.34$; $p < 0.05$) although the increase in grooming of rats exposed to female rat urine is not significant ($p > 0.05$), but also inverted the difference in rats' grooming duration between female rat urine and cat urine ($p > 0.05$). Muscimol injection into BLA could not diminish the decreased grooming duration induced by cat urine odor (interaction of drug and odor: $F_{(1,30)} = 2.02$; $p > 0.05$); the grooming duration of rats exposed to cat urine odor is shorter than that to female rat urine after the BLA blockage ($p < 0.05$).

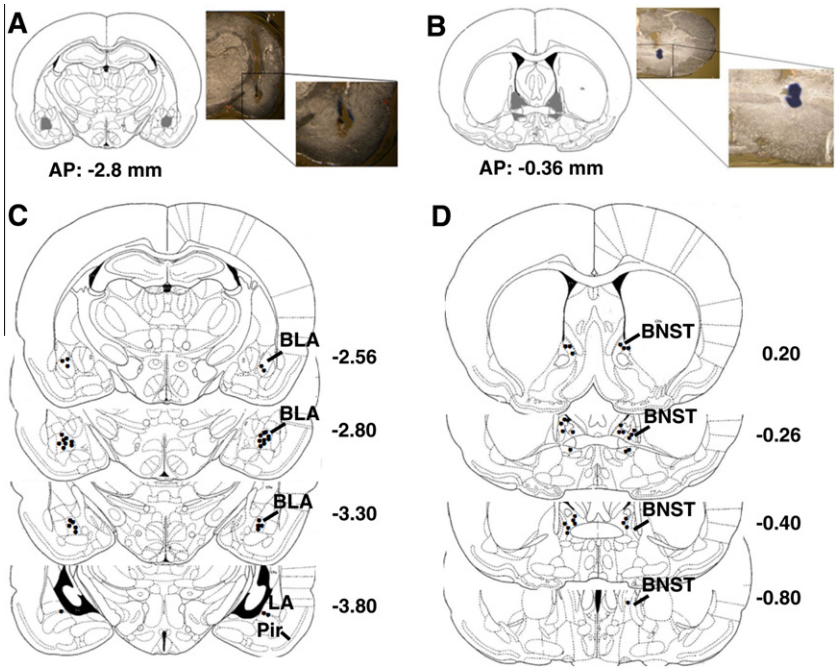


**Fig. 1.** Injection sites in the rat brain. (A and B) (Left) The gray shaded area indicates the target area for injection into the BLA and BNST; (Right) Representative photograph showing actual parts of the brain injected after staining with Evans blue dye. (C and D) The histological reconstructions of injection sites for rat receiving infusions of muscimol into the BLA and BNST, respectively. The numbers indicate the distance from bregma. *Abbreviations:* AP, anterior-posterior; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; LA, lateral nucleus of the amygdala; Pir, piriform cortex.

**Table 1.** The duration of the male rats injected into BNST and BLA with muscimol when exposed to either female rat urine or cat urine (unit: seconds)[a]

| Treatment | BNST | | BLA | |
|---|---|---|---|---|
| | Saline | Muscimol | Saline | Muscimol |
| Rat urine | 9.29 ± 2.97 | 13.01 ± 5.81 | 41.98 ± 3.89 | 44.11 ± 4.15 |
| Cat urine | 3.70 ± 0.70* | 15.56 ± 8.09# | 16.14 ± 5.51** | 18.80 ± 4.69** |

*Abbreviations:* BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis.

[a] The male rats injected with muscimol or saline into BLA or BNST were exposed to a petri dish containing female rat urine or cat urine and then the cumulative time of each rat spent in contact with the source of the stimuli, including chewing or touching with its vibrissae, during 10-minute periods was recorded. Data were expressed as mean ± SE. The data were analyzed by a two-way analysis of variance (ANOVA), followed by Tukey's tests for multiple comparisons between groups.

* Indicates significant differences from the female rat urine group (control) ($p < 0.05$).

** Indicate significant differences from the female rat urine group (control) ($p < 0.01$).

\# Indicates significant differences from injection of the saline group (control) ($p < 0.05$).

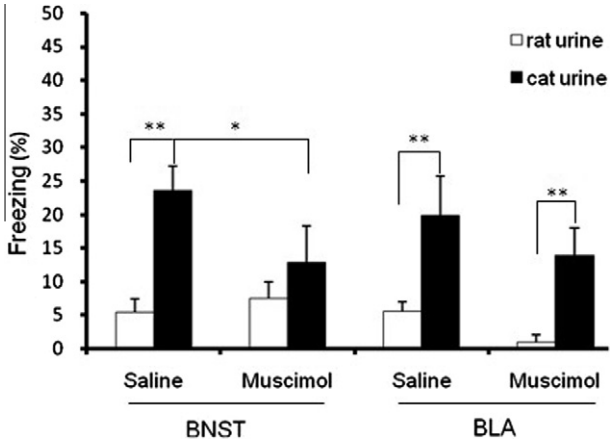


**Fig. 2.** Effects of muscimol injections into the BNST and BLA on the duration of the freezing response induced in rats by the odor of cat urine. The histograms show the percent of time spent motionless during each 10-min test after local injections of saline and muscimol. The data were analyzed by a two-way analysis of variance (ANOVA), followed by Tukey's tests for multiple comparisons between groups. * and ** indicate significant difference from saline injection group (control) ($p < 0.05$) and female rat urine group (control) ($p < 0.01$), respectively.

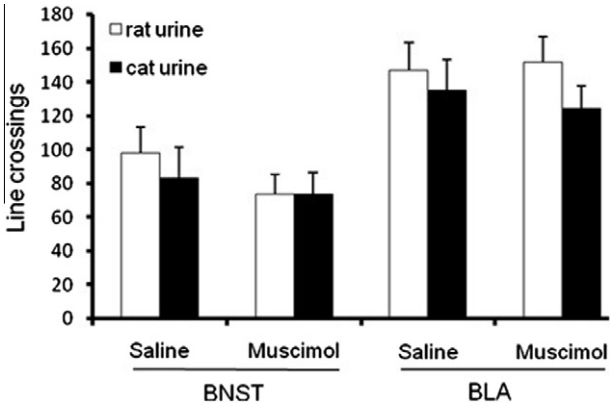


**Fig. 3.** Locomotion of male rats during 10-min periods under the indicated treatments and exposure conditions. The histograms show the number of grids on the floor of the experimental enclosure that were crossed by rats following local injections of saline and muscimol. The data were analyzed by a two-way analysis of variance (ANOVA). No significant difference among groups ($p > 0.05$) was found based on the F value obtained.

### Ambulation

It is possible that the attenuation of the freezing response induced by cat urine following muscimol injection into the BNST was due to increased locomotion. However, Fig. 3 indicates that injecting muscimol into the BNST did not affect rats' locomotion ($F_{(1,26)} = 1.52$; $p > 0.05$), irrespective of whether they were exposed to female rat urine or cat urine. A similar result was observed following injection of muscimol into the BLA.

## DISCUSSION

Our results demonstrate that exposure to cat urine induced a significantly greater frequency of freezing response in rats compared to female rat urine. To test the role of the BLA and BNST in mediating the behavioral responses of rats to these two odors, we temporarily inactivated these two nuclei by locally injecting the GABAa

**Table 2.** The duration of grooming of male rats injected into BNST and BLA with muscimol when exposed to either female rat urine or cat urine (unit: seconds)[a]

| Treatment | BNST | | BLA | |
|---|---|---|---|---|
| | Saline | Muscimol | Saline | Muscimol |
| Rat urine | 95.30 ± 12.60 | 86.12 ± 11.76 | 135.95 ± 20.02 | 186.13 ± 29.68 |
| Cat urine | 64.32 ± 6.70* | 120.10 ± 14.31# | 78.63 ± 16.64* | 106.22 ± 16.05* |

*Abbreviations:* BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis.

[a] The male rats injected with muscimol or saline into BLA or BNST were exposed to a petri dish containing female rat urine or cat urine and then the cumulative time of each rat that licks, scratches, or ''cleans'' its body with the head or forelimbs during 10-minute periods was recorded. Data were expressed as mean ± SE. It was found that the data were non-normally distributed. The data were converted to normal distribution by square root transformation and then subjected to a two-way analysis of variance (ANOVA), followed by Tukey's tests for multiple comparisons between groups.

* Indicates significant differences from the female rat urine group (control) ($p < 0.05$).

\# Indicates significant differences from injection of the saline group (control) ($p < 0.05$).

receptor agonist, muscimol. Rats exposed to female rat urine increased the duration of their contact with the odor source, indicating that our experiment included not only aversive, but also attractive, odors (Markham et al., 2004). Inactivation of the BNST not only increased the duration of contact with both female rat and cat urine, but also reduced the difference in contact duration between these two stimuli; in contrast, injecting muscimol into the BLA had no effect on rats' avoidance of cat urine, although there are differences of the basal values of both contact and grooming duration of rats exposed to female rat urine between the BNST and BLA saline injection treatments, which are probably due to error between different batches of injection experiments. Interestingly, we found that although temporary inactivation of the BLA had no effect on the duration of the freezing response induced by cat urine, temporary inactivation of the BNST did. Nevertheless, this effect of BNST blockage on freezing behavior was not caused by locomotion changes. This suggests that inactivation of the BNST may have blocked the innate response to the signal given by cat urine but not the sensation and perception of rats to odors. We all know that odor information into the brain is first perceived and discriminated by olfactorius bulbus, and is then transferred to the downstream areas. Also our behavioral results showed that inactivation of BLA did not impair the sensation and perception of rats to odors as well as the output of the behaviors detected. Similarly, we presumed that inactivation of BNST did not disturb the sensation of animal to odor either; however, probably, inactivation of BNST blocked the transmission of information to BNST and the release of certain neurotransmitters in BNST, which is important to regulate fear behaviors.

Our results indicate that cat urine induced a freezing response in rats. Similar results were reported by Bramley et al. (2000) who found that both a component of cat urine, *S*-methyl, methyl butanol, and cat urine itself, induced a stronger decrease in locomotion than cat feces in Kapiti rats. Other kinds of cat odor, e.g. that of a cat collar, significantly increased rats' blood pressure and decreased their activity. Rats avoided the odor stimulus and increased vigilance and risk-assessment measures (Dielenberg et al., 2001a; Blanchard et al., 2003). While there have been some contradictory results, there are a few studies in which cat urine has failed to induce defensive or fearful behavior in rodents (Blanchard et al., 2003; Fendt, 2006). The possible reason is that a predator urine odor implies the existence of a predator in the area, but it is not implicitly an indicator of acute and direct predatory threat. In addition, the quality or intensity of the stimuli or even the testing context is also involved in the effect of odor stimuli on the behaviors.

The results of our study indicated that the grooming behavior was enhanced in the rats exposed to cat urine odor after muscimol injection into BNST, but not BLA. It is known that novelty and other stressors can elicit grooming behavior in rats (Colbern et al., 1978). However, no relationship was found between grooming and anxiety-like behavior, such as urination, defecation, and freezing (File et al., 1988). Presumably, anxiolytic drugs depress novelty-induced grooming through attenuating the intensity of the perception of anxiogenic stimuli (Dunn et al.,1981; Molloy and Waddington, 1987). Rats are less likely to engage in maintenance behaviors when threatening stimuli occurred (Shepherd et al., 1992). The decreased grooming implied increased anxiety in the rats. In the present study, it seems that injection of muscimol into BNST, but not BLA apparently enhanced the grooming behavior in rats, and inverted the interaction of freezing response with grooming behavior during exposure of rats to cat urine odor after BNST blockage.

Within the last 10 years a number of laboratories have examined the neural correlates of fear responses induced by predator odors in adult rodents. An emerging consensus is that these fear responses are controlled by specific brain regions. Several brain regions have been found to be involved in the modulation of fear responses following exposure to cat odor and TMT. The present study explicitly demonstrates that temporary inactivation of the BLA does not affect the freezing response induced in rats by exposure to cat urine. Rats exposed to TMT or fox urine showed increased Fos positive neurons in the BLA (Funk and Amir, 2000). However, exposure to cat odor was not followed by an increase in c-fos activity within the central nuclei of the amygdale (CeA) and BLA (Dielenberg et al., 2001b). In contrast, ibotenic-acid lesions of the BLA caused a significant reduction in freezing behavior after the rats experienced the tone-footshock conditioning (Koo et al., 2004). Similarly, BLA-lesioned rats showed a significant reduction in motionlessness and avoidance behavior when exposed to a ball of cat fur (Vazdarjanova et al., 2001). All these inconsistent results may be attributed to the differential inactivation models of BLA. Excitotoxic lesions of the BLA destroy nerve fibers (Wallace and Rosen, 2001) whereas reversible inactivation of BLA does not. In addition to the BLA, the medial amygdala (MeA) is reported to have a critical role in the modulation of unconditioned fear induced by cat odor. Cat odor and TMT produce unique patterns of Fos expression in the MeA (Dielenberg et al., 2001b).

Our results indicate that inactivation of the BNST significantly decreases the freezing response of rats when exposed to cat urine. The BNST has been recognized as a fundamental structure that integrates and mediates emotional, cognitive, autonomic, endocrine, and behavioral responses to stress (Choi et al., 2007; Resstel et al., 2008; Alves et al., 2009; Crestani et al., 2009). Lesions, or pre-test infusions, into the BNST of compounds that interfere with its function decrease behavioral and autonomic responses to stress (Cecchi et al., 2002; Khoshbouei et al., 2002; Greenwood et al., 2005; Resstel et al., 2008; Crestani et al., 2009). Shekhar and Keim (2000) reported that pre-test infusions of muscimol directly into the BNST blocked behavioral, but not autonomic, responses to lactate infusions in rats that had received chronic L-allylglycine infusions into the dorsomedial hypothalamus. Interruption of BNST noradrenergic transmission attenuates immobilization stress-induced anxiogenic-like effects (Cecchi et al., 2002; Khoshbouei et al., 2002; Morilak et al., 2003) and reduces TMT-induced freezing response (Fendt et al., 2005). Similarly, BNST lesions attenuated the startle response induced

by bright light, an unconditioned stimulus that induces anxiety, which demonstrates that the BNST is involved in the modulation of unconditioned fear responses (Walker and Davis, 1997).

As one of the direct downstream structures from the BLA, BNST receives projections from BLA. Parts of fibers originating from posterior BLA stream pass directly through the central nucleus of amygdala to the anterior and posterior regions of the lateral BNST (Walker et al., 2003). The fibers form BNST projection to the other areas including the central gray which regulates freezing, social interaction, and hypoalgesia. In our opinion, there may be additional projection from other locations to BNST, which constitutes a circuit loop with central gray via BNST to regulate the anxiety behavior, and BNST is a critical hub in the anxiety-related signal transmission.

## CONCLUSIONS

Taken together, our results suggest that cat urine, an aversive odor, can induce a freezing response and avoidance behavior in rats. However, temporary inactivation of the BNST, but not the BLA, not only reduced the frequency of both these responses to cat urine, but also increased the grooming behavior. We conclude that the BNST is more important in the modulation of unconditional stress, while the BLA is less likely involved in the innate fear response.

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