Behavioral/Systems/Cognitive

Noradrenaline Transmission within the Ventral Bed Nucleus of the Stria Terminalis Is Critical for Fear Behavior Induced by Trimethylthiazoline, a Component of Fox Odor

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The bed nucleus of the stria terminalis (BNST) is involved in the mediation of fear behavior in rats. A previous study of our laboratory demonstrated that temporary inactivation of the BNST blocks fear behavior induced by exposure to trimethylthiazoline (TMT), a component of fox odor. The present study investigates whether noradrenaline release within the BNST is critical for TMT-induced fear behavior. First, we confirmed previous studies showing that the ventral BNST is the part of the BNST that receives the densest noradrenaline innervation. Second, using *in vivo* microdialysis, we showed that noradrenaline release within the BNST is strongly increased during TMT exposure, and that this increase can be blocked by local infusions of the α_2-receptor blocker clonidine. Third, using intracerebral injections, we showed that clonidine injections into the ventral BNST, but not into neighboring brain sites, completely blocked TMT-induced potentiation of freezing behavior. The present data clearly show that the noradrenergic innervation of the ventral BNST is important for the full expression of behavioral signs of fear to the predator odor TMT.

Key words: **amygdala; behavior; clonidine; freezing; noradrenaline; predator; rat**

Introduction

Fear is a behavioral response that helps animals and humans survive dangerous situations. Investigating the neuronal basis of fear can help identify new strategies for the treatment of pathological fear, such as anxiety disorders. In this scope, studies inducing fear in rodents by presenting stimuli that were paired with stressful events (e.g., foot shocks) highlighted the essential role of the amygdaloid complex in fear learning (Davis et al., 1993; Fendt and Fanselow, 1999; LeDoux, 2000).

The neuronal system underlying fear is innately activated by potentially dangerous stimuli, such as the presence of a predator (Griffith, 1920). To study the neurobiology of innate fear, cat odor was often used to induce fear in laboratory rodents (Zangrossi and File, 1992; Dielenberg et al., 2001; Blanchard et al., 2003b; Li et al., 2004). Alternatively, trimethylthiazoline (TMT), an ingredient in fox feces can be used (Vernet-Maury, 1980; Vernet-Maury et al., 1984; Blanchard et al., 2003a; Fendt et al., 2005). TMT exposure enhances risk-assessment behavior (Hebb et al., 2004), corticosterone release (Day et al., 2004), defensive burying (Holmes and Galea, 2002), and freezing behavior (Wallace and Rosen, 2000) and potentiates the startle response (Hebb et al., 2003) in rodents. These autonomic and behavioral changes are common to fear (Olivier et al., 1994; Rodgers, 1997; Fendt, 2005), indicating that TMT is, similar to cat odor, a natural stimulus, which innately induces fear in rodents (Fendt et al., 2005).

Interestingly, studies using TMT to investigate the neural basis of innate fear demonstrate a more prominent role of the bed nucleus of the stria terminalis (BNST) than of the lateral amygdala. For example, temporal inactivation of the BNST, but not of the lateral amygdala, blocked fear behavior induced by exposure to TMT (Fendt et al., 2003). This is supported by c-*fos* studies, demonstrating a stronger increase in c-*fos* expression within the BNST than within the lateral amygdala after exposure to TMT (Day et al., 2004) or to cat odor (Dielenberg et al., 2001).

Although these data demonstrate a crucial function of the BNST in innate fear, the underlying neurochemical processes are unknown. It can, however, be speculated that noradrenaline (NA) in the BNST is involved in the processing of TMT-induced fear, because the noradrenergic innervation of the BNST is very prominent (Moore and Card, 1984; Woulfe et al., 1990) and responds to stress (Pacak et al., 1995a; Wang et al., 2001),

The aim of the present study was to investigate NA within the BNST in TMT-induced fear behavior. First, using immunohistochemical staining of dopamine-β-hydroxylase (DBH), we identified the region of the BNST with the densest NA innervation. Second, the NA release within the ventral BNST (vBNST) was measured using *in vivo* microdialysis in freely moving rats. Furthermore, clonidine, a α_2-receptor agonist that blocks NA release, was infused into the BNST during TMT exposure. Last, animals were exposed to TMT, and the freezing behavior was measured after injections of saline or clonidine into the BNST.

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Materials and Methods

Animals

All experiments were performed on male Sprague Dawley rats (Charles River Wiga, Sulzfeld, Germany). Before surgery, all rats were housed in groups of four to eight in laboratory cages (under standard conditions; temperature, 22 ± 3°C; lights on from 6:00 A.M. to 6:00 P.M.). After surgery, animals used for microdialysis were housed individually, whereas all other animals were also group housed. Food was restricted to ~12 g/d. Tap water was available *ad libitum*.

Experiments were performed in accordance with the ethical guidelines regarding the care and use of animals and were approved by the local council of animal care (ZP1/00 and ZP4/02).

Immunohistochemistry

Three male Sprague Dawley rats (200–260 g) were anesthetized with chloral hydrate (420 mg/kg, i.p.) and perfused through the ascending aorta with 100 ml of 0.1 M PBS, followed by 500 ml of cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Thereafter, the brains were removed, stored for 1 h in 5% sucrose 4% PFA, and then placed in 20% sucrose in PBS until they sank. Coronal sections of 50 μm were taken on a freezing microtome and divided into two series. The first series was Nissl stained with thionine, and the second series was used for DBH immunohistochemistry. DBH is a catecholamine biosynthetic enzyme (Kozicz, 2002), and DBH immunoreactivity was used in the present study to localize terminals within the BNST using NA or adrenaline as transmitter.

To visualize neurons containing DBH, the sections were washed in Tris-buffered saline (TBS), pH 7.6, and preincubated for 1 h with 10% goat serum in TBS with 0.3% Triton X-100. Afterward, the sections were transferred into the primary antiserum, which contained rabbit anti-DBH antiserum (dilution, 1:500; Eugene Tech, Ramsey, NJ) and incubated at 4°C. After 24 h, the sections were washed several times in TBS and transferred into the secondary antiserum containing biotin-labeled goat anti-rabbit antiserum (dilution, 1:1000; Vector Laboratories, Burlingame, CA) for 1 h. After several washes in TBS, the sections were incubated in avidin–biotin complex (ABC) reagent (dilution of 1:100; Vectastain ABC-Elite kit; Vector Laboratories) for 1 h at room temperature. For the development of immunostaining, 0.02% 3–3′-diaminobenzidine (Sigma, Deisenhofen, Germany) in 0.005% H_2O_2 in TBS, pH 7.6, was used for ~10 min. The reaction was controlled under a stereomicroscope and stopped in TBS. After several washes in TBS, the sections were mounted on gelatin-coated slides and allowed to air-dry overnight. All sections were dehydrated in an ascending ethanol series, cleared in xylene, and coverslipped using DPX. For data analysis, representative sections were analyzed under a Reichert-Jung (Vienna, Austria) microscope.

Drugs

Microdialysis. The α_2-adrenoceptor agonist clonidine (100 μM; Biotrend, Cologne, Germany) was used. This dose has been shown to be effective in reducing NA levels when applied by reversed microdialysis into the locus ceruleus and the prefrontal cortex (Pudovkina et al., 2001). Clonidine was diluted in artificial CSF (aCSF) containing the following (in mM): 147 NaCl, 2.5 KCl, 1.3 $CaCl_2$, and 0.9 $MgCl_2$. Each day, aCSF as well as clonidine solutions were controlled to have a pH level of 7.2 ± 0.1.

Microinjections. Clonidine (5 mM) was used (i.e., 5 nmol/0.25 μl). This dose is one-half the amount that we used in previous experiments and that was effective in blocking fear after injections into the BNST (Schweimer et al., 2005). Clonidine was diluted in saline.

Surgery

Microdialysis. Rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and placed in a stereotaxic frame. Guide cannulas (CMA microdialysis; Semrau, Sprockhövel, Germany) were implanted unilaterally into the BNST (−0.5 mm rostral, −1.5 mm lateral, −5.8 mm ventral to bregma) (Paxinos and Watson, 1997) using standard stereotaxic procedures as described previously (Steiniger and Kretschmer, 2003).

Microinjections. Rats were anesthetized with ketamine/xylazine (9:1; 100 mg/kg, i.p.) and placed in a stereotaxic frame with blunt ear bars. Two stainless-steel guide cannulas (diameter, 0.7 mm) were implanted bilaterally into the brain aiming at the BNST (0.5 mm rostral, 1.4 mm lateral, 6.5 mm ventral to bregma) (Paxinos and Watson, 1997). The cannulas were fixed to the skull with dental cement and three anchoring screws. After surgery and between the tests, the cannulas were fitted with stiletts (diameter, 0.4 mm) to maintain patency. Rats were given 4–6 d to recover from surgery before testing.

Apparatus

Microdialysis. During microdialysis, the rats were attached to a tether, turning a liquid swivel (375/D/22QE; Instech Laboratories, Plymouth Meeting, PA) that was mounted to a counterbalanced arm. This set up allowed sampling in freely moving rats that were placed into an open field ($37 \times 47 \times 44$ cm^3) made of polyvinyl chloride. The perfusion medium (aCSF or aCSF plus clonidine) was delivered by using a CMA 200 pump and collected automatically by using the CMA 140 Microfraction Collector. The switching of the perfusion medium from aCSF to aCSF plus clonidine was done by using the CMA 111 syringe selector, if necessary.

TMT-exposure boxes. The animals were placed in one of four identical exposure boxes ($30 \times 30 \times 30$ cm^3) made of polyvinyl chloride to assess TMT-elicited freezing. The front doors of these chambers were constructed of Plexiglas to permit observation of the rats. The behavior of the animals was videotaped for later analysis. Each exposure box was connected via Teflon tubing to a generator supplying charcoal-filtered air; the outflow of the box was connected to an exhaust system. The air stream could be directed with the help of electrically operated three-way Teflon valves, either directly to the exposure boxes or indirectly through a glass bottle containing the odorant [5 μl of TMT (PheroTech, Delta, British Columbia, Canada) or 5 μl of saline on a piece of filter paper] and then to the boxes. In both cases, the airflow was regulated with needle valves (17 L/min) and monitored by flow meters.

Motor activity. Additionally, the effect of clonidine injections into the BNST on motor activity was measured. Therefore, rats were individually placed in a housing cage ($95 \times 44 \times 21$ cm^3). The activity of the rats was measured by an infrared detector using additional software (MOT version 1.2; TSE Systems, Bad Homburg, Germany).

Behavioral procedures

Effects of TMT on NA levels within the BNST. To test the effects of TMT on NA transmission within the BNST, *in vivo* microdialysis experiments were performed on freely moving rats. After a minimum recovery period of 72 h, a microdialysis probe (CMA/12, CMA microdialysis) with an active membrane length of 2 mm and a diameter of 0.5 mm was gently inserted into the BNST. Thereafter, rats were placed into the open field. The probes were perfused with aCSF at a flow rate of 1.8 μl/min.

After a 3 h equilibrium period, 10 20-min samples were collected. The first three samples were taken as baseline samples (aCSF perfusion and no odorant presentation). Subsequent to the last baseline sample, the odorant was presented by placing a piece of filter paper containing 5 μl of TMT into the open field. No changes regarding the perfusion medium were done in six animals (TMT-only group). On six additional animals, the effect of clonidine on TMT-induced NA release within the BNST was tested (TMT plus clonidine group). In the case of these animals, the perfusion medium was switched simultaneously with the TMT exposure from aCSF to aCSF plus 100 μM clonidine. The sampling continued under these conditions in both groups for an additional 140 min.

Effects of clonidine injections into the BNST on TMT-induced freezing. Twenty-five rats were used to test whether local microinjections of clonidine into the BNST affect TMT-induced freezing. First, each rat was placed in one of the exposure boxes on 2 consecutive days for 15 min to habituate the animals to the boxes. On the following 4 d, each animal received bilateral injections of saline or clonidine into the BNST. The solutions were infused bilaterally at a rate of 0.1 μl/10 s. After the injections, cannulas were left in place for an additional 2 min to allow the diffusion of the solution away from the tip of each cannula. Thereafter, rats were placed into the exposure boxes, and the freezing behavior was recorded during the next 15 min. After the first 4 min of clean airflow, the airflow was switched either to the TMT or to the saline-containing glass bottle. This airflow was presented to the individual animals for the next 11 min, respectively (Wallace and Rosen, 2000, 2001; Fendt et al., 2003).

After the test, animals were placed into a cage located in a fume hood for 2 h and then placed back to their home cage. All rats were repeatedly tested on 4 subsequent days, receiving both injections of saline and clonidine exposure to saline (fresh air) as well as TMT in a pseudorandomized order.

After each experimental session, the odor chamber and tubing were thoroughly washed with 70% ethanol and ventilated with clean air for 2 h. One observer who was not aware of the animal's condition analyzed the videotapes from all experiments. Freezing behavior was used as a measure of fear and is characterized by crouching, with cessation of movements except for those associated with breathing (cf. Blanchard and Blanchard, 1969). The percentage of time spent freezing was calculated for each rat, for every minute, for each test session.

Effects of clonidine injections into the BNST on motor activity. Sixteen rats received bilateral injections of saline or clonidine into the BNST (see above for injection procedure). Thereafter, they were placed into a laboratory cage, and the motor activity was recorded for the next 15 min. Each rat was tested once and received 0, 2.5, 5, or 10 nmol/0.25 μl of clonidine. For this experiment, rats from the TMT-exposure test were used 1 week after the TMT-exposure test ended.

Histology

After the experiments, the brain of each rat was removed and transferred to 4% PFA. The localization of the microdialysis probes as well as the injection cannulas was examined in Nissl-stained frontal brain sections (50 μM). The injection and microdialysis sites were drawn onto plates taken from the atlas used in the study by Paxinos and Watson (1997).

Biochemical analysis

NA was immediately analyzed using reverse-phase HPLC (Bischoff Chromatography, Leonberg, Germany) with electrochemical detection (Bischoff Chromatography; ESA, Leonberg, Germany). Briefly, an HPLC pump (Bischoff Chromatography) kept a constant flow of the mobile phase through a Prontosil 53 × 3 column (Bischoff Chromatography). The mobile phase contained 9.31 g of NaH_2PO_4, 37 mg of EDTA, and 200 mg of octanesulfonic acid diluted in 1 L of a 7% methanol solution. The pH was adjusted to 3.71. Electrochemical detection was performed with a two-electrode system (ESA Coulochem 5100; Bischoff Chromatography) with +175 mV at the first electrode and −200 mV at the second electrode. A detection limit of 0.1 nM NA was routinely achieved.

Statistics

The microdialysis data are expressed as concentrations found in the dialysates and transformed to percentages of the corresponding baseline values. Microdialysis data were assessed using nonparametric ANOVA with *post hoc* comparisons [Fisher's least significant difference (LSD) (protected *t*) test]. The data of the behavioral test (TMT exposure and motor activity) were analyzed with parametric ANOVAs using a repeated-measure design. The level of significance was set at $p < 0.05$. All data are presented as means ± SEM.

Results

Immunohistochemistry of catecholaminergic terminals within the BNST

In all three of the animals used for this experiment, DBH-immunopositive staining was only found in the ventral part of the BNST (Fig. 1). Closer inspections of this staining showed that terminals, not cell bodies, were DBH immunopositive. These results clearly indicate that noradrenergic terminals can only be located in this region. We therefore aimed to position injection cannulas and microdialysis probes used in the present experiments exactly in this region (Fig. 2*A*,*B*).

Measurement of NA release within the BNST during TMT exposure by microdialysis

All microdialysis probes were located within the BNST (Fig. 2*B*). Two locations were in the dorsal part of the BNST but still in a sufficient distance to the ventral part of the BNST, where the catecholaminergic terminals are located (compare Fig. 1).

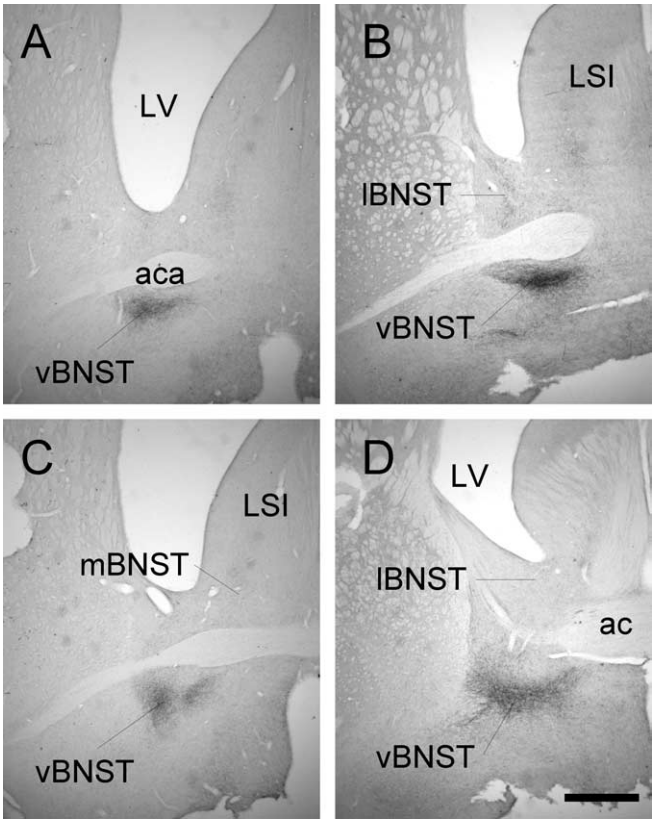

Figure 1. Photomicrograph demonstrating the localization of the noradrenergic terminals within the BNST (immunohistochemical staining of DBH). ac, Anterior commissure; aca, anterior part of the anterior commissure; lBNST, lateral part of the BNST; LSI, intermediate part of the lateral septum; LV, lateral ventricle; mBNST, medial part of the BNST. Scale bar, 1 mm.

TMT exposure produced a long-lasting increase of NA levels within the BNST, as shown in Figure 3*A* (repeated-measure ANOVA, $F_{(9,54)} = 6.50$; $p < 0.0001$). This increase in NA levels could not be observed after simultaneous clonidine infusion (100 μM) into the BNST (Fig. 3*B*); in this case, a delayed decrease of NA levels within the BNST was found ($F_{(9,54)} = 5.02$; $p < 0.0001$). *Post hoc* Fisher's LSD (protected *t*) test identified significant differences from basal mean levels at 160 and 180 min ($p > 0.05$).

Effects of clonidine injections into the BNST on TMT-induced freezing

The injection sites were mainly located in the dorsal or ventral part of the BNST but also in the globus pallidus, septofimbrial nucleus, and lateral septum (Fig. 2*A*). The behavioral data of the rats with injection sites within the ventral BNST were pooled and formed the vBNST group. The remaining rats represented the misplaced injections group. In both groups, we found a high percentage of animals in which the injection cannulas passed the ventricle on one or both sides. Therefore, one might assume that diffusion of the injected drug into the ventricle might be reasonable for some of the drug effects found in the present study. Statistically, there was no difference between the number of animals in both groups, in which cannulas passed through the ventricle (uncorrected χ^2 test: $\chi^2 = 0.49$; df = 1; $p = 0.48$). Thus, the drug diffusion into the ventricle cannot account for the behavioral difference found between the groups in this experiment.

To analyze the effects of TMT exposure on freezing behavior, ANOVAs with test phase (pre-odor, odor), odor (fresh air, TMT), and treatment (saline, 5 nmol/0.25 μl clonidine) as

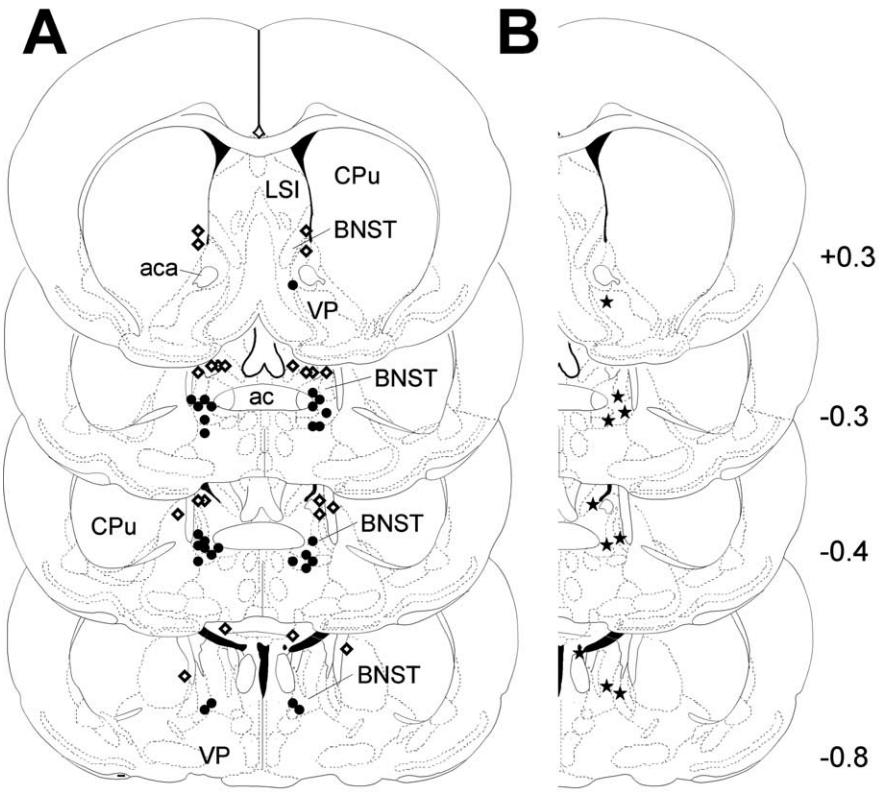

Figure 2. Reconstructions of the different injection sites of saline and clonidine into the BNST (***A***, filled circles, test on TMT-induced freezing and on motor activity; diamonds, misplaced injections) and the microdialysis sites (***B***, stars). The coronal sections were taken from the atlas of Paxinos and Watson (1997); numbers to the right indicate distance (in millimeters) from bregma. ac, Anterior commissure; aca, anterior part of the anterior commissure; CPu, caudate-putamen; LSI, intermediate part of the lateral septum; VP, ventral pallidum.

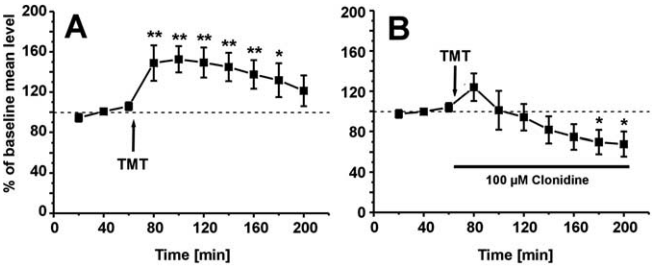

Figure 3. NA levels in the dialysates of the BNST. ***A***, Effect of TMT presentation on NA levels ($n = 6$). ***B***, Effects of local clonidine (100 μM) perfusion and simultaneously presented TMT on NA levels in the BNST ($n = 6$). The dashed line indicates 100% (i.e., the baseline mean level). The arrow indicates the time point of the TMT presentation. Clonidine (100 μM) was infused via reversed microdialysis as indicated by the black bar. Data are presented as a percentage of the basal mean level ± SEM. Data were analyzed by repeated-measures one-way ANOVA, followed by Fisher's LSD (protected *t*) test, if appropriate. $^*p < 0.05$, $^{**}p < 0.01$; significant differences from basal mean levels.

within-subject factors were performed for the vBNST and the misplaced injections groups. Under control conditions (saline injections), TMT exposure potentiated the freezing response in both groups (Fig. 4*A*,*B*) revealed by a significant effect of the factor odor (vBNST, $F_{(1,25)} = 58.4$, $p < 0.001$; misplaced, $F_{(1,14)} = 52.7$, $p < 0.001$) and a significant interaction phase × odor (vBNST, $F_{(1,25)} = 4.78$, $p = 0.038$; misplaced, $F_{(1,14)} = 6.12$, $p = 0.027$). Furthermore, paired *t* tests showed a significant increase of freezing by TMT in both groups (vBNST, $t = -3.58$, $p = 0.004$; misplaced, $t = -2.53$, $p = 0.035$).

This potentiation of freezing behavior by TMT could not be observed after injecting clonidine into the BNST (Fig. 4*A*). This was revealed by a significant interaction phase × odor × treatment ($F_{(1,25)} = 8.12$; $p = 0.009$) and confirmed by the fact that TMT did not further enhance freezing potentiation after clonidine injections given into the vBNST (paired *t* test, $t = 0.47$; $p = 0.65$). This effect of clonidine was not observed in the misplaced injections group, when clonidine had been injected in neighboring brain nuclei (dorsal BNST, globus pallidus, septofimbrial nucleus, and lateral septum). In these cases, no interaction of phase, odor, and treatment ($F_{(1,14)} = 0.008$; $p = 0.93$) was observed. Additionally, a potentiation of freezing behavior by TMT was detected (paired *t* test, $t = -3.28$; $p = 0.017$).

Effects of clonidine injections into the BNST on spontaneous motor activity

Clonidine injections into the BNST did not affect the spontaneous motor activity measured in a regular housing cage (Fig. 5). An ANOVA revealed no significant effect of the different doses of clonidine used in this experiment ($F_{(3,15)} = 0.50$; $p = 0.69$).

Discussion

In the present study, DBH-immunopositive terminals within the BNST were only found in the vBNST, demonstrating that a noradrenergic innervation of the BNST is restricted to the vBNST. Therefore, we aimed to position injection cannulas and microdialysis probes restrictively to the vBNST. These experiments clearly demonstrated an increase in NA transmission during TMT exposure. Moreover, clonidine infusions into the vBNST blocked this increase and the potentiation of freezing behavior induced by TMT. Interestingly, freezing behavior was not abolished by clonidine injections into neighboring brain areas (dorsal BNST, globus pallidus, septofimbrial nucleus, and lateral septum). Furthermore, clonidine injections into the vBNST did not affect spontaneous motor activity.

Our immunohistochemical data indicate that only the vBNST is densely innervated by noradrenergic and/or adrenergic fibers, whereas other regions of the BNST showed no or only weak DBH immunoreactivity. This finding confirms the results of previous studies, which report much higher NA levels in the vBNST, compared with other parts of the BNST (Brownstein and Palkovits, 1984; Moore and Card, 1984; Kilts and Anderson, 1986; Woulfe et al., 1990). Although we cannot distinguish between NA and adrenaline, it was shown previously that adrenaline is almost absent in the BNST (Kilts and Anderson, 1986), indicating that the stained terminals in the present study are mainly noradrenergic. This noradrenergic innervation of the BNST arises probably from the A1 and A2 medullary cell groups (Ungerstedt, 1971; Moore, 1978). These two cell groups are activated by threatening stimuli (Zhu and Onaka, 2002; Bailey et al., 2003). Thus, they might also be activated by predator odors. The aim of our immunohistochemical experiment was to identify the subregion of the

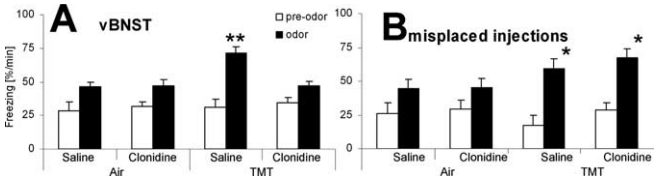

Figure 4. Mean percentage (±SEM) of time spent freezing to air and TMT in the pre-odor (min 1–4) and odor (min 5–15) condition. ***A***, Effects of clonidine injections into the ventral part of the BNST. ***B***, Effects of misplaced clonidine injections. *$p < 0.05$ and **$p < 0.01$ compared with the pre-odor condition (dependent *t* tests after ANOVA).

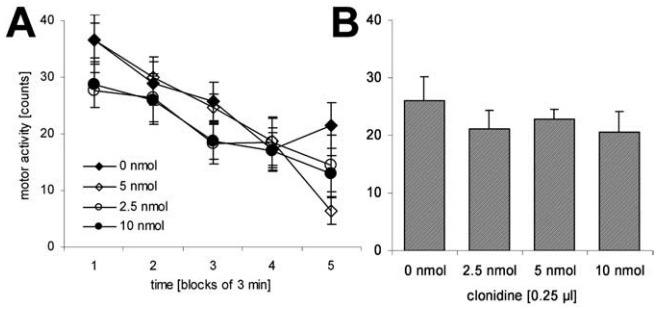

Figure 5. Time course of (***A***) or mean (***B***) spontaneous motor activity after clonidine injections into the ventral BNST measured by activity counts (±SEM) during 15 min in a cage.

BNST that is most densely innervated by NA. This subregion might be most responsive to manipulations of NA transmission in the following experiments.

Our microdialysis experiments confirm previous studies, demonstrating an increase in NA release within the BNST during the exposure of rats to stressful stimuli (Pacak et al., 1995a,b; Onaka and Yagi, 1998). To the best of our knowledge, the present experiment is the first to demonstrate that exposure to a predator odor enhances NA transmission within the BNST. Similar changes were also detected after stress induced by foot shocks or other stressors (Tsuda and Tanaka, 1985; Lorens et al., 1990).

In summary, our microdialysis data clearly support the idea that the NA release within the BNST is increased by stressful stimuli. Furthermore, the present study demonstrates that infusions of clonidine, a α_2-receptor agonist, into the BNST block this TMT-induced increase in NA release. These data elaborate studies showing that NA release can be blocked by local administration of clonidine into the BNST, as well as into other brain sites (van Veldhuizen et al., 1993; Forray et al., 1995; Carter, 1997; Erb et al., 2000; Garcia et al., 2004).

The introduction of the microdialysis probe into the brain causes a small lesion, which leads to a strong release of intracellular-stored substances, such as NA. Eventually, the extracellular concentration of NA reaches stable baseline concentration. This condition is mandatory to measure neurochemical effects of an experimental manipulation, for example exposure to TMT. As a consequence, the animals in this experiment were placed into the set up several hours before TMT presentation and are therefore habituated and mostly inactive after this time. Hence, this situation is not suitable for measuring immobility behavior, such as freezing. Therefore, we performed a separate behavioral experiment, in which the effects of clonidine injections into the BNST on TMT-induced freezing behavior were investigated. In previous experiments, we showed that freezing is the most affected behavior by TMT exposure (Fendt et al., 2003; Laska et al., 2004).

Clonidine injections into the vBNST clearly blocked the potentiation of freezing behavior induced by TMT. This effect of clonidine is specific to the vBNST, because injections of clonidine into the dorsal part of the BNST, as well as into the neighboring areas, did not affect TMT-induced freezing. One might speculate that the blockage of freezing behavior is caused by a motor stimulating effect of clonidine, because it was reported that systemic clonidine injections have this effect (Spyraki and Fibiger, 1982; Millan et al., 2000). However, in our study, clonidine injections into the vBNST did not increase spontaneous motor activity (compare Fig. 5).

The present study is one of the first behavioral studies investigating the neural basis of TMT-induced behavioral changes. It is known that TMT exposure stimulates a number of brain sites, which are involved in fear processing, such as the BNST or parts of the amygdala (Day et al., 2004). Surprisingly, lesions of the amygdala, especially of its basolateral complex, did not affect TMT-induced fear behavior (Wallace and Rosen, 2001; Fendt et al., 2003). On the contrary, temporary inactivation of the BNST blocked TMT-induced freezing (Fendt et al., 2003). In this study, muscimol (a $GABA_A$ receptor agonist) was locally infused to induce a transient inactivation of the BNST. Because muscimol leads to an unselective and almost complete blockade of neural activity (Edeline et al., 2002), it was not possible to conclude on the transmitter system within the BNST that is critical for TMT-induced fear behavior. Because NA is found in very large concentrations within the BNST, we hypothesized that NA may mediate TMT-induced fear behavior. The data of the present study clearly support this hypothesis.

From the present data it is not possible to conclude on the neural pathway responsible for the NA release within the BNST in our study. Neuroanatomical studies demonstrate that the A1 and A2 medullary cell groups are the source of the NA innervation of the vBNST (Ungerstedt, 1971; Moore, 1978). However, these nuclei do not receive direct input from the olfactory system (Shipley et al., 1995), which would be a direct link between TMT presentation and NA release in the BNST. Therefore, indirect mechanisms need to drive the activity of the NA nuclei. The BNST itself, as well as the amygdala, receive glutamatergic input from the accessory olfactory system and project to the A1 and A2 medullary cell groups (Schwaber et al., 1980, 1982; Shipley et al., 1995). Thus, there are two possible pathways through which TMT exposure influences noradrenaline transmission within the BNST. First, the glutamatergic projection from the olfactory system could affect noradrenaline transmission via presynaptic facilitation. This is supported by the presence of presynaptic NMDA receptors on NA terminals within the BNST (Aliaga et al., 1995; Forray et al., 1997, 2000). Second, the medullary NA cell group could be positioned as a feedback or a long loop to the BNST. A long loop is supported by the fact that especially the projection from the medial amygdala to the medullary NA cell group is important for a full-NA response to a stressor (Dayas and Day, 2002). TMT could activate NA release within the BNST via an activation of the medial amygdala, which in turn excites the medullary NA cell groups and leads to an increased NA release within the BNST. Additional studies have to clarify which of these two possibilities are effective.

The enhancement of NA release may be necessary to fully express a behavioral response to TMT exposure (in this study, an induction of freezing behavior). This role of NA is also observed in other functions of the BNST as well as in other parts of the fear system. For example, a blockade of NA release within the BNST blocks behavioral symptoms of opiate withdrawal (Delfs et al.,

2000), place aversion learning (Wang et al., 2001), anxiety-like behavior in the elevated plus-maze (Cecchi et al., 2002), and behavioral responses to foot shocks (Onaka and Yagi, 1998).

It is unclear which output structures are involved in mediating BNST-dependent fear behavior. Principally, the BNST has a similar output pattern as the amygdala, which is known to be involved in fear processing (Davis, 1996). It is therefore reasonable to assume that the same structures (e.g., the periaqueductal gray, which are implicated in mediating amygdala-dependent fear behavior) are also implicated in mediating BNST-dependent fear behavior (e.g., TMT-induced freezing). In this respect, it is also important to note that the BNST is not only involved in unlearned fear behavior but also in learned fear (Sullivan et al., 2004; Schweimer et al., 2005). Clearly, more data are needed to clarify the exact role of the BNST in the neural circuitry underlying unlearned and learned fear behavior.

In summary, the present data clearly show that NA transmission within the vBNST is enhanced by exposure to the predator odor, TMT. Local infusions of clonidine into the vBNST block the aforementioned TMT-induced enhancement of NA release. Furthermore, fear behavior (here, freezing) is blocked by clonidine infusions into the vBNST. We hypothesize that an enhancement of the NA release within the BNST is necessary to fully express fear behavior during TMT exposure.

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