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Role of norepinephrine in the dorsomedial hypothalamic panic response An in vivo microdialysis study

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Abstract

Blockade of gamma-aminobutyric acid-A ($GABA_A$) receptors in the dorsomedial hypothalamus (DMH) elicits a panic-like response that includes increases in heart rate (HR), blood pressure (BP), respiration rate (RR), and anxiety. Norepinephrine (NE) has been postulated to be critical in regulating panic and anxiety responses. Therefore, the first study sought to determine changes in extracellular NE levels within the DMH following acute blockade of $GABA_A$ receptors in the DMH using in vivo microdialysis. Rats were implanted with femoral arterial catheters and microdialysis probes into the DMH. Following recovery, the DMH of conscious rats were perfused with 100, 150, or 200 μM solutions of the $GABA_A$ receptor antagonist bicuculline methiodide (BMI) via the microdialysis probe. HR and BP responses were recorded and the changes in extracellular levels of NE in the dialysate samples from the DMH were determined by HPLC. Rats receiving BMI injections showed dose-dependent increases in both the extracellular NE levels in the DMH as well as HR and BP. The second study was conducted to test the functional importance of NE in the DMH to the BMI-induced physiological responses. The effects of BMI microinjection into the DMH were measured at baseline and 10 days after local injection of either vehicle or two doses of 6-hydroxydopamine (6-OHDA), a neurotoxin known to lesion NE terminals. There was a significant loss of tissue NE levels as well as BMI-induced HR, BP and RR responses in the 6-OHDA-treated but not vehicle-treated rats. Thus, blockade of $GABA_A$ receptors in the DMH results in NE release and the presence of NE appears to be necessary for eliciting the physiological components of the panic-like responses in this region.

Keywords: Anxiety; Bicuculline; Blood pressure; Heart rate; Locus ceruleus; Panic; Stress

1. Introduction

The dorsomedial hypothalamus (DMH) is one of the forebrain sites thought to play a major role in the regulation of panic anxiety (Shekhar, 1994). Blockade of gamma-aminobutyric acid-A ($GABA_A$) receptors in the DMH of rats elicits several physiological responses such as increases in heart rate (HR), blood pressure (BP), and respiration rate (RR; DiMicco et al., 1992; Shekhar et al., 1993), corticosterone release (Keim and Shekhar, 1996) as well as behavioral measures of anxiety in a variety of tests (Shekhar, 1993; Shekhar and Katner, 1995; Shekhar et al., 1990). Thus, $GABA_A$ receptors in the DMH appear to tonically inhibit a coordinated physiological and behavioral response suggesting panic anxiety and may be an important neuroanatomical substrate in human panic disorder (Shekhar et al., 1996).

Activation of norepinephrine (NE) neurons, particularly in the locus ceruleus (LC), is also postulated to underlie the production of anxiety and panic responses (Redmond and Huang, 1979; Charney et al., 1995). Clinical studies in patients with panic disorder also suggest an overactivity of the NE system (Ko et al., 1983; Charney et al., 1984). The DMH has one of the highest contents of NE within

Abbreviations: BMI, bicuculline methiodide; BP, blood pressure; DMH, dorsomedial hypothalamus; HR, heart rate; NE, norepinephrine; RR, respiratory rate

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the hypothalamus (Bernardis and Bellinger, 1987) receiving afferents from many brain stem NE groups, including the LC (Moore and Bloom, 1979). Rats experiencing "anxiety" in a fear-potentiated startle test show increases in NE levels in the DMH (Shekhar et al., 1994). Therefore, NE neurotransmission in the DMH may also be associated with the generation of anxiety responses.

Since both the GABA neurons and NE terminals in the DMH appear to be involved in anxiety responses, it is hypothesized that there may exist a relationship with local GABA inhibition and NE release within the DMH. This report presents the results of two studies conducted to further clarify the interactions between the tonic GABA inhibition and NE neurotransmission in the DMH of rats. The first study measured, using in vivo microdialysis, changes in extracellular NE levels accompanying the physiological responses following perfusion with different concentrations of the $GABA_A$ receptor antagonist, bicuculline methiodide (BMI), into the DMH. In the second study, the effects of lesioning the NE terminals in the DMH with local injections of the neurotoxin 6-hydroxydopamine (6-OHDA) on the BMI-induced physiological responses were examined.

2. Materials and methods

Experiments were performed on male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing between 300 and 350 g. Rats were housed individually in plastic cages in a temperature-controlled room (72 °F), and kept on a 12-h day/night cycle (the day cycle beginning at 0700 h). They were given free access to food and water.

2.1. Construction of the microdialysis probe

Microdialysis probes were constructed according to procedures described previously (Perry and Fuller, 1992). Briefly, loop-style probes were made from Intramedic polyethylene (PE-10, PE-20) tubing (Clay Adams, Parsippany, NJ) contained in 19-gauge thin-wall stainless steel tubing. The dialysis membrane was approximately 1 mm in width and the measurement from the tip of the membrane to the stainless steel tubing was 2 mm in length. The in vitro recovery of monoamines by these probes was in the range of 9–12%.

2.2. Surgical methods

Rats were first given a 1 mg/kg ip injection of atropine (Sigma, St. Louis, MO) and were then anesthetized with a 50 mg/kg ip injection of sodium pentobarbital (Abbott laboratories, Chicago, IL). A catheter made of Tygon microbore tubing (Fisher Scientific, Pittsburgh, PA) was inserted into the femoral artery of the anesthetized rat. The free end of this catheter was then routed subcutaneously to the dorsal aspect of the neck and stabilized with a leather vest as described elsewhere (Shekhar et al., 1993).

In order to measure HR and BP during stereotaxic surgery, catheters were flushed with saline and connected to a Beckman R511 Dynograph (Schiller Park, IL). Rats were then placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the incisor bar set at +5°. The skull was exposed and a small hole was drilled above the area to be perfused. After removal of the dura mater, a microdialysis probe was fastened to a stereotaxic manipulator arm that was set at an angle of 10° and carefully lowered into the DMH on one side of the brain. The coordinates for the target site in the DMH in relation to bregma were 1.2 mm posterior, 0.5 mm lateral, and 9.0 mm ventral (Paxinos and Watson, 1986).

Next, a 1-cc syringe was filled with artificial cerebrospinal fluid (a-CSF; NaCl 0.124 M, KCl 0.002 M, $MgCl_2$ 0.002 M, KH_2PO_4 0.00125 M, $NaHCO_3$ 0.026 M, $CaCl_2$ 0.002 M, glucose 0.011 M) and connected to PE-20 tubing. The syringe was placed on a Harvard Apparatus syringe infusion pump 22 (South Natick, MA) and initially set to deliver 5 μl/min. For implanting the microdialysis probes (Experiment 1), the probe was connected to the PE tubing containing a-CSF and allowed to perfuse for 15 min at the abovementioned rate. After 15 min, the infusion pump rate was lowered to 1.5 μl/min and baseline HR and BP were established for 20 min. Next, the a-CSF infusion was disconnected and a separate 1-cc syringe and tubing that was filled with a 200 μM solution of BMI was connected to the microdialysis probe. At the same infusion rate of 1.5 μl/min, the 200 μM BMI was infused into the DMH via the probe for 3 min and 20 s (i.e., total volume of 5 μl). At the end of this time, the tubing containing BMI was disconnected and the tubing containing a-CSF was reconnected. HR, BP and respiration were recorded prior to BMI injection, and any changes following the injection were noted. For implanting microinjection cannulae (Experiment 2), the guide cannulae were lowered into the DMH and a 100 nl solution of BMI (20 pmol/100 nl) was injected via the injection cannulae. An increase in HR of at least 50 beats per minute was considered a reactive site in both experiments. Upon establishment of a reactive site, the implanted dialysis probe or guide cannula was anchored to the skull with the aid of three stainless steel screws and cranioplastic cement (Plastics One, Roanoke, VA).

2.3. Experiment 1: Microdialysis protocol

Experiments were performed in conscious, freely moving animals 24 h after probe implantation. Sixteen rats were implanted successfully with microdialysis probes in the reactive site at the DMH. The femoral arterial catheter of the rat was again flushed with saline, con-

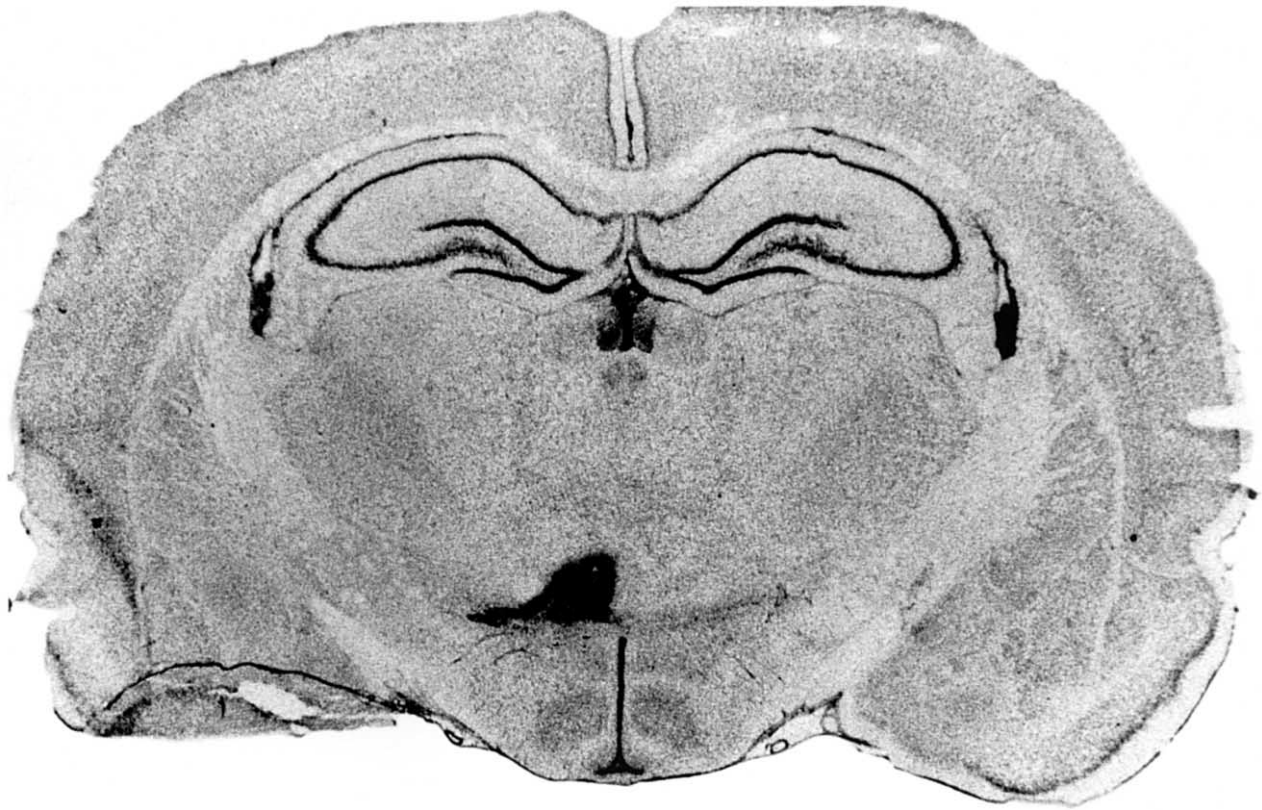

Fig. 1. Photomicrograph of an actual histological section from the brain of an animal that had the microdialysis probe implanted in the DMH. The site of implantation was marked by infusing India ink solution through the dialysis probe.

nected via transducer to a Beckman R511 Dynograph, and HR and BP were measured continuously throughout the experiment.

The microdialysis probe was then connected to a syringe and pump assembly infusing a-CSF via a CMA/120 swivel assembly (CMA/Microdialysis, Acton, MA). After an initial washout period of 15 min at 5 μl/min, the infusion pump rate was lowered to 1.5 μl/min for the remainder of the experiment. A separate syringe and tubing filled with one of the BMI (200, 150, or 100 μM) solutions was also attached to the pump. The dialysate was collected into 0.5-ml polyethylene tubes (Sarstedt, West Germany) containing 5 μl of 0.5 M HCl for preservation of NE.

During infusion of a-CSF, three 20-min baseline dialysate samples were collected. The infusate was then switched to one of the BMI solutions. The infusate containing BMI was again delivered in 3 min and 20 s (i.e., a total of 5 μl). Each rat received only one of the BMI doses. Following the injection, the tubing containing BMI was disconnected and the a-CSF infusion was reconnected. At least four 20-min post-BMI dialysate samples were then collected. Samples were immediately frozen in a container filled with dry ice, transferred to a −70 °C freezer and the NE measured by HPLC analysis within 2 weeks following the experiment.

2.4. Experiment 2: Microinjection and 6-OHDA protocol

Another 16 rats were implanted successfully with microinjection cannulae in the reactive site at the DMH. Experiments were begun 48 h after cannulae implantation by injecting BMI in conscious, freely moving animals. The femoral arterial catheter of the rat was again flushed with saline, connected via transducer to a Beckman R511 Dynograph, and HR, BP, and RR were measured continuously throughout the experiment. All rats were injected with vehicle (100 nl of a-CSF) and BMI (20 pmol/100 nl) and the increases in HR, BP, and RR elicited by BMI compared to vehicle were noted. The animals were then injected into the DMH with either freshly prepared vehicle (1 μl of 1% ascorbic acid, $n=4$) or two doses of 6-OHDA (8 and 12 μg/μl, $n=6$ each) and allowed to recover for 10 days. After 10 days, they were all retested with the injection of BMI into the DMH and the HR, BP and RR responses were again recorded.

2.5. Histology

At the end of each experiment, microdialysis probes or the guide cannulae were perfused with 50% India ink to verify placement in the DMH. The rats were injected a lethal dose of pentobarbital intraperitoneally, their brains

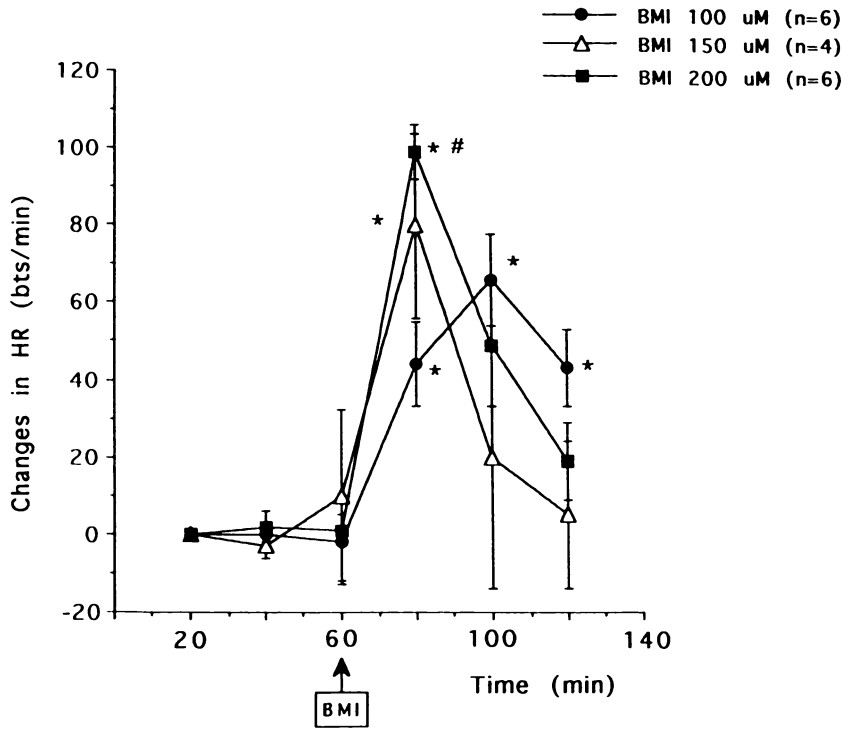

Fig. 2. Changes in HR following infusions of different concentration of BMI (5 μl of 100, 150 or 200 μM solution) in rats that were implanted unilaterally with a microdialysis probe in the DMH. Data are presented as mean ± S.E.M. The baseline HR for the three groups were (beats/min): BMI 100 μM — 371 ± 32; BMI 150 μM — 365 ± 30; BMI 200 μM — 395 ± 26. * Significantly different from baseline, $P < .05$ (repeated-measures ANOVA with Fisher's LSD test).

removed and those from the microdialysis study were sectioned into 40-μm slices. The sections were stained with Neutral Red and the exact site of injection was then determined by comparing them with the atlas of Paxinos

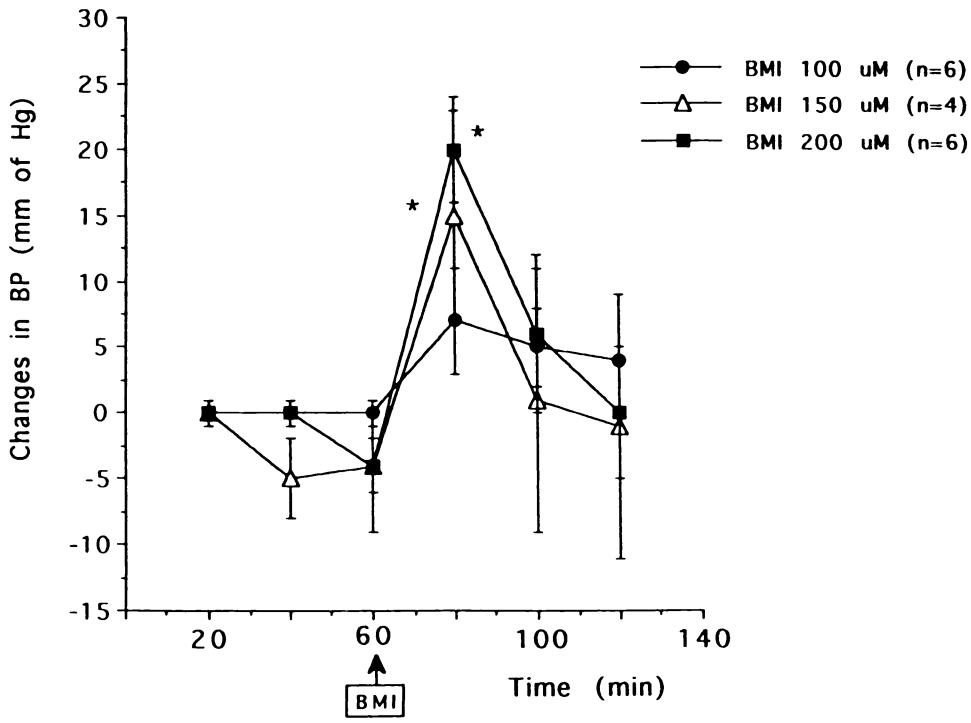

Fig. 3. Changes in BP elicited by infusions of different concentration of BMI in rats that were implanted unilaterally with a microdialysis probe in the DMH. Data are presented as mean ± S.E.M. The baseline BP for the three groups were (mmHg): BMI 100 μM — 96 ± 20; BMI 150 μM — 105 ± 13; BMI 200 μM — 102 ± 17. * Significantly different from baseline, $P < .05$ (repeated-measures ANOVA with Fisher's LSD test).

and Watson (1986). In the 6-OHDA study, the brains were dissected, the sites were visually verified and the DMH was microdissected as previously described (Shekhar et al., 1994) for tissue NE assay.

2.6. Norepinephrine assay

NE levels in the DMH were measured using HPLC equipment (Shekhar et al., 1994) consisting of an ISCO model 2350 pump, an EG&G Princeton Applied Research model 400 EC detector, a Hewlett Packard HP3396 Series II integrator, and a 3 μM ODS hypersil column (4.6 × 150 mm; Keystone Scientific, Bellefonte, PA). Mobile phase (1875 ml of 0.83 M sodium phosphate buffer, 75 ml of acetonitrile, 300 mg/l of 1-octanesulfonic acid at pH 3.1; and 800 μl of 0.1 M ethylenediaminetetraacetic acid [EDTA]) was pumped at 1 ml/min. NE was detected electrochemically using a glassy carbon electrode set at a potential of 0.7 V. The lower limit of detection by the assay was 50 fmol/25 μl.

2.7. Data analysis

The data were expressed as mean ± standard error of the mean (S.E.M.). The comparison of means in the microdialysis study were made by using a mixed ANOVA with the BMI concentrations being an independent variable, the microdialysate collection times being a repeated-measure variable and the NE, HR or BP being the dependent variable. Statistical significance was accepted at $P < .05$ and the post hoc comparisons were made using the Fisher's least significant difference (LSD) test. Student's t test was used to determine significant differences in the results obtained in the 6-OHDA experiments.

3. Results

3.1. Experiment 1

Fig. 1 shows an actual histological section from a rat implanted with a microdialysis probe within the DMH. All rats used in this study had microdialysis probes implanted within the region of the DMH.

Injection of 100, 150, and 200 μM BMI into the DMH of rats elicited a concentration dependent increases in HR. The increases in HR were maximal with the first dialysate sample (0–20 min) collected after BMI perfusion (Fig. 2).

Infusing the different BMI solutions also elicited concentration-dependent increases in BP in the animals. The 150 and 200 μM solutions of BMI into the DMH elicited the maximum BP response during the first dialysate sample collected after BMI perfusion and was

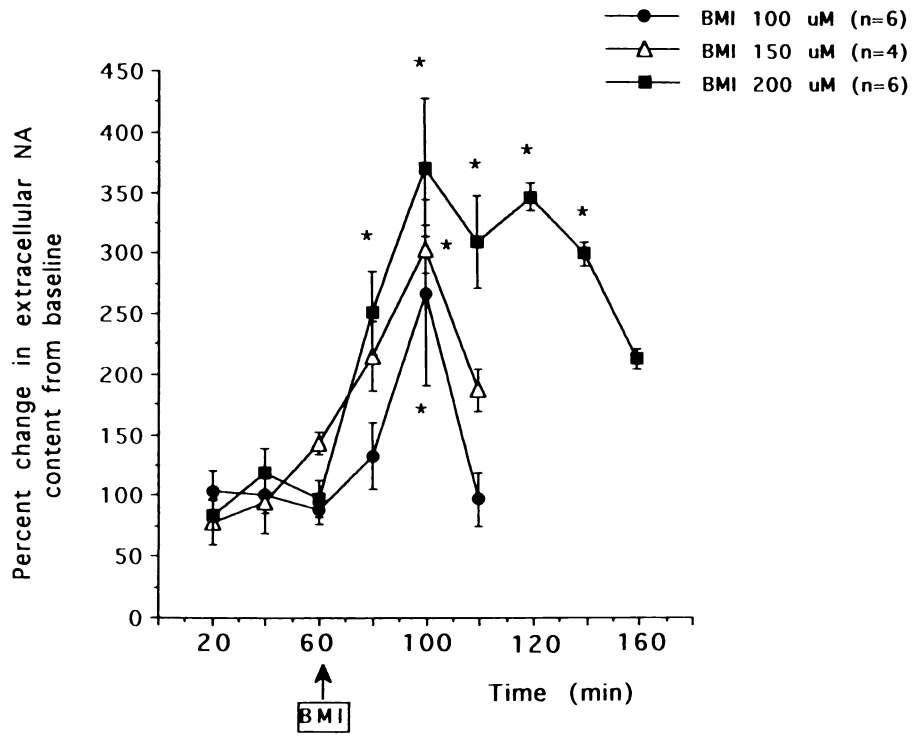

Fig. 4. Changes in extracellular fluid levels of NE following infusions of different doses of BMI via the microdialysis probe implanted in the DMH of conscious rats. Dialysates were collected in 20-min aliquots and assayed by HPLC. Data are presented as mean ± S.E.M. The basal levels of NE in the extracellular fluid for the three groups were (fmol/25 μl): BMI 100 μM — 73 ± 21; BMI 150 μM — 65 ± 12; BMI 200 μM — 75 ± 23. * Significantly different from baseline by repeated-measures ANOVA with Fisher's LSD test, $P < .05$.

Table 1
Effects of injecting either vehicle or two doses of 6-OHDA into the DMH of rats on the physiological responses to BMI injections

Treatment	HR, beats/min (baselines in parenthesis)	BP, mmHg (baselines in parenthesis)	Respiration, breaths/min (baselines in parenthesis)
Control (n = 4)			
Day 1 (Pre)	85 ± 17 (365 ± 26)	16 ± 3 (109 ± 11)	38 ± 8 (72 ± 9)
Day 10 (Post)	95 ± 18 (350 ± 30)	15 ± 4 (111 ± 19)	16 ± 5* (64 ± 10)
6-OHDA 8 μg (n = 6)			
Pre	77 ± 9 (385 ± 44)	16 ± 4 (119 ± 17)	41 ± 15 (88 ± 7)
Post	31 ± 9* (370 ± 27)	8 ± 3* (98 ± 16)	8 ± 3* (76 ± 14)
6-OHDA 12 μg (n = 6)			
Pre	69 ± 13 (343 ± 32)	14 ± 3 (129 ± 20)	42 ± 11 (80 ± 10)
Post	13 ± 12* (331 ± 19)	6 ± 2* (106 ± 21)	10 ± 7* (74 ± 6)

Ten days after microinjection, 6-OHDA injections significantly decreased the HR, BP and respiratory rate responses to BMI injections into the DMH.
* Significantly different from vehicle group by ANOVA coupled with Fishers LSD, $P < .05$.

significantly different from baseline. The 100 μM solution of BMI was not significantly different from baseline at any time period (Fig. 3).

Changes in the extracellular levels of NE following perfusion of various concentrations of BMI can be seen in Fig. 4. The mean NE amounts in the baseline collection fractions for the different BMI groups were as follows: BMI 100 μM—73 ± 21 fmol/25 μl; BMI 150 μM—65 ± 12 fmol/25 μl; and BMI 200 μM—75 ± 23 fmol/25 μl. There were concentration-dependent increases in extracellular NE following BMI infusions in the DMH. The time course of BMI-stimulated NE release indicated that the maximum response occurred during the second dialysate sample (20–40 min) collected after BMI perfusion. The increases in NE release continued for much longer than the increases in HR and BP following the infusion of BMI (see Figs. 2–4).

3.2. Experiment 2

At baseline, all the rats showed a significant increase in HR, BP and RR following microinjection of BMI (20 pmol/100 nl) into the DMH (Table 1). When they were retested with BMI injections 10 days following either vehicle or 6-OHDA treatments, the animals injected with 6-OHDA had significant loss of the BMI response compared to the vehicle-treated animals.

When the DMH tissue levels of monoamines were determined, microinjection of 6-OHDA and not vehicle resulted in a dose-dependent loss of NE and DA content in these rats (Table 2). The tissue levels of serotonin were unaffected in all groups, suggesting that this dose of 6-OHDA was selectively toxic to the catecholamine terminals in the DMH.

Table 2
Effects of injecting either vehicle or two doses of 6-OHDA into the DMH of rats

Treatment	NE (pmol/mg protein)	DA (pmol/mg protein)	5-HT (pmol/mg protein)
Vehicle ($n = 4$)	16.6 ± 2.6	2.1 ± 0.4	2.2 ± 0.6
6-OHDA 8 μg ($n = 6$)	6.7 ± 0.9*	1.0 ± 0.2	1.8 ± 1.0
6-OHDA 12 μg ($n = 6$)	4.6 ± 1.2*	0.6 ± 0.2*	1.7 ± 0.2

Ten days after microinjection, 6-OHDA significantly decreased the NE and dopamine but not serotonin levels in the DMH.
* Significantly different from vehicle group by ANOVA coupled with Fishers LSD, $P < .05$.

4. Discussion

The data presented above suggest that blocking the $GABA_A$ receptors in the DMH of rats with the selective antagonist BMI results in a dose-dependent increase in the extracellular fluid levels of NE, suggesting an increased neuronal release of NE. The release of NE appears to follow the increases in HR and BP seen after injection of BMI. There is a close parallel between the physiological changes and the NE release induced by BMI injections in the DMH (Figs. 2–4). It appears that the $GABA_A$ receptors in the DMH regulate the panic-like response that may be associated with a local release of NE. A similar release of NE in the hypothalamus has been reported in rats subjected to conditioned fear and other stress responses (Tanaka et al., 1983; Nakata et al., 1990; Ida et al., 1991). Blockade of $GABA_A$ receptors in the DMH significantly increases fear and anxiety responses in addition to activating the autonomic responses. Thus, an increase in local NE release would be consistent with an increase in anxiety and physiological responses.

The DMH has one of the highest contents of NE within the hypothalamus and appears to receive afferents from all brain stem NE groups (Moore and Bloom, 1979; Palkovits et al., 1974, 1980). The DMH has strong projections to the LC and the nucleus subceruleus (Ter Horst and Luiten, 1986). Physiological responses elicited by LC stimulation are attenuated in hypothalamus lesioned rats (Przuntek and Philippu, 1973). The autonomic and

behavioral responses elicited by hypothalamic stimulation is accompanied by a reduction in brain NE content (Reis et al., 1967) suggesting an interaction between the diencephalic areas and the LC. The NE terminals in the DMH appear to be regulated in a complex manner such that only certain types of stress paradigms elicit changes in the tissue levels (Shekhar et al., 1994) as well as the rates of basal and stimulated release of NE (Sajdyk et al., 1997). Similarly, the integrity of the catecholamine terminals appears be a necessary condition for the physiological responses elicited by GABA blockade in the DMH (Tables 1 and 2), while the GABA inhibition in turn may be important in regulating NE release in this region.

The exact role of NE in the anxiety response elicited by activating the DMH is not known. Lesioning the NE terminals in the DMH by local infusion of 6-OH-dopamine resulting in a significant attenuation of the increases in HR and BP elicited by BMI injections into the DMH clearly suggests a facilitatory role for NE in this DMH response. However, injections of 6-OH-dopamine into the DMH also reduced the dopamine content in the DMH, confounding the interpretation of the effects of the lesions to NE loss alone. More selective lesioning of the NE terminals only or infusions of NE receptor antagonists along with BMI into the DMH would further clarify the role of NE in this response. Increases in BP have been observed by infusing hypertonic saline into the hypothalamus that is associated with increased release of NE. This pressor response appears to depend on the NE released in the hypothalamus and may be acting via the alpha-2 adrenergic receptors (Nakata et al., 1990, 1993). Thus, it is likely that NE may be critical for modulating the different stress-induced responses in the DMH region. Therefore, the GABA neurons in the DMH, which tonically inhibit the stress and panic responses (DiMicco et al., 1992; Shekhar, 1994), could also be important in regulating the local release of NE.

In addition to possible regulation of the NE release by GABA, other explanations need to be considered. It is possible that the NE release is secondary to the HR and BP changes induced by the BMI injections into the DMH. The peak increase in NE levels occurred during the second collection fraction while the peak HR and BP effects took place during the first collection fraction after BMI injections, suggesting a delay in the NE response. It is unlikely that some of this delay could be mechanical since the delay in collection time (due to the movement of the infusate through the collection tubes) is accounted for by matching the collection tube numbering to the actual fraction collection time in this study. While it is possible that this correction may not be completely accurate, especially when the animal is agitated, it is unlikely to result in such a slower peak of the NE response in BMI-treated animals. In addition, after BMI injections into the DMH, the increases in NE release occur for a much longer interval than the increases in HR and BP. A similar phenomenon has been noted in other studies observing the increases in NE release following exposure to stressful stimuli (Nakata et al., 1990; Yokoo et al., 1990). This prolonged increase in NE release was particularly evident in the posterior regions of the hypothalamus much more than the paraventricular nucleus (PVN) of the hypothalamus (Nakata et al., 1990). Therefore, even within the hypothalamus, there may be differential local regulation of NE release. Injection of the $GABA_A$ agonist muscimol into the DMH and not PVN blocked the stress-induced tachycardia in rats (Stotz-Potter et al., 1996), suggesting that the $GABA_A$ receptors in the DMH may have a different role in stress responses than the PVN. The differences in NE release in the DMH compared to those in the PVN appear consistent with such regional differences in function within the hypothalamus.

In summary, blocking $GABA_A$ receptor-mediated inhibition by infusing BMI results in concentration-dependent increases in the extracellular fluid levels of NE in the DMH of conscious rats that parallel the increases in HR and BP. Lesioning the catecholamine terminals of the DMH significantly attenuated the increases in HR, BP and RR induced by BMI injections into the DMH. Thus, increased release of NE may be an important determinant of the panic-like response elicited by $GABA_A$ receptor blockade in the DMH of rats.

Acknowledgments

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