EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 36, pp. 3549–3558, 2012 doi:10.1111/j.1460-9568.2012.08277.x

BEHAVIORAL NEUROSCIENCE

Regulation of the ventral tegmental area by the bed nucleus of the stria terminalis is required for expression of cocaine preference

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Keywords: bed nucleus of the stria terminalis, cocaine, conditioned place preference, lateral hypothalamus, orexin, ventral tegmental area

Abstract

Lateral hypothalamus (LH) orexin neurons are essential for the expression of a cocaine place preference. However, the afferents that regulate the activity of these orexin neurons during reward behaviors are not completely understood. Using tract tracing combined with Fos staining, we examined LH afferents for Fos induction during cocaine preference in rats. We found that the ventral bed nucleus of the stria terminalis (vBNST) was a major input to the LH orexin cell field that was significantly Fos-activated during cocaine conditioned place preference (CPP). Inactivation of the vBNST with baclofen plus muscimol blocked expression of cocaine CPP. Surprisingly, such inactivation of the vBNST also increased Fos induction in LH orexin neurons; as activity in these cells is normally associated with increased preference, this result indicates that a vBNST–orexin connection is unlikely to be responsible for CPP that is dependent on vBNST activity. Because previous studies have revealed that vBNST regulates dopamine cells in the ventral tegmental area (VTA), which is known to be involved in CPP and other reward functions, we tested whether vBNST afferents to the VTA are necessary for cocaine CPP. We found that disconnection of the vBNST and VTA (using local microinjections of baclofen plus muscimol unilaterally into the vBNST and contralateral VTA) significantly attenuated expression of cocaine preference. However, blocking ionotropic glutamatergic afferents to the VTA from the vBNST did not significantly reduce cocaine preference. These results indicate that a non-glutamatergic vBNST–VTA projection is involved in expression of cocaine preference.

Introduction

Orexins (also named hypocretins) have been shown to play a role in mediating an array of behaviors related to reward and motivation. In particular, inhibition of the orexin 1 receptor (OX1R) attenuates seeking behaviors for alcohol (Lawrence *et al.*, 2006), cocaine (Smith *et al.*, 2009a,b), nicotine (Hollander *et al.*, 2008), amphetamine (Hutcheson *et al.*, 2011), and high fat (Borgland *et al.*, 2009). However, the neural circuitry underlying orexin's role in reward-seeking and related behaviors is poorly understood.

Orexin neurons, particularly in the lateral hypothalamus (LH), potentially have ideal neuronal connections to regulate reward processing. For example, they project to areas involved in motivation and reward, such as the nucleus accumbens and ventral tegmental area (VTA) (Peyron *et al.*, 1998; Fadel & Deutch, 2002; Baldo *et al.*, 2003), and these areas express high levels of orexin receptors (Trivedi *et al.*, 1998; Marcus *et al.*, 2001; Korotkova *et al.*, 2003). Several studies have shown that orexin input to the VTA is important in reward-related behaviors (Narita *et al.*, 2006; Harris *et al.*, 2007; Espana *et al.*, 2011; James *et al.*, 2011; Mahler *et al.*, 2012), and recent evidence indicates that orexin projections to other brain areas are also involved in drug seeking (Schneider *et al.*, 2007; Hollander *et al.*, 2008).

Although orexin projections to reward-related areas appear to play a significant role in reward and motivation, until recently very little was known about the inputs to orexin neurons that regulate their activity during drug seeking. LH orexin neurons receive afferents from several reward-related brain regions, such as the nucleus accumbens shell (NAcS), bed nucleus of the stria terminalis (BNST), central amygdala, lateral septum (LS), and prelimbic and infralimbic cortices (Sakurai *et al.*, 2005; Yoshida *et al.*, 2006; Marchant *et al.*, 2009). LH orexin inputs from the LS and NAcS have been implicated in cocaine conditioned place preference (CPP) (Sartor & Aston- Jones, 2012), beer seeking (Millan *et al.*, 2010), and food intake (Baldo *et al.*, 2004). However, it remains to be determined whether other afferents are also important in regulating LH orexin activity during reward-related behaviors.

In the current study, a combination of tract tracing, cocaine CPP and staining for the immediate early gene product, Fos, was used to identify brain regions that may be important in activating LH orexin

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Received 7 June 2012, revised 31 July 2012, accepted 3 August 2012

neurons and associated cocaine preference. We found that inputs to the LH from the ventral BNST (vBNST) show Fos activation during cocaine CPP in proportion to the preference expressed, and that inactivation of the vBNST blocked cocaine preference. Surprisingly, however, vBNST inactivation increased Fos expression in orexin neurons in the LH, indicating that activation of these cells was unlikely to mediate the role of the vBNST in cocaine preference.

Several studies have revealed a strong functional and anatomical projection from the vBNST to the VTA (Georges & Aston-Jones, 2001, 2002; Massi *et al.*, 2008; Briand *et al.*, 2010). Therefore, in the second group of experiments, we determined whether vBNST afferents to the VTA are necessary for expression of cocaine place preference. Using a vBNST–VTA bilateral disconnection approach, we found that vBNST afferents to the VTA are critical for cocaine preference.

Materials and methods

Animals

Male Sprague Dawley rats (initial weight approximately 300–325 g; Charles River, Raleigh, NC, USA) were pair-housed under a reversed 12-h light/dark cycle, and had *ad libitum* access to food and water. Rats were housed in a temperature-controlled and humidity-controlled animal facility at the Medical University of South Carolina (AAALAC-accredited). All experiments were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and conducted according to the specifications of the NIH as outlined in the *Guide for the Care and Use of Laboratory Animals*. A total of 110 rats were used in these experiments.

Stereotaxic surgery

In experiment 1, microinjections of cholera toxin b subunit (CTb) were made into the LH, using methods similar to those described in previous publications (Delfs *et al.*, 1998; Chen *et al.*, 1999; Sartor & Aston- Jones, 2012). In brief, rats were anesthetized with intraperitoneal ketamine/xylazine (56.5/8.7 mL/kg) and placed in a stereotaxic apparatus. A glass pipette (tip diameter, 10 μm) was lowered into the LH orexin field (from skull surface: AP, −2.8; ML, 1.7; DV, −8.8), and 30 nL of CTb (0.5% dissolved in 0.1 M phosphate buffer; Sigma) was unilaterally delivered via pressure injection. The pipette was left in place for 15 min after the injection to allow for CTb diffusion and to minimize backflow up the pipette tract.

In experiments 2, 3, and 4, guide cannulae (22 gauge; Plastics One) were implanted unilaterally or bilaterally 2 mm above the vBNST (15° angle lateral to medial: ML, ± 3.5; AP, −0.1; DV, −6.1). The guide cannulae were fastened to the skull with acrylic cement, and obturators were inserted to prevent blockage. Five minutes prior to the preference test, injector cannulae (28 gauge; Plastics One) were lowered through and 2 mm below the guide cannulae, and one of the following was infused: artificial cerebrospinal fluid (ACSF), a cocktail of the GABA receptor agonists baclofen and muscimol (B-M) (0.3 and 0.03 nmol, respectively), or the OX1R antagonist 1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride (SB-334867; 1 mM; Tocris). In experiment 3, unilateral B-M injections outside of the vBNST ($n = 5$) and ACSF injection into the vBNST ($n = 4$) were combined and referred to as control injections, because there were no differences in CPP scores or Fos expression between these two groups (t-values of < 1.2, $P > 0.05$).

In the bilateral disconnection studies (experiments 5 and 6), guide cannulae were implanted unilaterally above the vBNST and the contralateral VTA (10° angle ML: ML, ± 2.1; AP, −5.2; DV, −6.9). Five minutes before the preference test, injector cannulae (28 gauge; Plastics One) were lowered through and 2 mm below the guide cannulae, and one of the following was infused: ACSF, the GABA receptor agonist cocktail B-M (0.3 and 0.03 nmol, respectively), or a cocktail of the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 0.7 mM) and (2*R*)-amino-5-phosphonovaleric acid (AP5; 1.6 mM). All drugs were dissolved in ACSF, and injection volumes were 0.3 μL. The injector cannulae were kept in place for 1 min to allow for diffusion of drug. Animals with B-M or CNQX–AP5 injections outside of the vBNST or VTA (missed injections) were used for control data.

Cocaine conditioned place preference

Cocaine conditioned place preference experiments utilized methods previously employed by our laboratory (Harris & Aston-Jones, 2003; Harris *et al.*, 2004, 2005; Sartor & Aston- Jones, 2012). Briefly, the CPP apparatus consisted of two distinct compartments that were separated by a removable partition. In a pre-test acclimation session, rats were allowed free access to both sides of the chamber for 15 min via a doorway in the partition. The time spent on each side of the chamber was recorded automatically. None of the rats had an initial bias in the pre-test (< 100-s time difference). Two days later, rats were conditioned for three consecutive days. During conditioning, the rats were injected with cocaine (10 mg/kg; NIDA, Research Triangle Park, NC, USA) and confined to one side of the chamber by a solid partition for 30 min, or injected with saline and confined to the other side of the chamber for 30 min. Additional rats received saline injections on both sides of the chamber, and were used as controls in experiment 1 (non-conditioned, $n = 6$). As in our previous publication (Sartor & Aston- Jones, 2012), cocaine-conditioned rats that did not show a preference on the test day (< 75 s more on the cocaine side) were also included in the non-conditioned group in experiment 1 ($n = 7$). Conditioning occurred in morning and afternoon sessions (at least 4 h apart). Two days after the last conditioning day, rats were tested for cocaine preference in a 15-min session with free access to both sides of the chamber via a doorway in the partition. The time spent on both sides of the chamber was automatically measured via photobeam breaks and custom software. Rats that were saline-conditioned or cocaine-conditioned and did not show a preference on the test day were combined, because there were no differences in preference scores ($t_{11} = 0.46$, $P = 0.7$) or CTb/Fos labeling in each brain region (t-values of < 2.0, $P > 0.05$) between these groups. Where mentioned, a counterbalanced within-subject design was used to test for cocaine preference in some groups.

Immunohistochemistry

Fos was visualized in orexin-positive and CTb-positive neurons with a double-labeling immunohistochemical procedure. For this, rats were deeply anesthetized with intraperitoneal ketamine and xylazine (100 and 20 mg/kg, respectively) 90 min after the CPP test, and then perfused transcardially with cold 0.9% saline followed by 4% paraformaldehyde. Brains were cut into 40-μm-thick tissue sections, and then processed for visualization of Fos and orexin. Visualization of Fos was performed by incubating the sections in rabbit anti-Fos (1 : 5000; Santa Cruz) overnight, in biotinylated donkey anti-rabbit secondary antibody (1 : 500; Jackson) for 2 h, and finally in

avidin–biotin complex (ABC) (1 : 500) for 1.5 h. Detection of Fos was performed with 3,3′-diaminobenzidine (DAB) (Sigma) followed by nickel ammonium sulfate, producing a purple–black reaction product in the nucleus. Subsequently, in the same sections, orexin was visualized by incubation in goat anti-orexin (1 : 1000; Santa Cruz) overnight, biotinylated donkey anti-goat secondary antibody (1 : 500) for 2 h, and ABC (1 : 500) for 1.5 h. Finally, sections were incubated in DAB to stain the cytoplasm brown in orexin neurons.

For visualization of Fos in CTb neurons, Fos was stained as described above, and this was followed by incubation of the same tissue sections in goat anti-CTb (1 : 20 000; List Labs) overnight, in biotinylated donkey anti-goat secondary antibody (1 : 500; Jackson), and ABC (1 : 500). Finally, sections were incubated in DAB to yield a brown reaction product in the cytoplasm.

Histology

Following the CPP test, each rat was deeply anesthetized with ketamine–xylazine, and pontamine sky blue (2% in 0.5 M sodium acetate, 300 nL) was injected through the cannulae before the rat was perfused transcardially as described above. Brains were cut into 40-μm-thick coronal sections through the vBNST and VTA, and these were mounted directly on slides and counterstained with neutral red to confirm cannula placements.

Data analysis

Preference scores were calculated as the time spent in the cocaine-paired side minus the time spent in the saline-paired side on the CPP test. The resulting preference scores were compared within or between groups by use of Student's *t*-test or one-way ANOVA. When a significant *F*-value was obtained, pairwise comparisons were carried out with a Newman–Keuls test. GRAPHPAD PRISM V5 was used for statistical analysis. The risk of type 1 error (α) was set at $P < 0.05$.

The percentages of CTb or orexin neurons with Fos expression (doubly labeled neurons) were averaged across two sections per rat, and compared between groups by use of Student's *t*-test. Correlations between percentages of CTb neurons that were Fos-positive and preference scores were performed with Pearson's correlation coefficient test. An observer blind to the experimental conditions manually counted cells with a point-counter tool, using OPENLAB image processing software (Improvision).

Results

Experiment 1

A combination of tract tracing, cocaine CPP and staining for Fos was used to identify LH afferents activated during cocaine preference. Consistent with previously published data (Sakurai *et al.*, 2005; Yoshida *et al.*, 2006), we identified several brain areas that contained numerous retrogradely labeled (CTb-positive) neurons after injection of CTb into the LH (Table 1). Many retrogradely labeled neurons in the LS were Fos-activated during cocaine preference, as recently described (Sartor & Aston- Jones, 2012). We also found that vBNST inputs to the LH were significantly Fos-activated by cocaine CPP expression: 17% of CTb-positive (LH-projecting) neurons in the vBNST expressed Fos during cocaine preference (cocaine-conditioned, $n = 8$), as compared with 7% in control, non-conditioned rats ($n = 13$) ($t_{19} = 3.4$, $P = 0.003$) (Fig. 1A,C). The prelimbic cortex (PrL), infralimbic cortex (IL), NAcS and lateral preoptic nucleus (LPO) all contained $\geq$ 5% CTb-positive neurons that were Fos-positive during the cocaine CPP; however, none of these areas showed a significant increase in Fos expression in CTb-positive neurons after the CPP test ($P > 0.05$ each). The insula cortex, central and medial amygdala and VTA contained < 1% CTb-positive neurons with Fos positivity after the CPP test (data not shown), indicating that these areas may not be involved in regulating LH orexin neurons during cocaine preference.

TABLE 1. Mean (± standard error of the mean) numbers of CTb-positive and CTb-positive/Fos-positive neurons after CTb injections into the LH in non-conditioned and cocaine-conditioned rats

	Number of CTb^+ neurons		Number of CTb^+/Fos^+ neurons	
	Non-conditioned	Cocaine-conditioned	Non-conditioned	Cocaine-conditioned
PrL	14.8 (2.0)	22.4 (3.7)	1.6 (0.5)	3.1 (0.8)
IL	19.8 (2.1)	26.3 (3.3)	2.6 (0.6)	1.9 (0.5)
NAcS	29.9 (2.4)	35.0 (6.5)	1.7 (0.3)	1.7 (0.7)
vBNST	30.4 (4.1)	28.3 (4.3)	1.6 (0.3)	5.4 (1.7)*
dBNST	32.7 (3.4)	41.1 (8.1)	0.8 (0.3)	1.1 (0.4)
LPO	29.5 (1.7)	23.4 (3.2)	3.3 (0.5)	3.6 (1.4)

IL; PrL. *vBNST cocaine-conditioned > vBNST non-conditioned in number of CTb^+/Fos^+ neurons, $P < 0.05$.

Further analysis revealed a significant correlation between the percentage of LH-projecting neurons in the vBNST that were Fos-positive and the corresponding preference score for each rat ($r = 0.52$, $P = 0.02$) (Fig. 1B). Together, these results indicated that the vBNST, but not several other afferents to the LH, are activated in proportion to the degree of preference that rats exhibit for cocaine, and may play a role in driving this preference. Thus, vBNST inputs to the LH were studied in greater detail.

Experiment 2

To determine whether the vBNST is essential for the expression of cocaine preference, additional rats were conditioned with cocaine CPP and then given bilateral injections of the $GABA_{A/B}$ receptor cocktail B-M or ACSF into the vBNST immediately prior to the CPP preference test, using a within-subject, counterbalanced design ($n = 6$). Figure 2 shows that bilateral microinjections of B-M into the vBNST significantly attenuated cocaine preference as compared with ACSF injections into the vBNST or injections of B-M nearby but outside of the vBNST (missed B-M, $n = 6$) ($F_{2,15} = 15.06$, $P = 0.0003$). Injections of B-M outside of the vBNST (missed B-M preference scores: 160 ± 10 and 215 ± 95 for the dBNST and LPO, respectively) did not significantly affect expression of cocaine preference as compared with ACSF injections into the vBNST ($t_{10} = 1.8$, $P = 0.1$). Microinjections of B-M had no significant effect on general motor activity during the CPP test, as measured by the number of crosses between the two chambers ($F_{2,15} = 0.3$, $P = 0.9$). Interestingly, a significant trend and non-significant trend in Fos induction were found in non-orexin neurons (ACSF, 70 ± 11.5; B-M, 117 ± 12.3; $t_4 = 2.7$, $P = 0.04$) and orexin neurons in the LH (ACSF, 12 ± 2.1; B-M, 19.8 ± 3.3; $P > 0.05$), respectively, following this bilateral vBNST inactivation.

Experiment 3

To determine whether vBNST connections regulate LH orexin activity during expression of cocaine CPP (as indexed by Fos induction),

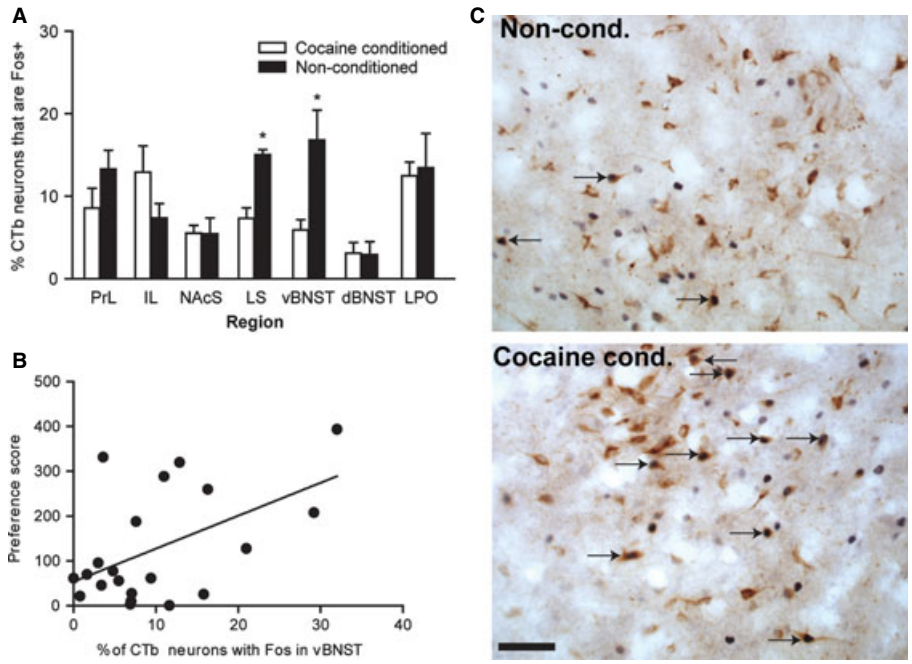

FIG. 1. Lateral hypothalamus afferents activated during cocaine place preference. (A) LH inputs from several brain regions are activated during cocaine preference. Note that the percentages of Fos-activated neurons in the vBNST that project to the LH orexin area were significantly increased during cocaine preference (cocaine-conditioned) as compared with non-conditioned rats (non-conditioned: saline-conditioned or cocaine-conditioned rats without a preference) (non-conditioned, $n = 13$; cocaine-conditioned, $n = 8$; $^*P < 0.05$ indicates a significant difference from non-conditioned by t-test). The data shown here for Fos activation of LS afferents to the LH orexin area are from our recent report (Sartor & Aston- Jones, 2012). (B) Significant positive correlations between percentages of CTb-positive neurons with Fos expression in the vBNST and preference scores (Pearson's correlation, $r = 0.52$, $^*P < 0.05$). (C) Representative immunohistochemical staining for CTb and Fos in the vBNST in cocaine-conditioned and non-conditioned rats. Note the substantial increase in the number of Fos-activated neurons in vBNST that project to the LH (CTb/Fos doubly labeled neurons) during cocaine preference as compared with non-conditioned control rats. Arrows indicate CTb (brown neurons) and Fos (black nuclei) doubly labeled neurons. Scale bar: 100 μm.

additional rats received a unilateral microinjection of B-M or vehicle into the vBNST immediately prior to the preference test. The unilateral approach was utilized because bilateral inactivation of the vBNST decreases preference, and such altered behavior could affect Fos expression in orexin neurons, thereby confounding the interpretation of Fos changes. Because almost all vBNST neurons that project to the LH are ipsilateral, inactivating the vBNST unilaterally should reveal effects in ipsilateral orexin neurons without affecting contralateral orexin neurons or CPP behavior. As shown in Fig. 3, we found that unilateral microinjections of B-M into the vBNST ($n = 7$) significantly increased Fos expression in orexin and non-orexin neurons in the ipsilateral LH and perifornical area/

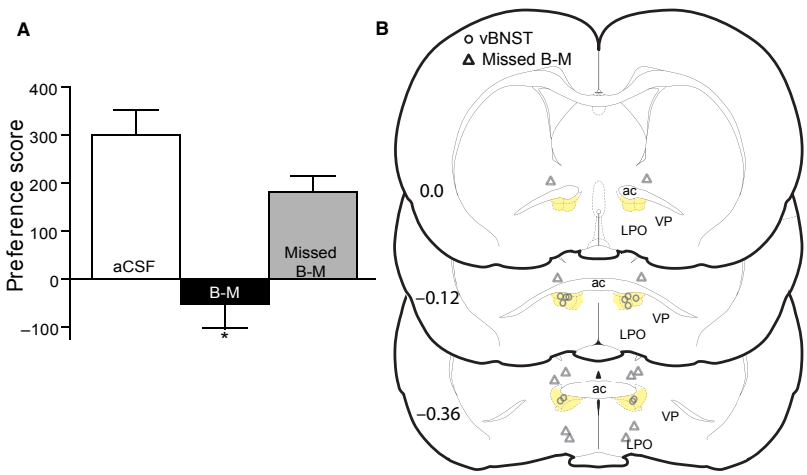

FIG. 2. Bilateral inactivation of the vBNST attenuated cocaine preference. (A) Bilateral microinjections of B-M into the vBNST (B-M), but not outside of the vBNST (Missed B-M) or vehicle injections into the vBNST (ACSF), significantly attenuated cocaine preference ($n = 6$; $^*P < 0.01$ indicates a significant difference by Newman–Keuls *post hoc* test). (B) Cannula placements in the vBNST (open circles) and B-M injections outside of the vBNST (Missed B-M, triangles). The vBNST is shaded. ac, anterior commissure; VP, ventral pallidum.

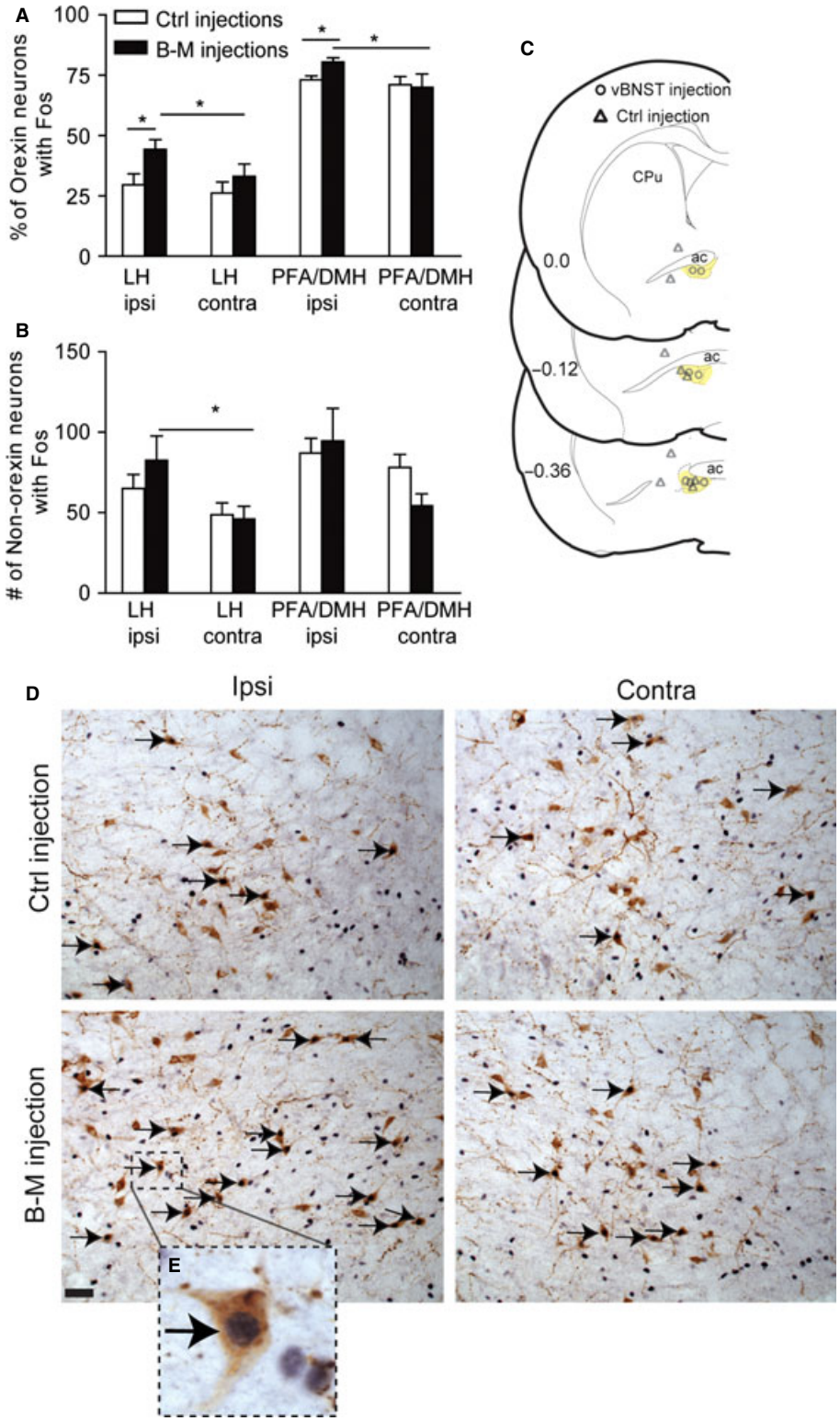

FIG. 3. Unilateral inactivation of the vBNST and orexin/Fos staining in the LH and PFA/DMH. (A and B) Percentages of orexin neurons with Fos expression and numbers of non-orexin neurons with Fos expression following unilateral B-M microinjection into the vBNST. Ipsilateral (ipsi) and contralateral (contra) are in reference to the unilateral B-M injection into the vBNST. Significant increases in the percentage of Fos-activated orexin neurons and the number of Fos-positive non-orexin neurons were found following injection of B-M into the vBNST (*$P < 0.05$ indicates a significant difference by *t*-test). (C) Unilateral B-M cannula placements in the vBNST (vBNST injections, open circles, $n = 7$) and outside of the vBNST, or ACSF microinjection placements in the vBNST (control injections, triangles, $n = 9$). (D) Representative staining for orexin (brown cytoplasm) and Fos (black nuclei) in the LH following unilateral vBNST inactivation. Note the increased number of orexin neurons with Fos expression (double-labeled) and non-orexin neurons with Fos expression (Fos only) in the LH, ipsilateral to B-M injection into the vBNST. Double-labeled orexin/Fos neurons are indicated by black arrows. Scale bar: 100 μm. ac, anterior commissure; CPu, caudate putamen.

dorsomedial hypothalamus as compared with injections of B-M outside of the vBNST, with ACSF injections into the vBNST (control injections, $n = 9$), or with orexin neurons in the non-injected, contralateral hemisphere (t-values of > 2.1, $P < 0.05$) (Fig. 3–a–c). Unilateral microinjections of B-M into the vBNST, however, did not alter preference scores (control preference score of 167.3 ± 28.8 s vs. B-M preference score of 152.4 ± 47.6 s, $t_{14} = 0.3$, $P = 0.8$). These manipulations did not alter general exploration of the chamber as measured by the number of crosses between chambers ($t_{14} = 0.04$, $P = 0.9$).

Experiment 4

As described above, bilateral inactivation of the vBNST during CPP blocked cocaine preference, but increased Fos expression in the LH and PFA/DMH. Previous results from our laboratory showed that LH orexin neurons are activated with cocaine preference (Harris *et al.*, 2005). Together, these findings indicate that LH orexin neurons are unlikely to be involved in the ability of vBNST inactivation to decrease cocaine preference. Given that high levels of OX1Rs and orexin fibers are present in the vBNST (Trivedi *et al.*, 1998; Cutler *et al.*, 1999; Marcus *et al.*, 2001), it seemed possible that orexin-mediated activation of the vBNST may be involved in cocaine preference, and that an influence of elevated orexin activity after vBNST inactivation would be obviated by vBNST inactivation. If that were the case, we would expect OX1R antagonism in the vBNST to decrease cocaine preference. To examine this hypothesis, the OX1R antagonist SB-334867 was bilaterally microinjected into the vBNST after CPP conditioning, prior to the CPP preference test ($n = 6$). As shown in Fig. 4, blocking OX1Rs in the vBNST in this way had no effect on cocaine preference as compared with vehicle injections (paired t-test, $t_5 = 0.3$, $P = 0.8$).

Experiment 5

We reasoned that bilateral vBNST inactivation might block cocaine CPP expression but also increase Fos expression in LH orexin neurons (which is normally associated with increased CPP) (Harris *et al.*, 2005) if vBNST projection to a non-hypothalamic region is necessary for cocaine CPP (see Discussion for a discussion of the possible mechanisms involved). Our laboratory previously reported a functional and anatomical projection from the vBNST to the VTA (Georges & Aston-Jones, 2001, 2002). To determine whether afferents to the VTA from the vBNST are necessary for expression of cocaine preference, we utilized a bilateral disconnection technique in which cocaine-conditioned rats received a unilateral microinjection of B-M into the vBNST and also into the contralateral VTA (B-M, $n = 11$) immediately prior to the CPP preference test (Fig. 5). Unilateral injections of ACSF into the vBNST and the contralateral VTA were used as vehicle controls (ACSF, $n = 8$). Figure 6 shows that bilateral disconnection of the vBNST and VTA significantly attenuated cocaine preference as compared with ACSF injections

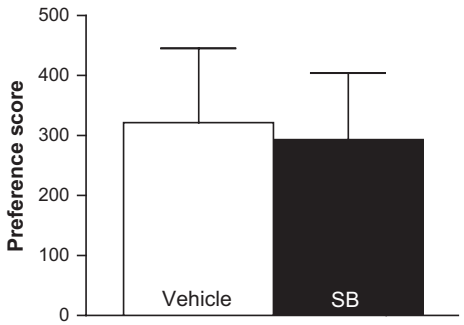

FIG. 4. Orexin-1 receptor antagonist injections into the vBNST. Bilateral injections of the OX1R antagonist SB-334867 (SB) had no effect on cocaine preference as compared with vehicle injections ($n = 6$, $P > 0.05$).

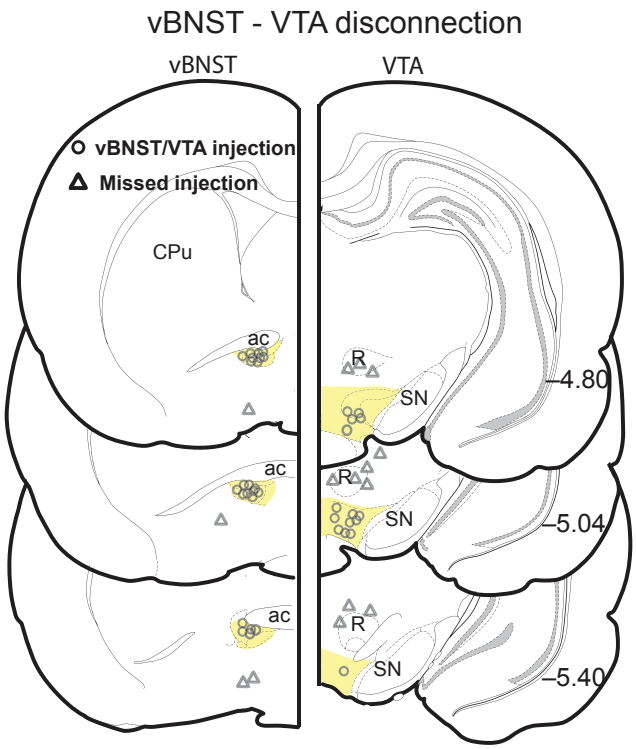

FIG. 5. Cannula placements for vBNST–VTA disconnection studies. Cannula placements within the vBNST and the contralateral VTA are indicated by open circles (vBNST/VTA injection), and placements outside of the vBNST or VTA are indicated by by open triangles (Missed injection). ac, anterior commissure; CPu, caudate putamen; R, red nucleus; SN, substantia nigra.

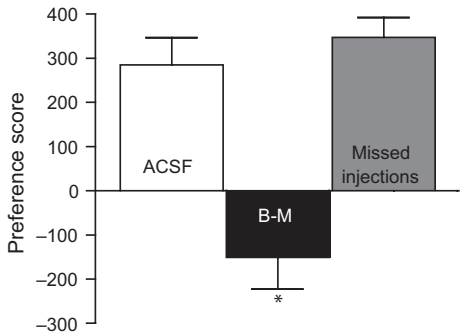

FIG. 6. vBNST–VTA disconnection eliminates cocaine preference. Unilateral injections of B-M into the vBNST and contralateral VTA (B-M, $n = 11$) significantly attenuated cocaine preference as compared with injections of ACSF into the vBNST and VTA (ACSF, $n = 8$) or with injections of B-M outside of the vBNST and/or VTA (Missed injections, $n = 10$) (*$P < 0.001$ indicates a significant difference by Newman–Keuls *post hoc* test). Note that vBNST–VTA disconnection also caused a significant avoidance of the cocaine paired side. Injections of B-M outside of the vBNST, VTA or both had no effect on CPP as compared with ACSF microinjections into the vBNST and VTA.

into the vBNST and VTA or with B-M injections outside of either the vBNST or VTA (missed injections, $n = 10$) ($F_{2,26} = 20.55$, $P < 0.0001$). Moreover, the accurately placed B-M microinjection into the vBNST and VTA produced a significant avoidance of the cocaine-paired side as compared with ACSF injections ($t_{17} = 4.3$, $P = 0.0004$). This bilateral disconnection of the vBNST and VTA had no significant effect on general motor activity, as measured by the number of crosses between the two CPP chambers (crosses after ACSF, 49.2 ± 9.6; crosses after B-M, 34.5 ± 6.8; $t_{17} = 1.5$, $P = 0.14$).

Experiment 6

Previous studies have indicated that the VTA receives glutamatergic inputs from the vBNST (Georges & Aston-Jones, 2001, 2002). To determine whether glutamatergic afferents to the VTA from the vBNST are critical for expression of cocaine preference, we again utilized a bilateral disconnection method. In these experiments, conditioned rats received a unilateral microinjection of B-M into the vBNST and a microinjection of a cocktail of the AMPA/*N*-methyl-D-aspartate (NMDA) glutamate receptor antagonists CNQX and AP5 into the contralateral VTA prior to the CPP preference test (B-M CNQX/AP5, $n = 11$). Unilateral microinjections of ACSF into the vBNST and contralateral VTA were used as vehicle controls (ACSF, $n = 8$). As shown in Fig. 7, unilateral microinjection of B-M into the vBNST and of glutamate receptor antagonists into the contralateral VTA did not significantly attenuate cocaine preference ($F_{2,23} = 1.2$, $P = 0.3$), although a noticeable reduction in the average preference score was apparent following this manipulation. These injections into the vBNST and VTA had no significant effect on the number of crosses during the CPP test (crosses after ACSF, 57 ± 11.3; crosses after after B-M/glutamate antagonists, 55.4 ± 7.8). In addition, microinjections of B-M or CNQX/AP5 outside of the vBNST or VTA (missed injections, $n = 7$) had no significant effect on preference scores as compared with ACSF ($t_{13} = 0.3$, $P = 0.8$) (Fig. 7).

Discussion

Summary of the present findings

Although there is mounting evidence for a role for orexin in reward processing, little has been known about the circuitry that regulates orexin neurons during reward-related behaviors. Recently, we found that LS afferents to LH orexin neurons are important for expression of cocaine place preference (Sartor & Aston-Jones, 2012). However, orexin neurons in the LH receive inputs from many reward-related brain regions (Sakurai *et al.*, 2005; Yoshida *et al.*, 2006; Marchant *et al.*, 2009), and it is unknown whether inputs in addition to those from the LS are important in cocaine preference. In the current study, we surveyed LH afferents for Fos activation during cocaine CPP. Consistent with previous results (Sakurai *et al.*, 2005; Yoshida *et al.*, 2006), we found that the LH receives strong inputs from several brain regions, including the PrL, IL, NAcS, LS, vBNST, and LPO. Despite the many retrogradely labeled afferents to the LH, we found that only the LS and the vBNST exhibited significant Fos induction with cocaine CPP. As the role of LS afferents to the LH in cocaine preference has been previously reported (Sartor & Aston-Jones, 2012), here we focused on the vBNST afferents.

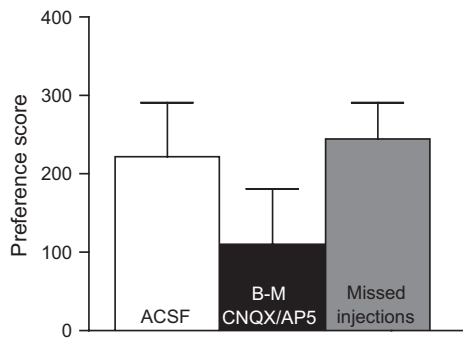

FIG. 7. Glutamatergic afferents to the VTA from the vBNST and cocaine preference. Unilateral inactivation of the vBNST with B-M and antagonism of NMDA and non-NMDA receptors in the contralateral VTA with CNQX/AP5 (B-M CNQX/AP5, $n = 11$) did not significantly attenuate cocaine preference as compared with ACSF injections into the vBNST (ACSF, $n = 8$), or with B-M and CNQX/AP5 injections outside of the vBNST or VTA (Missed injections, $n = 7$).

The percentages of CTb-positive neurons (retrogradely labeled from the LH) that were also Fos-activated in the vBNST were positively correlated with the amount of preference exhibited on the CPP test day. In addition, using B-M to locally inactivate neurons, we found that vBNST activity is necessary for cocaine CPP. We also unilaterally inactivated vBNST neurons during CPP expression, to evaluate the effect of vBNST projections to the LH on Fos expression in orexin cells. Unexpectedly, this manipulation increased Fos expression in ipsilateral LH orexin neurons during cocaine preference.

As previous studies showed that Fos activation in LH orexin neurons is positively correlated with increased cocaine preference (Harris *et al.*, 2005), the finding that inactivation of the vBNST increased Fos expression in these cells but also attenuated cocaine preference indicated that LH orexin neurons are unlikely to be a target of the vBNST that is responsible for its role in cocaine preference. There are several possible mechanisms by which inhibition of the vBNST could reduce preference but also increase Fos expression in orexin neurons. First, expression of Fos was elevated in lateral and medial orexin neurons following B-M injections into the vBNST. Fos activation in medial orexin neurons has been associated with stress/anxiety/negative attributes (Harris & Aston-Jones, 2006). Such elevated negative effects could potentially overcome the positive effects of the LH orexin activation, leading to reduced CPP. Second, it is possible that the LH orexin neurons that are activated by vBNST inhibition are not those that project to the primary mediators of preference (e.g. the VTA), and therefore play less of a role in preference overall. Third, Fos induction, an often-used marker of neuronal activation, can also be an indication of neuronal inhibition or alterations in signaling cascades without a change in neuronal firing (Morgan & Curran, 1991; Mikkelsen *et al.*, 2005). Therefore, it is possible that Fos activation of the LH in this study does not reflect increased firing of LH orexin neurons. Finally, it is plausible that orexin is not a primary mediator of preference, but rather is necessary to modulate other areas that are more primary mediators of CPP (e.g. the VTA). For example, orexin may serve to modulate responses of VTA dopamine neurons to glutamate inputs that drive the specific behavioral responses (as we have recently resported) (Mahler *et al.*, 2012). Thus, LH orexin may be necessary but not sufficient for reward-related behaviors. Therefore, although we have demonstrated that the vBNST plays a significant role in cocaine CPP, more work is needed to understand how BNST neurons interact with orexin neurons during reward-related behaviors.

Given the high level of expression of OX1Rs in the vBNST (Trivedi *et al.*, 1998; Marcus *et al.*, 2001), one possible explanation for this puzzling situation was that orexin neurotransmission within

the vBNST may be important for cocaine preference, and that inactivation of the vBNST blocked this pathway. However, OX1R antagonist injections into the vBNST did not attenuate cocaine preference, indicating that orexin input to the vBNST was not critical for this behavior.

Therefore, we sought targets other than LH orexin neurons where the vBNST might act to support cocaine preference. Our laboratory previously showed that the vBNST acts as a potent regulator of dopamine neurons in the VTA (Georges & Aston-Jones, 2002), but it was unknown whether this connection is critical during reward-related behaviors. Using a bilateral disconnection technique, we found that afferents to the VTA from the vBNST are essential for cocaine preference. However, we also found, with local microinjections of glutamate antagonists, that this projection does not purely depend on activation of inotropic glutamate receptors in the VTA.

The present study utilized a bilateral disconnection technique to determine whether vBNST afferents to VTA neurons are necessary for cocaine preference. One caveat of this technique is that it does not differentiate between afferents and efferents. Therefore, one might argue that our results were attributable to interruption of projections from the VTA to the vBNST that are important in cocaine preference. However, this alternative explanation is not likely, in view of anatomical evidence showing that there are almost no VTA projections to the vBNST (Shin *et al.*, 2008). Dopamine neurons from the VTA do, however, project heavily to the dorsal BNST (dBNST) (Risold & Swanson, 1997), and this region is strongly interconnected with the vBNST (Shin *et al.*, 2008); other indirect pathways could exist. Therefore, it is possible that indirect connections from the VTA to the vBNST could be affected by the disconnection technique used in these experiments.

Role of the BNST in reward behaviors

Several studies have identified roles for the vBNST and dBNST in reward-related behaviors (Aston-Jones *et al.*, 1999; Leri *et al.*, 2002; Buffalari & See, 2011) and drug-induced neuroplasticity (Erb *et al.*, 2004; Dumont *et al.*, 2005, 2008; Grueter *et al.*, 2006, 2008; Colussi-Mas *et al.*, 2007; Francesconi *et al.*, 2009; Krawczyk *et al.*, 2011; Nobis *et al.*, 2011). We targeted neurons in the vBNST because of its strong connections with the LH, Fos activation of LH-projecting neurons in this area during cocaine preference, and strong projections from this area to the VTA (Georges & Aston-Jones, 2002). In addition, we showed that inactivation of the vBNST, but not of the dBNST, attenuated cocaine CPP. Therefore, although the vBNST and dBNST have been shown to be important in other drug-seeking behaviors (Briand *et al.*, 2010; Buffalari & See, 2011; Wenzel *et al.*, 2011), only the vBNST and its projections to the VTA appear to play an important role in cocaine preference.

Relapse to drug-seeking can be evoked by discrete cues, stresses, or contexts. Inactivation of the BNST has been shown to attenuate cue-induced and stress-induced relapse to cocaine-seeking behaviors (Buffalari & See, 2011). Although recent data have implicated the BNST in contextual fear learning (Sullivan *et al.*, 2004), it was unknown whether the BNST is involved in contextual drug-seeking behaviors. We showed that the vBNST is critical for the expression of cocaine place preference, a behavior that reflects context-reward associations (Bardo & Bevins, 2000; Cunningham *et al.*, 2006). In addition, the BNST receives strong projections from the ventral subiculum (vSUB) (Shin *et al.*, 2008), a region that has been implicated in context-induced drug-seeking behaviors (Lasseter *et al.*, 2010). Therefore, it seems possible that the vSUB influences BNST activity and cocaine preference. Consistent with this view, Massi *et al.* (2008) reported that the vSUB exerts a strong excitatory influence on BNST neurons that project to the VTA. However, further research is needed to determine whether a vSUB–BNST, or perhaps a vSUB–BNST–VTA, circuit is involved in context-induced drug-seeking behaviors.

vBNST projection to the VTA: role in cocaine preference

Many studies have demonstrated that glutamatergic afferents to the VTA are important in regulating the activity of dopamine neurons (Georges & Aston-Jones, 2001, 2002; Dumont & Williams, 2004; Massi *et al.*, 2008). Furthermore, glutamatergic inputs to VTA dopamine neurons are involved in neural plasticity that could eventually lead to drug addiction (Geisler & Wise, 2008). Our laboratory reported that activation of NMDA and non-NMDA receptors in the VTA is necessary for the acquisition and expression of cocaine and morphine CPP (Harris & Aston-Jones, 2003; Harris *et al.*, 2004), but the source of this glutamatergic input is unknown. Anatomical and functional data indicate that the vBNST is one possible source of the glutamatergic input to the VTA (Georges & Aston-Jones, 2002). We found that vBNST afferents to the VTA are essential for cocaine preference, but although preference was somewhat decreased, it did not appear to be solely mediated by glutamatergic projections. However, it is also possible that our glutamate antagonist microinjections did not effectively cover all of the VTA, so that glutamate plays more of a role in the preference mediated by this projection than indicated by these results. Recently, Geisler *et al.* (2007) reported staining for vesicular glutamate transporters, a marker for glutamatergic neurons, in vBNST neurons that largely did not project to the VTA. Therefore, it seems likely that the primary source of input to the VTA from the vBNST that is involved in cocaine preference involves pathways in addition to glutamatergic projections.

Recent data indicate that the BNST is important in stress and reward (Leri *et al.*, 2002; Briand *et al.*, 2010; Buffalari & See, 2011). In the present study, unilateral inactivation of the vBNST and the contralateral VTA during a CPP test not only significantly attenuated preference but also caused avoidance of the cocaine-paired side. Cocaine is known to have both rewarding and anxiogenic effects (Yang *et al.*, 1992; Blanchard *et al.*, 1999; Rogers & See, 2007). Therefore, it is possible that inactivating the vBNST–VTA circuit reduces reward–context associations of cocaine, revealing unopposed anxiogenic properties of cocaine associations, and resulting in an avoidance behavior. Consistent with the idea of a vBNST–VTA circuit being involved in anxiety and reward, Briand *et al.* (2010) recently reported that vBNST projections to the VTA were activated following exposure to cocaine and stress. A study by Wenzel *et al.* (2011), however, found that inactivation of the dBNST reduced avoidance of cocaine, using a runway self-administration procedure. Although there are several differences between these findings and those of the current study (inactivation of the dBNST vs. the vBNST–VTA connection, Pavlovian vs. instrumental behaviors, etc.), these data indicate that subregions of the BNST may be involved in the anxiogenic behavioral effects of cocaine.

In summary, this study extends our present understanding of the BNST in reward processing by showing that the vBNST and its connections with the VTA are necessary for cocaine place preference. Although strong interconnections between the vBNST and the LH exist, the current study indicates that vBNST afferents to the

LH do not selectively regulate orexin neurons during cocaine preference. Instead, our results show that vBNST projections to the VTA are necessary for cocaine preference. Therefore, further studies are needed to determine the significance of vBNST–LH connections and what role orexin plays in this circuit during drug-seeking behaviors.

Acknowledgements

This research was supported by National Institute on Drug Abuse Grants R01DA017289, R37DA06214, and T32 DA007288. We thank Michael Smith for his excellent technical assistance.

Abbreviations

ABC, avidin–biotin complex; ACSF, artificial cerebrospinal fluid; AP5, (2*R*)-amino-5-phosphonovaleric acid; B-M, baclofen plus muscimol; BNST, bed nucleus of the stria terminalis; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, conditioned place preference; CTb, cholera toxin b subunit; DAB, 3,3′-diaminobenzidine; dBNST, dorsal bed nucleus of the stria terminalis; IL, infralimbic cortex; LH, lateral hypothalamus; LPO, lateral preoptic area; LS, lateral septum; NAcS, nucleus accumbens shell; NMDA, *N*-methyl-D-aspartate; OX1R, orexin 1 receptor; PrL, prelimbic cortex; vBNST, ventral bed nucleus of the stria terminalis; vSUB, ventral subiculum; VTA, ventral tegmental area.

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