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Noradrenaline in the bed nucleus of the stria terminalis is critical for stress-induced reactivation of morphine-conditioned place preference in rats

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Abstract

The effect of noradrenaline in the bed nucleus of the stria terminalis and locus coeruleus on maintenance and reactivation of morphine-conditioned place preference induced by footshock stress was investigated in rats. After receiving alternate injection of morphine (10 mg/kg) and saline for 6 consecutive days, the rats spent more time in the drug-paired compartment (morphine-conditioned place preference) on day 7. These animals did not show morphine-conditioned place preference on day 37 following sham-footshock once every 3 days from days 8 to 36 (28 days drug-free). However, 15 min of intermittent footshock once every 3 days could induce the maintenance of morphine-conditioned place preference on day 37 with significantly more time spent in the drug-paired compartment than on day 0. Microinjection of the α_2-adrenoceptor agonist, clonidine (0.1 or 1 μg), into the locus coeruleus 30 min before footshock did not affect stress-induced maintenance of conditioned place preference. However, infusions of clonidine (1 μg) into the bed nucleus of the stria terminalis significantly attenuated the maintenance of conditioned place preference induced by footshock stress. In another experiment, after a 21-day extinction of morphine-conditioned place preference, a single footshock could reactivate the morphine place preference that was significantly blocked by pretreatment with infusion of clonidine (0.1 or 1 μg) into the bed nucleus of the stria terminalis but not the locus coeruleus. Reactivation of morphine-conditioned place preference elicited by footshock stress was significantly inhibited by 6-hydroxydopamine-induced lesions in the ventral noradrenergic bundle, most of the norepinephrine input to the bed nucleus of the stria terminalis arising from caudal brain stem noradrenergic cell groups. In contrast, chemical lesions of the dorsal noradrenergic bundle that arises from the locus coeruleus had no such effects. These findings suggest that noradrenergic neurons in locus coeruleus are not involved in stress-induced reinstatement of drug-seeking and further clearly demonstrate that noradrenaline in the bed nucleus of the stria terminalis plays a critical role in mediating this effect. Comprehension of the neurochemical events underlying the stress-induced and the bed nucleus of the stria terminalis-mediated reinstatement of drug-seeking may, therefore, throw more light on the biological bases of drug dependence and addictive behavior © 2001 Published by Elsevier Science B.V.

Keywords: Noradrenaline; Clonidine; 6-Hydroxydopamine; Opiate dependence; Conditioned place preference; Relapse; Stress; Bed nucleus of the stria terminalis; Locus coeruleus

1. Introduction

Relapse is a major characteristic of drug addiction and remains to be the primary problem in treating drug abuse. Despite a great deal of research, the exact factors that determine renewed drug-seeking, the urge to use drugs and persistent craving for them remain unclear. Among the several possible factors inducing relapse to drug dependence, environmental stress has received considerable attention in recent years (Koob, 1999; Stewart, 2000). Stress experiences appear to have a strong influence on susceptibility to drug-taking in various animal models (Erb et al., 1998; Highfield et al., 2000), which has also been observed in clinical populations suffering from substance abuse. Animal data make it evident that repeated stress not only increases the propensity to drug-taking, but also augments the psychomotor effects of drugs, a phenomenon termed

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behavioral sensitization (Deroche et al., 1992; Pierec and Kalivas, 1994). However, the exact mechanisms underlying stress-induced relapse are not understood.

Until recently, other and our studies have shown that the neurochemical events involved in stress- and in drug-induced reinstatement in an animal model of relapse are not identical (Shaham and Stewart, 1996; Lu et al., 2000a). Stress-induced reinstatement is unaffected by opioid receptor antagonists and is relatively insensitive to dopamine receptor antagonists, whereas both the opioid and dopamine systems are critical for drug-induced relapse. In contrast, the corticotropin-releasing factor (CRF) system appears to contribute to stress- but not drug-induced reinstatement of opiate- and psychostimulant-seeking (Shaham et al., 1997). In addition, CRF receptor antagonists can attenuate but not completely block stress-induced relapse to drug dependence, which indicates that other neuronal pathways may contribute to this effect.

The noradrenergic system in the brain, such as the locus coeruleus, clusters of noradrenergic cell bodies, is thought to mediate the response to stress (Tanaka et al., 1990; Koolhaas et al., 1998). Previous evidence has shown that physiological (internal) and environmental (external) stress activate the major source of norepinephrine projection to the forebrain, the cells of the locus coeruleus in the pons (Valentino et al., 1993) and that electrical stimulation of the locus coeruleus results in anxiogenic-like responses and activation of the autonomic nervous system, whereas local injections of an α_2-adrenoceptor agonist that inhibits norepinephrine cell firing and release can decrease various responses to stress (Bremner et al., 1996). However, the role of the noradrenergic system in stress-induced relapse to drug dependence is still unexplored. Hyperactivity of brain norepinephrine has long been implicated in mechanisms of opiate withdrawal (Aghajanians, 1978; Nestler, 1992), and recent evidence shows that locus coeruleus noradrenergic neurons are not involved in attenuation of stress-induced reinstatement of opiate seeking by clonidine, an α_2-adrenoceptor agonist (Shaham et al., 2000). Another important brain structure with dense norepinephrine innervation is the bed nucleus of the stria terminalis that is an anatomically and functionally heterogeneous brain region (Alheid and Heimer, 1988; Brog et al., 1993). The medial aspect of the bed nucleus of the stria terminalis that connects with the lateral preoptic area, posterior pituitary and associated nuclei is thought to be involved in stress and neuroendocrine-related functions (Casada and Dafny, 1992; Gray et al., 1993). The norepinephrine innervation of the bed nucleus of the stria terminalis is particularly prominent in its ventral and medial subdivisions. Ascending axons of the caudal brain stem norepinephrine cell groups (A1 and A2) combine to form the ventral noradrenergic bundle, whereas those of the locus coeruleus form the dorsal noradrenergic bundle (Moore, 1978; Sahakian et al., 1983; Phelix et al., 1992). Recent evidence demonstrated that lesions of the ventral but not dorsal noradrenergic bundle inhibit withdrawal-induced place aversion and that infusion of clonidine into the bed nucleus of the stria terminalis attenuates aversive or somatic opiate-withdrawal signs (Aston-Jones et al., 1999). The CRF system is known to interact with the norepinephrine system in the bed nucleus of the stria terminalis in modulation of the stress response (Francis et al., 1999), and CRF injection in the bed nucleus of the stria terminalis reinstates cocaine-seeking (Erb and Stewart, 1999). However, no studies have been performed to determine the role of noradrenaline in the bed nucleus of the stria terminalis in stress-induced relapse to opiate dependence. Thus, we now investigated the possible effects of noradrenergic innervation of the bed nucleus of the stria terminalis on stress-induced reinstatement of opiate seeking by microinjection of clonidine into the bed nucleus of the stria terminalis or locus coeruleus and infusion of 6-hydroxydopamine into the ventral or dorsal noradrenergic bundle.

2. Materials and methods

2.1. Animals and drugs

Male Sprague–Dawley rats (body weight 250–280 g) were housed in groups and maintained on a 12-h light/dark cycle with access to food and water ad libitum. The experimental animals were housed in the colony room for at least 1 week before surgery and were allowed to recover for 6–7 days after surgery. All animal treatments were strictly in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals. The drugs used were clonidine HCl (Sigma, USA), 6-hydroxydopamine hydrobromide (Sigma) and morphine HCl (Qinghai Pharmaceutical, China).

2.2. Intracerebral cannulae

Cannulae were implanted under sodium pentobarbital anesthesia (60 mg/kg, i.p.) and affixed to the skull with dental cement. The cannulae (Plastic One) consisted of a double-guided cannula, dummy and cap. The cannulae were implanted bilaterally into the locus coeruleus as follows: -9.8 mm from bregma, ± 1.1 mm lateral from the midline and -6.8 mm below the surface of the dura (Paxinos and Watson, 1997). The coordinates for bilateral implantation into the bed nucleus of the stria terminalis were: AP: -0.4 mm, ML: ± 3.5 mm, DV: -6.3 mm from the skull surface, incisor -3.1. The stock solutions were prepared in 10 mM acetic acid. Final dilutions in twofold-concentrated artificial cerebrospinal fluid (CSF) containing (in mM) NaCl (125.0), KCl (2.5), $MgCl_2$ (0.9), $CaCl_2$ (1.2) and NaH_2PO_4 (1.2) were prepared immediately before the experiments. The final pH of the drug solutions was 7.4. Vehicle solutions were prepared by diluting 10 mM acetic acid in artificial CSF in an identical manner. Infusion of 0.5 μl per site was made through 28-gauge injector cannulae over 60 s, and the

injector was left in place for an additional 60 s. Cannula placement was verified for each animal by histological examination of the brains after methylene blue injection (0.25 μl per site). The result showed that there were 8–10 rats with correct inserted cannulae in each group containing 10–11 rats (Fig. 1), and only the data obtained from rats with correctly inserted cannulae were included in the statistical analysis.

2.3. 6-Hydroxydopamine lesions

The 6-hydroxydopamine lesion procedure was based on previous reports (Hansen et al., 1980; Aston-Jones et al., 1999). The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic apparatus. Bilateral infusion cannulae (24 gauge) were directed toward the dorsal or ventral noradrenergic bundle in separate groups of animals with the following coordinates: the ventral noradrenergic bundle, AP: −6.5 mm, ML: ±2.0 mm, DV: −8.8 mm from skull surface, incisor +5.1 mm; the dorsal noradrenergic bundle, AP: −6.1 mm, ML: ±0.8 mm, DV: −6.4 mm from skull surface, incisor −2.4 mm. Infusions of 2 μl 6-hydroxydopamine (3 μg/2 μl in CSF) or vehicle were made over 5 min using a Hamilton syringe connected to a Harvard apparatus infusion pump. The injector remained in place for an additional 5 min to allow tissues to absorb the infusion and limit diffusion upwards along the cannula tract.

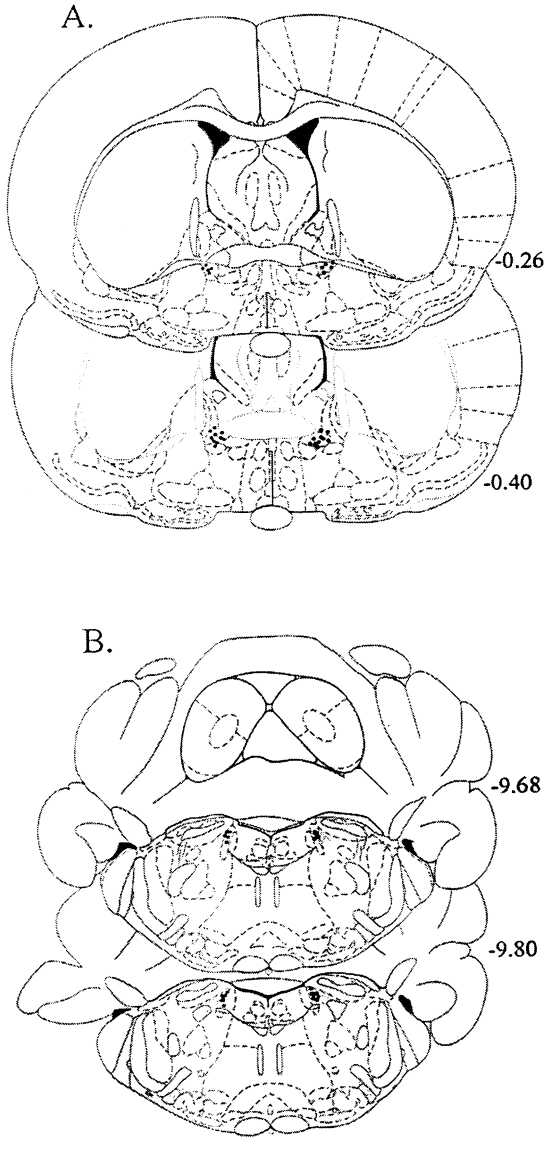

Fig. 1. The top panel shows placement of injector tips for animals with cannula implants in the bed nucleus of the stria terminalis for clonidine and vehicle. The bottom panel shows placement of injector tips for animals with locus coeruleus cannula implants for clonidine and vehicle. Values on the right represent millimetres from bregma. Drawings are adapted from Paxinos and Watson (1997).

2.4. Tissue assays

At the end of the experiments, after the last behavioral test was performed, lesion and vehicle (sham-operated) rats were killed by decapitation. The brains were rapidly removed and the frontal cortex, hippocampus, the bed nucleus of the stria terminalis and hypothalamus were dissected bilaterally at 4 °C. Based on previous reports that 6-hydroxydopamine lesions of the ventral noradrenergic bundle markedly reduced the norepinephrine level in the bed nucleus of the stria terminalis and in hypothalamus, and that lesions of dorsal noradrenergic bundle caused significant reduction of the norepinephrine level in the cortex and hippocampus (Hansen et al., 1980; Delfs et al., 2000), the above four brain regions (because of their reciprocal connections with the ventral noradrenergic bundle or dorsal noradrenergic bundle) were selected to determine the effect of lesions in the ventral noradrenergic bundle or in the dorsal noradrenergic bundle. Dissected structures were immediately frozen on dry ice and stored at −75 °C until biochemical assay. Norepinephrine contents were measured in the dissected brain regions by high-performance liquid chromatography (HPLC) coupled with electrochemical detection. Tissues were weighed and homogenized in 0.1 M perchloric acid containing 1 mM EDTA, 2 mM $Na_2S_2O_4$. Chloroform (300 μl) was added to the 600 μl homogenates. The mixture was vortexed and centrifuged at 11,800 × *g* for 45 min at 4 °C. The supernatant was removed and 120 μl with 1 ng hydrocaffeic acid as internal standard was injected into a reverse phase high-performance liquid chromatography with electrochemical detection (HPLC-ECD). The HPLC system consisted of an EP-300 pump, an ODS C18 reverse-phase column, an ECD-300 electrochemical detector and an automated sample injector. The mobile phase was 0.035 M sodium acetate, 0.05 M citric acid containing 1.1 mM octanesulfonate, 8.3 μM EDTA and 15% methanol (v/v). Analysis was performed at a flow rate of 1.0 ml/min at room temperature. Contents of norepinephrine in different brain regions were calculated with respect to the mean peak height values obtained from standard solutions using an internal standard correction procedure.

2.5. Procedure

2.5.1. Apparatus for conditioned place preference

The test box for conditioned place preference consisted of a shuttle Plexiglas chamber (30 × 60, 30 high) that was divided into two equal-sized compartments by insertion of a removable Plexiglas wall. The test box was black. One compartment had white stripes on the wall and a textured floor and the other had walls with white dots and a smooth floor. The test box was placed under conditions of dim illumination (40 lx) and masking white noise (Lu et al., 2000b).

2.5.2. Induction of morphine-conditioned place preference

Conditioned place preference was tested as described previously (Maldonado et al., 1997; Lu et al., 2001). Briefly, the place-preference conditioning schedule consisted of three phases. In the preconditioning phase, rats were placed in the middle of the neutral area of the place-preference box and allowed to move freely in the two compartments of the box for 15 min. The time spent in each compartment during the 15-min session was recorded, and rats that spent more than 500 s in one side of the box were excluded. During the conditioning phase, animals were randomly paired to drug or saline administration and assigned to a compartment. Each rat was treated for 6 consecutive days with alternate injections of morphine HCl (10 mg/kg, s.c.) and saline, and confined to the conditioning compartment for 50 min, after which it was returned to the home cage. During the test phase, the barrier was removed and the rats were placed on the floor of the box and allowed to move freely for 15 min. The time spent in each compartment during the 15-min session was recorded and preference for the drug-paired compartment was determined as time spent by each animal in the drug-paired side during the session (conditioned place preference test).

2.5.3. Maintenance of morphine-conditioned place preference

After rats had been given alternate injections of morphine HCl (10 mg/kg, s.c.) and saline for 6 consecutive days (from days 1 to 6) to induce morphine-conditioned place preference, they randomly received 15 min of intermittent footshock (0.5 mA, 0.5 s on with a mean off period of 40 s) or sham-footshock once every 3 days from days 8 to 36 (28-day drug-free period). Our previous study had shown that this regime of intermittent footshock could induce maintenance of morphine-conditioned place preference on day 37 (Lu et al., 2000a). Thus, to investigate whether the stress-induced maintenance of morphine-conditioned place preference is affected by noradrenaline in the bed nucleus of the stria terminalis or locus coeruleus, clonidine (0.1 or 1 μg) and vehicle were injected, 0.5 μl per site, into the bed nucleus of the stria terminalis or locus coeruleus 30 min prior to each footshock, respectively. Every animal was tested for conditioned place preference on days 0, 7 and 37, respectively.

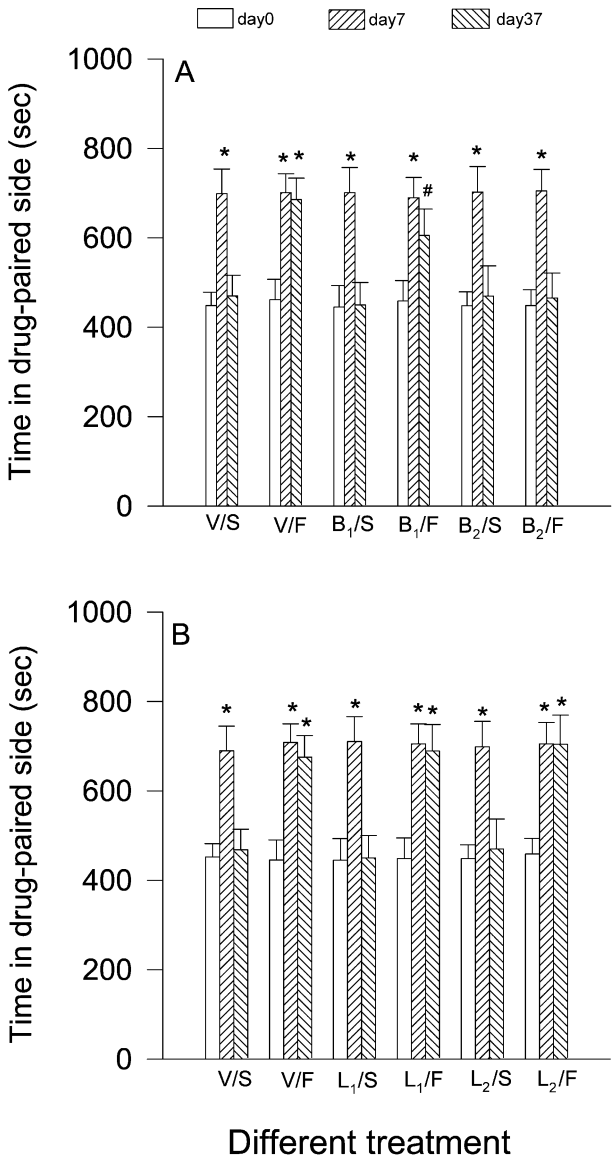

Fig. 2. Effect of pretreatment with clonidine infusion into the bed nucleus of the stria terminalis or locus coeruleus on the footshock-induced maintenance of morphine-conditioned place preference. The top panel shows the effect of pretreatment with infusion of vehicle (V) and 0.1 μg (B_1) or 1 μg (B_2) clonidine into the bed nucleus of the stria terminalis on footshock (F)- or sham stress (S)-induced maintenance of morphine-conditioned place preference. The bottom panel shows the effect of pretreatment with infusion of vehicle (V) and 0.1 μg (L_1) or 1 μg (L_2) clonidine into locus coeruleus on footshock (F)- or sham stress (S)-induced maintenance of morphine-conditioned place preference. The rats were given alternate injections of morphine HCl (10 mg/kg, s.c.) and saline for 6 consecutive days (from days 1 to 6) and then randomly treated with 15 min of intermittent footshock or sham stress once every 3 days from days 8 to 36 (28-day drug-free period). Clonidine (0.1 or 1 μg) and vehicle were injected in 0.5 μl per site into the bed nucleus of the stria terminalis or locus coeruleus 30 min prior to each footshock or sham footshock, respectively. Time spent in the drug-paired side for each rat was measured for a 15-min test on days 0, 7 and 37, respectively. Each column represents the mean with S.E.M. for 8–10 animals. $^{\#}P<0.05$ and $^{*}P<0.01$ vs. time spent in drug-paired side on day 0.

2.5.4. Reactivation of morphine-conditioned place preference

In the extinction phase of morphine-conditioned place preference, after the rats had been injected alternately with morphine HCl (10 mg/kg, s.c.) and saline for 6 consecutive days (from days 1 to 6) to induce morphine-conditioned place preference, they were given a saline injection and randomly confined to the previous drug- or saline-paired compartment for 50 min daily from days 8 to 29 (21-day extinction). On day 31, these animals received a single 15 min of intermittent footshock to reactivate the morphine place preference (Lu et al., 2000a). To investigate the effect of stress-induced reactivation of morphine-conditioned place preference by noradrenaline in the bed nucleus of the stria terminalis or locus coeruleus, clonidine (0.1 and 1 μg) and vehicle were injected, 0.5 μl per site, into the bed nucleus of the stria terminalis or locus coeruleus 30 min prior to the single footshock. These rats received the conditioned place preference test on days 0, 7 30 and 31, respectively. In another experiment, morphine-conditioned place preference was induced (from days 1 to 6) and the rats were given the extinction treatment (from days 8 to 29) as above, then received intracerebral infusions of 6-hydroxydopamine (3 μg per site) or vehicle into the ventral noradrenergic bundle or the dorsal ventral noradrenergic bundle on day 31, respectively. After a week recovery from 6-hydroxydopamine infusions, the rats received a single 15 min of intermittent footshock to determine the effects of stress-induced reactivation of morphine-conditioned place preference by lesions of the ventral or dorsal noradrenergic bundle. These rats received the conditioned place preference test on days 0, 7, 30 and 38, respectively. All rats were given the footshock or sham stress 60 min before the conditioned place preference test outside the box.

2.6. Statistical analysis

The time spent in the drug-paired side in the conditioned place preference test is expressed as means ± S.E.M. and was analyzed using the GB-STAT statistical package. Repeated-measures two-way analysis of variance (ANOVA) with (footshock × treatment) the Newman–Keuls post hoc test was used to evaluate the difference in time in the drug-paired side between day 0 and the other days, and the norepinephrine level in two groups was compared using Student's *t*-test. Statistical differences with $P < 0.05$ were considered significant.

3. Results

3.1. Effects of microinjection of clonidine into the bed nucleus of the stria terminalis or locus coeruleus on stress-induced maintenance of morphine-conditioned place preference

As shown in Fig. 2, alternate injections of morphine (10 mg/kg) and saline for 6 consecutive days significantly

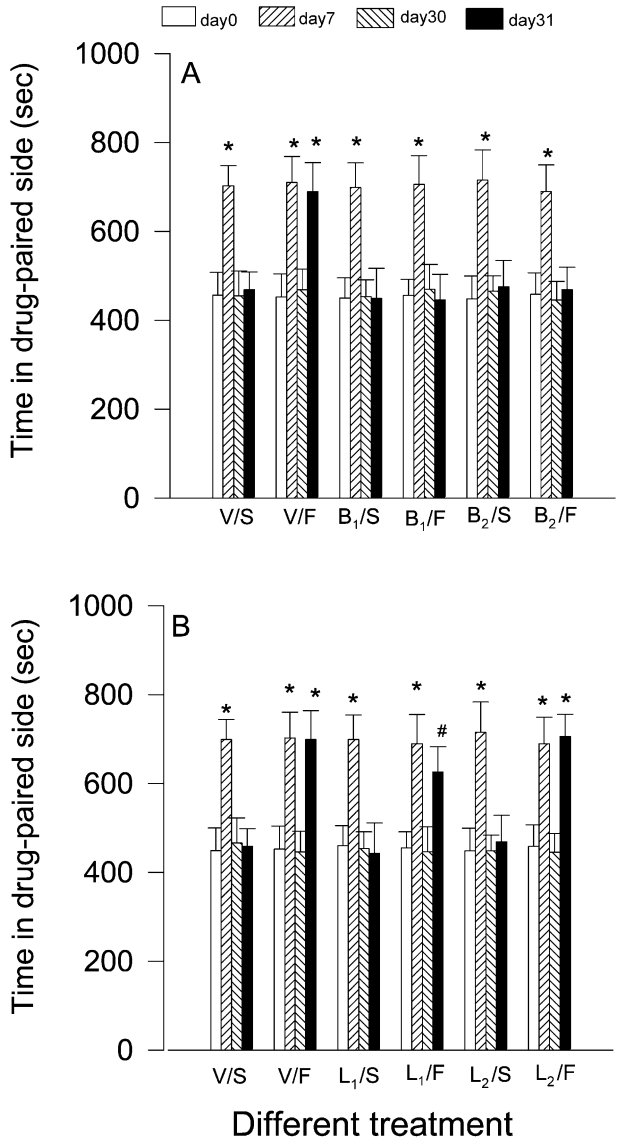

Fig. 3. Effect of pretreatment with clonidine infusion into the bed nucleus of the stria terminalis or locus coeruleus on the footshock-induced reactivation of morphine-conditioned place preference. The top panel shows the effect of pretreatment with infusion of vehicle (V) and 0.1 μg (B_1) or 1 μg (B_2) clonidine into the bed nucleus of the stria terminalis on footshock (F)- or sham stress (S)-induced reactivation of morphine-conditioned place preference. The bottom panel shows the effect of pretreatment with infusion of vehicle (V) and 0.1 μg (L_1) or 1 μg (L_2) clonidine into the locus coeruleus on footshock (F)- or sham stress (S)-induced reactivation of morphine-conditioned place preference. The animals were alternately injected with morphine and saline for 6 consecutive days (from days 1 to 6) to induce morphine-conditioned place preference, and then given extinction treatment daily from days 8 to 29 (21-day extinction). On day 31, these animals received a single footshock to reactivate the morphine-conditioned place preference. The infusions of clonidine or vehicle were administered 30 min prior to the footshock or sham stress. Time spent in the morphine-paired side for each rat was measured for a 15-min test on days 0, 7, 30 and 31, respectively. Each column represents the mean with S.E.M. for 8–10 animals. $^{\#}P < 0.05$ and $^{*}P < 0.01$ vs. time spent in drug-paired side on day 0.

increased the time spent in the drug-paired compartment (morphine-conditioned place preference) in all groups of animals on day 7, and the rats did not show morphine-conditioned place preference on day 37 following sham-footshock once every 3 days from days 8 to 36. However, 15 min of intermittent footshock once every 3 days could induce maintenance of morphine-conditioned place preference on day 37 with a significantly increased time spent in the drug-paired compartment compared with that on day 0. Interestingly, there was a significant effect of clonidine injection in the bed nucleus of the stria terminalis on footshock-induced maintenance of conditioned place preference [$F(2,48) = 16.23$, $P < 0.01$]. Fig. 2A shows that pretreatment with an infusion of 1 μg clonidine in the bed nucleus of the stria terminalis significantly blocked the footshock-induced maintenance of conditioned place preference, indicated by the lack of a significant increase in time spent in the drug-paired compartment on day 37 in comparison with that on day 0 (465.2 ± 55.8 vs. 448.4 ± 35.6, $P > 0.05$). However, infusion of 0.1 μg clonidine into the bed nucleus of the stria terminalis tended to attenuate footshock-induced maintenance of morphine-conditioned place preference, although this failed to reach statistical significance (Fig. 2A). In contrast, pretreatment with microinjection of 0.1 or 1 μg clonidine in the locus coeruleus did not affect the maintenance of conditioned place preference induced by footshock [$F(2,49) = 1.63$, $P > 0.05$] (Fig. 2B).

3.2. Effects of microinjection of clonidine into the bed nucleus of the stria terminalis or locus coeruleus on stress-induced reactivation of morphine-conditioned place preference

As shown in Fig. 3, no animals showed the morphine-conditioned place preference on day 30 after they were given the extinction treatment from days 8 to 29 (21-day extinction). A single footshock stress 1 h before the conditioned place preference test on day 31 could reactivate the place preference with a significant increase in time spent in the drug-paired side in comparison with that on day 0. However, there was a statistically significant effect of clonidine injection in the bed nucleus of the stria terminalis on stress-induced reactivation of conditioned place preference [$F(2,47) = 18.25$, $P < 0.01$]. As shown in Fig. 3A, microinjection of both 0.1 and 1 μg clonidine into the bed nucleus of the stria terminalis significantly blocked the stress-induced reactivation of conditioned place preference (Fig. 3A), as indicated by the lack of a significant difference in the time spent in the drug-paired compartment between days 31 and 0 (468.9 ± 50.2 vs. 458.1 ± 48.5, $P > 0.05$ for 0.1 μg group; and 445.8 ± 57.0 vs. 455.2 ± 35.6, $P > 0.05$ for 1 μg group). In contrast, microinjection of 0.1 or 1 μg clonidine into the locus coeruleus did not also affect the expression of morphine-conditioned place preference induced by a single footshock stress [$F(2,49) = 1.28$, $P > 0.05$] (Fig. 3B).

3.3. Effect of 6-hydroxydopamine lesions of the ventral noradrenergic bundle or dorsal noradrenergic bundle on norepinephrine levels in various brain areas

As shown in Table 1, in comparison with vehicle infusion, intracerebral infusion of 6-hydroxydopamine into dorsal noradrenergic bundle resulted in a significant decrease in norepinephrine levels in the frontal cortex ($t_{16} = 11.9$, $P < 0.01$) and hippocampus ($t_{16} = 15.16$, $P < 0.01$), but not in the bed nucleus of the stria terminalis and hypothalamus. Intercerebral infusion of 6-hydroxydopamine into the ventral noradrenergic bundle induced a clear decrease in norepinephrine levels in the bed nucleus of the stria terminalis ($t_{17} = 14.76$, $P < 0.01$) and hypothalamus ($t_{17} = 8.77$, $P < 0.01$), whereas lesioning of the ventral noradrenergic bundle did not significantly affect the norepinephrine contents in the frontal cortex and hippocampus (Table 1). In addition, after 6-hydroxydopamine-induced lesion of the ventral noradrenergic bundle or dorsal noradrenergic bundle, no obvious behavioral abnormality (gross observation) was found in rats with a decreased norepinephrine level in the above brain area (data not shown).

3.4. Effects of 6-hydroxydopamine lesions of the ventral noradrenergic bundle or dorsal ventral noradrenergic bundle on stress-induced reactivation of conditioned place preference

As shown in Fig. 4, no animals showed the morphine-conditioned place preference on day 30 after they received the extinction treatment from days 8 to 29. The morphine-

Table 1
Effects of 6-hydroxydopamine-induced lesions in dorsal ventral noradrenergic bundle or the ventral noradrenergic bundle on norepinephrine levels in various brain areas

Infusion	Noradrenaline levels (pg/g)			
	Frontal cortex	Hippocampus	The bed nucleus of the stria terminalis	Hypothalamus
Dorsal noradrenergic bundle (vehicle)	11.3 ± 1.6	12.2 ± 1.5	65.8 ± 10.2	85.2 ± 11.2
Dorsal noradrenergic bundle (lesions)	3.1 ± 1.3[a]	2.8 ± 1.1[a]	60.2 ± 6.8	74.5 ± 12.8
Ventral noradrenergic bundle (vehicle)	17.2 ± 2.8	10.2 ± 2.5	97.1 ± 16.4	64.4 ± 13.5
Ventral noradrenergic bundle (lesions)	15.2 ± 02.5	9.3 ± 2.2	18.5 ± 3.6[a]	25.5 ± 3.6[a]

All animals were killed immediately after the final behavioral test. Data are means ± S.E.M. for 8–10 animals per group.
[a] $P < 0.01$ versus vehicle group.

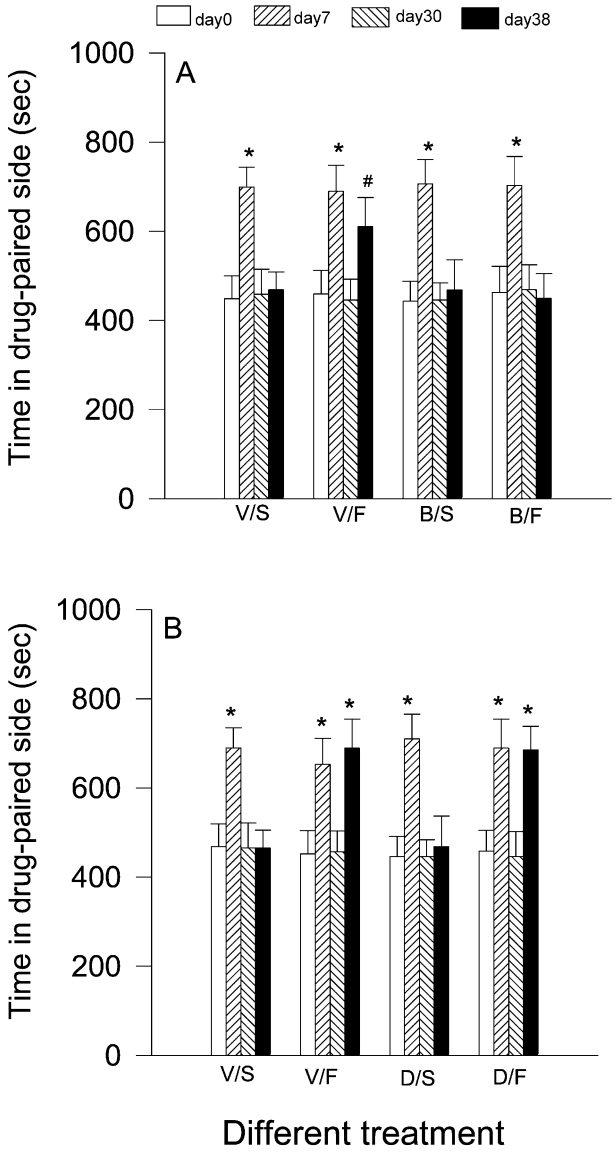

Fig. 4. Effect of the 6-hydroxydopamine-induced lesions of the ventral noradrenergic bundle or the dorsal noradrenergic bundle on the footshock-induced reactivation of morphine-conditioned place preference. The top panel shows the effect of infusion of 6-hydroxydopamine (B) or vehicle (V) into the ventral noradrenergic bundle on footshock (F)- or sham stress (S)-induced reactivation of morphine-conditioned place preference. The bottom panel shows the effect of infusion of 6-hydroxydopamine (D) or vehicle (V) into dorsal ventral noradrenergic bundle on footshock (F)- or sham stress (S)-induced reactivation of morphine-conditioned place preference. The animals were alternately treated with morphine and saline for 6 consecutive days (from days 1 to 6) to induce morphine-conditioned place preference, and then given the extinction treatment daily from days 8 to 29 (21-day extinction). On day 31, these animals received intracerebral infusions of 3 μg 6-hydroxydopamine or vehicle into the ventral noradrenergic bundle or dorsal ventral noradrenergic bundle, respectively. After a week's recovery from 6-hydroxydopamine infusion, the rats were given a single 15 min of intermittent footshock to reactivate the morphine-conditioned place preference. The time spent in the morphine-paired side for each rat was measured for a 15-min test on days 0, 7, 30 and 38, respectively. Each column represents the mean with S.E.M. for 8–9 animals. $^{\#}P<0.05$ and $^{*}P<0.01$ vs. time spent in drug-paired side on day 0.

conditioned place preference could be reactivated by a single footshock on day 38 when the animal received infusion of vehicle into the ventral noradrenergic bundle or dorsal ventral noradrenergic bundle on day 31. However, there was a significant effect of lesioning in the ventral noradrenergic bundle [$F(1,38)=11.46$, $P<0.01$]. As shown in Fig. 4A, infusion of 6-hydroxydopamine into the ventral noradrenergic bundle significantly inhibited footshock-induced reactivation of conditioned place preference: the rats spent the same time in the drug-paired compartment on days 38 and 0 (448.9 ± 55.8 vs. 462.3 ± 58.4, $P>0.05$). In contrast, no significant effect of lesioning in the dorsal noradrenergic bundle was observed [$F(1,37)=2.04$, $P>0.05$]. As shown in Fig. 4B, the footshock-induced reactivation of morphine-conditioned place preference was not affected by the infusion of 6-hydroxydopamine into the dorsal ventral noradrenergic bundle, as indicated by a significant difference in time spent in the drug-paired compartment on day 38 compared with that on day 0 ($685.3\pm52.7.0$ vs. 458.2 ± 46.5, $P<0.01$).

4. Discussion

Environmental stressors have an important effect on the sensitivity of an individual to drugs of abuse. Studies of intravenous and oral drug administration in laboratory animals have shown that both physical and psychological stressors facilitate the acquisition of drug dependence, probably by increasing the reinforcing efficacy of drugs of abuse (Wallace, 1989; Piazza and Le Moal, 1996). In the present study, repeated footshock could prevent the extinction of morphine-conditioned place preference, and a single footshock could elicit reactivation of place preference following 21-day extinction. The results are consistent with those described previously (Erb et al., 1996; Shaham et al., 1997), suggesting that stress may interact with drug craving. Recent efforts to understand the role of brain stress systems in drug addiction have focused on the effect of amygdala and its related brain regions (Self and Nestler, 1998; Erb and Stewart, 1999). This system is part of a neural circuitry regulating emotional response to stress, including anxiety. Stress-like symptoms and anxiety are an integral part of acute and protracted drug and alcohol withdrawal syndromes (Koob and Le Moal, 1997). Evidence is accumulating to suggest that these withdrawal-induced signs involve activation of noradrenergic neurons in the bed nucleus of the stria terminalis (Aston-Jones et al., 1999; Delfs et al., 2000) and this effect may be a common neurobiological element in acute withdrawal from all drugs of abuse (Aston-Jones et al., 1999; Schulteis et al., 2000). However, it has not been determined whether noradrenaline in the bed nucleus of the stria terminalis acts to contribute to stress-induced reinforcement of drug seeking.

In our present experiments, following pretreatment with an infusion of clonidine into the bed nucleus of the stria terminalis, stress-induced maintenance and reactivation of

morphine-conditioned place preference was significantly attenuated or blocked, respectively. However, microinjection of clonidine into locus coeruleus had no such effect. These results extend a previous report that nordrenaline in the locus coeruleus is not involved in stress-induced reinstatement of drug seeking (Shaham et al., 2000) and further clearly demonstrate that noradrenaline in the bed nucleus of the stria terminalis mediates this effect. Moreover, 6-hydroxydopamine-induced lesions of the ventral noradrenergic bundle but not of the dorsal ventral noradrenergic bundle significantly inhibited reactivation of morphine place preference elicited by footshock stress. These findings also support previous evidence that noradrenaline from the locus coeruleus, long believed to be critical for opiate withdrawal, is not necessary for the stress-induced relapse to opiate dependence (Maldonado, 1997; Erb and Stewart, 1999), and are the first to suggest that the noradrenergic afferents to the bed nucleus of the stria terminalis through the ventral noradrenergic bundle originating in the A2 and A1 cell groups may mediate the rewarding process of opiate dependence.

The bed nucleus of the stria terminalis is strongly and reciprocally connected with the amygdala and has been thought to be a key component of the extended amygdala (Alheid and Heimer, 1988; Drolet et al., 1992). In addition, the bed nucleus of the stria terminalis has wide connections with other limbic and autonomic structures such as the nucleus accumbens, hypothalamus and nucleus tractus solitarius (Delfs et al., 2000). These brain regions, particularly the nucleus accumbens, play a critical role in the reinforcing and behavioral-activating effect of such opiate drugs as heroin and morphine (Rossetti et al., 1992; Kelsey and Arnold, 1994). The bed nucleus of the stria terminalis has also received considerable attention for its role in the expression of the stress response. Previous evidence suggests that noradrenergic fibers in the bed nucleus of the stria terminalis mediate the suppressive vasopressin but not the augmentative oxytocin response to nonassociatively applied fear stimuli and that the bed nucleus of the stria terminalis and amygdala are differently involved in fear versus anxiety, respectively (Davis et al., 1997; Van Bockstaele et al., 1999). Despite the density of noradrenaline in the bed nucleus of the stria terminalis and its relationship to the nucleus accumbens and amygdala, this structure has been largely overlooked with respect to its potential role in opiate dependence. However, the present findings indicate that the bed nucleus of the stria terminalis may be involved in stress-induced reinstatement of drug craving through its noradrenaline input. From a clinical perspective, environmental stress and related cues are significant motivating factors for drug-seeking in addicts (Sinha et al., 1999). Our present results clearly demonstrate that the bed nucleus of the stria terminalis may be an important component of the brain system involved in the stress-induced maintenance and reactivation of opiate craving. Further comprehension of the neurochemical events underlying the bed nucleus of the stria terminalis' mediating relapse to drug dependence induced by stress may, therefore, throw light on the biological bases of drug taking and addictive behavior.

In summary, our results provide evidence that pretreatment with clonidine infusion into the bed nucleus of the stria terminalis but not the locus coeruleus could significantly attenuate the maintenance of morphine-conditioned place preference induced by footshock. Following 21-day extinction, the morphine-conditioned place preference could be reactivated by a single footshock, and premicroinjection of clonidine into the bed nucleus of the stria terminalis suppressed this stress-induced reactivation of conditioned place preference, whereas microinjection of clonidine into the locus coeruleus did not show any effects. Furthermore, reactivation of morphine-conditioned place preference elicited by stress was significantly inhibited by 6-hydroxydopamine-induced lesions in the ventral noradrenergic bundle, most of the norepinephrine input to the bed nucleus of the stria terminalis arising from caudal brain stem noradrenergic cell groups. In contrast, chemical lesions of the dorsal noradrenergic bundle that arises from the locus coeruleus had no such effects. These findings suggest that nordrenaline in the locus coeruleus is not involved in reinstatement of drug-seeking induced by stress and further clearly demonstrate that noradrenaline in the bed nucleus of the stria terminalis plays a critical role in mediating this effect.

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