IN VOLVEMENT OF THE BED NUCLEUS OF THE STRI A TERMINALIS ACTIVATED BY THE CENTRAL NUCLEUS OF THE AMYGDALA IN THE NEGATIVE AFFECTIVE COMPONENT OF MORPHINE WITHDRAWAL IN RATS

T. NAKAGAWA,1a R. YAMAMOTO,1a M. FUJIO,1a Y. SUZUKI,1a M. MINAMI,1a M. SATOH,2b AND S. KANEKO2a

1Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan
2Nara Institute of Science and Technology, Ikoma 630-0101, Japan

Abstract—The central nucleus of the amygdala (Ce) and the bed nucleus of the stria terminalis (BST) are key structures of the extended amygdala, which is suggested to be involved in drug addiction and reward. We have previously reported that the Ce plays a crucial role in the negative affective component of morphine withdrawal. In the present study, we examined the involvement of the neural pathway between the Ce and the BST in the negative affective component of morphine withdrawal in rats. Rats were rendered morphine dependent by s.c. implantation of a 75-mg morphine pellet for 3 days, and morphine withdrawal was precipitated by an i.p. injection of naloxone (0.3 mg/kg). In the place-conditioning paradigm, discrete bilateral excitotoxic lesions of the Ce or the BST significantly reduced naloxone-precipitated morphine withdrawal-induced conditioned place aversion. On the other hand, they had little effect on morphine withdrawal-induced somatic signs. In an immunohistochemical study for c-Fos protein, naloxone-precipitated morphine withdrawal dramatically induced c-Fos-immunoreactive neurons in the capsular part of the Ce, and the lateral and medial divisions of the BST. Bilateral excitotoxic lesion of the Ce reduced the number of morphine withdrawal-induced c-Fos-immunoreactive neurons in the lateral and medial BST, with significant decreases in the posterior, ventral and juxtacapsular parts of lateral division, and anterior part of the medial division, but not in the ventral part of the medial division of the BST. On the other hand, bilateral excitotoxic lesion of the BST had no effect on such c-Fos induction within the capsular part, nor the ventral and medial divisions of the Ce. These results suggest that activation of the BST mediated through the neural pathway from the Ce contributes to the negative affective component of morphine withdrawal. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: conditioned place aversion, drug dependence, extended amygdala, c-Fos, immunohistochemistry, lesion.

Chronic use of morphine is well known to lead to physical and psychological dependence, characterized by the expression of withdrawal symptoms including both somatic and affective components, upon cessation of drug administration. In animals, morphine withdrawal produces various characteristic somatic signs, as well as disruption of schedule-controlled operant responses for food (Higgins and Sellers, 1994; Koob et al., 1989), elevation of intracranial self-stimulation thresholds (Schaefer and Michael, 1986) and aversive avoidance behavior from the environment previously associated with morphine withdrawal (conditioned place aversion; CPA) (Mucha, 1987; Stinus et al., 1990). These behavioral changes are thought to reflect the negative affective component of morphine withdrawal, such as dysphoria, irritability and anxiety, which might contribute to aversively motivated drug seeking.

The extended amygdala is a neuronal continuum within the basal forebrain with considerable morphological, neurochemical and neuroanatomical similarities, and seems to contribute to motivational and emotional responses. The extended amygdala is divided into medial and central components. The central extended amygdala includes the central nucleus of the amygdala (Ce), the lateral bed nucleus of the stria terminalis (BST), and the central sublenticular extended amygdala, which is a part of the dorsal substantia innominata, while the medial extended amygdala includes the medial nucleus of the amygdala and the medial BST (Alheid et al., 1995). Recently, it has been suggested that the extended amygdala is involved in both the acute positive reinforcing effects of drugs of abuse and the negative reinforcement associated with drug withdrawal (Koob, 1999; Koob and Le Moal, 2001). A number of studies have shown that morphine withdrawal increases the expression of a transcription factor, c-Fos protein, as a marker of neural activation (Kovacs, 1998), in several components of the extended amygdala, including the Ce and the BST (Frenois et al., 2002; Gracy et al., 2001; Le Guen et al., 2001; Stornetta et al., 1993; Veinante et al., 2003). The c-Fos induction in them was reported to correlate with the negative affective, rather than somatic, component of morphine withdrawal (Frenois et al., 2002; Gracy et al., 2001). Indeed, it was shown that microinjection of opioid antagonists into the amygdala, as well as the nucleus accumbens, of morphine-dependent rats produced mor-
phine withdrawal-induced CPA (Maldonado et al., 1992; Stinus et al., 1990). Furthermore, we and other groups reported that excitotoxic lesion of the Ce (Kelsey and Arnold, 1994; Watanabe et al., 2002b), or microinjection of β-adrenoceptor antagonists into the Ce (Watanabe et al., 2003) or the BST (Delfs et al., 2000) attenuated the morphine withdrawal-induced CPA, while they had no or weaker effects on the morphine withdrawal-induced somatic signs. These findings support the notion that the Ce and the BST play an important role in the negative affective, rather than somatic, component of morphine withdrawal.

It has been reported that the Ce and the BST are reciprocally connected through the stria terminals (Dong and Swanson, 2004; Han and Ju, 1989; Ju and Swanson, 1989; Krettek and Price, 1978; Sun et al., 1991; Sun and Cassell, 1993; Veinante and Freund-Mercier, 1998). Furthermore, it has been suggested that they have common physiological roles in autonomic and behavioral responses associated with motivational and emotional responses (Delfs et al., 2000; Dunn, 1987; Dunn and Whitener, 1986; Watanabe et al., 2003). However, whether the neural pathway between them is involved in the negative affective component of morphine withdrawal has not been established. In this study, we examined the effects of bilateral excitotoxic lesions of the Ce and the BST on the naloxone-precipitated morphine withdrawal-induced CPA, somatic signs, and c-Fos expression in the subdivisions of the BST and the Ce in rats. We show here that activation of the neural pathway from the Ce to the BST plays a crucial role in the negative affective component of morphine withdrawal.

**EXPERIMENTAL PROCEDURES**

The present study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee and the Japanese Pharmacological Society and was in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and limit experimentation to what was necessary to produce reliable scientific information.

**Animals**

Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) initially weighing 180–220 g were used. They were kept at a constant ambient temperature of 24±1 °C under a 12-h light/dark cycle with free access to food and water. After arrival, rats were individually housed in plastic cages with woodchip bedding for at least 1 day before surgery.

**Materials**

Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). Morphine pellets each containing 75 mg of morphine base were prepared according to the method of Gibson and Tingsstad (1970). N-methyl-D-aspartic acid (NMDA) and naloxone hydrochloride were purchased from Sigma (St. Louis, MO, USA).

**Surgery**

Bilateral excitotoxic lesions of the Ce or the BST were made as previously described (Watanabe et al., 2002b) with slight modifications. Briefly, under thiamylal sodium anesthesia (50 mg/kg, i.p.), 0.3 M NMDA dissolved in phosphate-buffered saline (PBS) was bilaterally infused into the Ce (1.8 mm caudal to bregma, 3.8 mm lateral to the midline, and 7.0 mm below the surface of the skull) or the BST (0.3 mm caudal to bregma, 1.6 mm lateral to the midline, and 7.0 mm below the surface of the skull) according to the atlas of Paxinos and Watson (1998) in a volume of 0.3 μl/side over 3 min via stainless steel injection cannulas (33 gauge, o.d. 0.2 mm). The injection cannulas were left in place for an additional 2 min to prevent backflow. Sham-operated rats received identical surgical treatment except that PBS alone was infused. Then, the rats were individually returned to their respective cages and left for at least 1 week before the experiment. A total of 140 rats were divided five groups, i.e. two non-operated, 24 Ce-sham-operated, 46 Ce-lesioned, 24 BST-sham-operated and 44 BST-lesioned animals.

**Induction of morphine dependence and precipitation of morphine withdrawal**

Rats were rendered morphine dependent by s.c. implantation of a morphine pellet as previously described (Watanabe et al., 2002a). Under light ether anesthesia, rats had either a morphine or placebo pellet implanted s.c. in the back of the neck. Three days later, the animals received a single i.p. injection of naloxone (0.3 mg/kg) dissolved in saline without removing the implanted pellet.

**CPA paradigm**

Twelve Ce-sham-operated, 20 Ce-lesioned, 12 BST-sham-operated and 20 BST-lesioned animals, which were implanted with a morphine pellet, were used in the CPA experiments.

**Apparatus.** CPA was conducted as previously described (Watanabe et al., 2002a). The place conditioning apparatus consisted of a shuttle box (30×60×30 cm: width×length×height) divided into two equal-sized compartments. The inner surface of one compartment was black with a smooth floor; and the other was white with a textured floor. The time spent in each compartment during a period of 15 min (800 s) was measured automatically in a blind fashion using a computer system (KN-80; Natsume Seisakusho, Tokyo, Japan). The apparatus was enclosed by a sound- and light-attenuated box under conditions of dim illumination and masking white noise.

**Preconditioning session.** On the first day (day 1), the partition separating the two compartments was raised 12 cm above the floor, and a metal neutral platform (5×2×12 cm) was inserted along the seam separating the compartments. The rats were individually placed on the platform and allowed to climb down and freely explore the two compartments for 900 s, so that they could habituate themselves to the apparatus. On day 2 (preconditioning session), the same trial was performed, and the time spent in each compartment for 900 s was measured. There was no significant difference between the time spent in the black compartment with a smooth floor (455±19.9 s, n=36) and time in the white compartment with a textured floor (444±19.9 s, n=36), indicating that there was no preference bias before conditioning in the apparatus. We determined the preferred compartment for each rat in which the rats spent greater than 50% of the total time (i.e. 450 s). Four Ce- and BST-operated rats, a Ce-lesioned and a BST-sham-operated rat that spent more than 80% of the time (i.e. 720 s) in one side on day 3, or that spent more than 600 s in one side on day 2 and more than 600 s on the other side on day 3 were excluded from further analysis.

**Conditioning session.** On day 3, place conditioning was performed as follows: each rat was injected i.p. with saline and confined to its non-preferred compartment for 1 h. After at least 3 h, the rat was injected i.p. with naloxone (0.3 mg/kg) and then confined to its preferred compartment for 1 h.
Measurement of naloxone-precipitated morphine withdrawal-induced somatic signs

Twelve Ce-sham-operated, 26 Ce-lesioned, 12 BST-sham-operated and 24 BST-lesioned animals, which were implanted with a morphine pellet, were used in the experiments for somatic signs assessment. Measurement of naloxone-precipitated morphine withdrawal-induced somatic signs was performed as previously described (Watanabe et al., 2002a). Briefly, each morphine-dependent rat was placed in a Plexiglas cylinder to acclimate it to the experimental environment. After a 60-min habituation period, naloxone (0.3 mg/kg) was injected i.p., and then behavior was observed for 60 min. Body weight was measured just before and 1 h after naloxone injection, and is presented as the means±S.E.M. of percentage body weight loss. The number of occurrences of stretching, wet-dog shaking, teeth chattering, jumping, paw shaking, head shaking, ejaculation, penile grooming and grooming was counted, and data are presented as means±S.E.M. of total numbers. The occurrence of diarrhea, salivation, lacrimation, rhinorrhea and plosis was monitored and is presented as the number of rats showing positive signs divided by the total number of rats tested.

Immunohistochemistry for c-Fos

Four Ce-sham-operated, four Ce-lesioned, four BST-sham-operated and four BST-lesioned rats, which were used for measurement of morphine withdrawal-induced somatic signs, and two non-operated and placebo pellet-implanted rats were used in the immunohistochemical experiments. Ninety minutes after the i.p. injection of naloxone (0.3 mg/kg), the animals were deeply anesthetized with thiamylal sodium and perfused transcardially through the ascending aorta with 0.1 M PBS, immediately followed by 4% paraformaldehyde in PBS. Brains were removed, post-fixed in the same fixative for 3–4 h, cryoprotected with 15% sucrose in PBS for 48 h at 4 °C, and then frozen in powdered dry ice. Coronal sections (25 μm) including the Ce or the BST were prepared on a cryostat, thaw-mounted onto gelatin-coated slides and stored at –80 °C until use. The sections were stained with Cresyl Violet and each section was examined by microscopy (×400). Lesion boundaries were identified on the basis of neuronal loss and reactive gliosis. Only rats with bilateral excitotoxic lesions of the Ce or the BST were included in the analysis.

RESULTS

Histology of the bilateral excitotoxic lesions of the Ce and the BST

The neuronal cells were clearly retained in the Ce, BST and their surroundings of the 20 Ce- and 23 BST-sham-operated control rats.

Illustrations of excitotoxin-induced lesion sites in 19 of the rats that received bilateral infusion of NMDA into the Ce are shown in Fig. 1A. The excitotoxic lesion encompassed a significant amount of the capsular part (CeC) and the lateral (CeL) and medial (CeM) divisions of the Ce, although the neuronal cells in the surrounding sites were not damaged. In some animals, there was neuronal damage in portions of the lateral globus pallidus and substantia innominata. In a few animals, there was neuronal damage in portions of the lateral or dorsomedial nucleus of the amygdala. The data from 26 rats were excluded from the final analysis, i.e. in 19 cases neuronal damage sub-
stantially extended into the lateral, basolateral and dorsomedial nucleus of the amygdala or Meynert basal nucleus, and in the other seven cases neuronal damage to the Ce was incomplete.

Illustrations of excitotoxin-induced lesion sites in 17 of the rats that received bilateral infusion of NMDA into the BST are shown in Fig. 1B. The excitotoxic lesions encompassed a significant amount of the dorsal (BSTLD), posterior (BSTLP), ventral (BSTLV) and juxtacapsular (BSTLJ) parts of the lateral division, and the anterior (BSTMA) and ventral (BSTMV) parts of the medial division of the BST, including the anterior commissure and internal capsule, although the neuronal cells in the surrounding sites were not damaged. In some animals, there was neuronal damage in portions of the caudate putamen, lateral globus pallidus, ventral pallidum, substantia innominata and lateral septal nucleus. The data from 27 rats were excluded from the final analysis, i.e. in 20 cases neuronal damage substantially extended into these surrounding sites, and in the other seven cases neuronal damage to the BST was incomplete.

Effects of Ce- or BST-lesions on morphine withdrawal-induced CPA

The effects of bilateral excitotoxic lesions of the Ce or the BST on naloxone-precipitated morphine withdrawal-induced CPA are shown in Fig. 2.

As we previously reported (Watanabe et al., 2002b), Ce-lesions significantly attenuated the morphine withdrawal-induced CPA. In the present study, the time that Ce-sham-operated control rats spent in the morphine-withdrawal paired compartment in the test session was 310±38.8 s, which was significantly shorter than that in the preconditioning session (521±23.4 s, P<0.001). On the other hand, the time that Ce-lesioned rats spent in the morphine-withdrawal paired compartment in the test session was 472±49.8 s, which was not significantly different compared with that in the preconditioning session (550±27.2 s). The CPA score of Ce-lesioned rats was significantly smaller than that of Ce-sham-operated control rats (Fig. 2A).

Similarly, the time that BST-sham-operated control rats spent in the morphine-withdrawal paired compartment in the test session was 330±20.3 s, which was significantly shorter than that in the preconditioning session (511±16.8 s, P<0.001). The time that BST-lesioned rats spent in the morphine-withdrawal paired compartment in the test session was 395±26.6 s, which was significantly shorter than that in the preconditioning session. However, the CPA score of BST-lesioned rats was significantly smaller than that of BST-sham-operated control rats (Fig. 2B).

Effects of Ce- or BST-lesions on morphine withdrawal-induced somatic signs

The effects of bilateral excitotoxic lesions of the Ce or BST on the naloxone-precipitated morphine withdrawal-induced somatic signs are shown in Table 1. In the Ce- and BST-sham-operated control rats, i.p. injection of naloxone elicted char-

---

Fig. 1. Schematic representation of bilateral excitotoxic lesions of the Ce (A) and the BST (B). Shaded areas represent the smallest (black) and largest (gray) extent of neuronal damage. Outlines represent sections ranging from −1.8 to −2.8 mm (A) and from 0.20 to −1.40 mm (B) from bregma (Paxinos and Watson, 1998).
The effects of bilateral excitotoxic lesions of the Ce on the naloxone-precipitated morphine withdrawal-induced CPA in rats. Sham-operated (open bars) or lesioned (closed bars) rats were given a morphine pellet for 3 days, and then were injected i.p. with naloxone (0.3 mg/kg) under the place-conditioning paradigm. Each column represents the CPA score expressed as mean ± S.E.M. * P<0.05, ** P<0.01 compared with respective sham-operated group by Student’s t-test. Numerals in parentheses indicate the numbers of rats used.

**Fig. 2.** Effect of bilateral excitotoxic lesion of the Ce (A) or the BST (B) on the naloxone-precipitated morphine withdrawal-induced CPA in rats. Sham-operated (open bars) or lesioned (closed bars) rats were given a morphine pellet for 3 days, and then were injected i.p. with naloxone (0.3 mg/kg) under the place-conditioning paradigm. Each column represents the CPA score expressed as mean ± S.E.M. * P<0.05, ** P<0.01 compared with respective sham-operated group by Student’s t-test. Numerals in parentheses indicate the numbers of rats used.

**Table 1. Effects of Ce- or BLA-lesions on the somatic signs induced by naloxone-precipitated morphine withdrawal**

<table>
<thead>
<tr>
<th>Withdrawal signs</th>
<th>Ce</th>
<th>BST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (12)</td>
<td>Lesion (10)</td>
</tr>
<tr>
<td></td>
<td>Lesion (9)</td>
<td>Sham (12)</td>
</tr>
<tr>
<td></td>
<td>Lesion (9)</td>
<td></td>
</tr>
<tr>
<td>Weight loss (%)</td>
<td>2.9 ± 0.3</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3.1 ± 0.4</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>Stretching</td>
<td>4.9 ± 1.5</td>
<td>2.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>5.3 ± 1.3</td>
<td>6.0 ± 2.6</td>
</tr>
<tr>
<td>Wet-dog shaking</td>
<td>9.8 ± 1.5</td>
<td>16.0 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>11.0 ± 1.9</td>
<td>15.6 ± 4.3</td>
</tr>
<tr>
<td>Teeth chattering</td>
<td>70.7 ± 6.5</td>
<td>57.7 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>106.7 ± 13.6</td>
<td>63.7 ± 9.0</td>
</tr>
<tr>
<td>Jumping</td>
<td>0.3 ± 0.3</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>Paw shaking</td>
<td>4.0 ± 1.2</td>
<td>10.7 ± 2.1**</td>
</tr>
<tr>
<td></td>
<td>6.1 ± 1.3</td>
<td>14.0 ± 2.0**</td>
</tr>
<tr>
<td>Head shaking</td>
<td>11.5 ± 2.1</td>
<td>13.8 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>10.6 ± 1.9</td>
<td>12.2 ± 3.9</td>
</tr>
<tr>
<td>Ejaculation</td>
<td>1.6 ± 0.4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.9 ± 0.3</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Penile grooming</td>
<td>3.8 ± 0.8</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>4.3 ± 0.8</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>Grooming</td>
<td>7.3 ± 1.8</td>
<td>7.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>11.3 ± 2.4</td>
<td>11.8 ± 1.9</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>11/12</td>
<td>9/10</td>
</tr>
<tr>
<td></td>
<td>11/12</td>
<td>9/9</td>
</tr>
<tr>
<td>Salivation</td>
<td>2/12</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>2/12</td>
<td>4/9</td>
</tr>
<tr>
<td>Lacermination</td>
<td>6/12</td>
<td>4/10</td>
</tr>
<tr>
<td></td>
<td>3/12</td>
<td>2/9</td>
</tr>
<tr>
<td>Rhinorrhea</td>
<td>10/12</td>
<td>6/10</td>
</tr>
<tr>
<td></td>
<td>8/12</td>
<td>6/9</td>
</tr>
<tr>
<td>Ptosis</td>
<td>12/12</td>
<td>8/10</td>
</tr>
<tr>
<td></td>
<td>11/12</td>
<td>8/9</td>
</tr>
</tbody>
</table>

Weight loss is presented as the mean ± S.E.M of percentage body weight loss during 1 h. Signs are shown as the means ± S.E.M of total numbers for 1 h. The occurrence of diarrhea, salivation, lacrimation, rhinorrhea and ptosis is expressed as the number of rats showing positive signs divided by the total number of rats tested. * P<0.05, ** P<0.01 compared with respective sham operated group by Student’s t-test. Numerals in parentheses indicate the numbers of rats used.

**Effect of BST-lesions on morphine withdrawal-induced c-Fos expression in the Ce**

The effects of bilateral excitotoxic lesion of the BST on the naloxone-precipitated morphine withdrawal-induced c-Fos expression in the Ce are shown in Fig. 4 and Table 3. In the non-operated and placebo pellet-implanted naive rats injected i.p. with naloxone, only a few c-Fos-immunoreactive neurons were observed in any subdivision of the Ce (data not shown). On the other hand, in the BST-sham-operated and morphine-dependent rats, i.p. injection of naloxone dramatically induced c-Fos-immunoreactive neurons in the CeC, while it induced only a few c-Fos-immunoreactive neurons in the CeL and CeM (Fig. 4A, C). In the BST-lesioned and morphine-dependent rats, the induction of c-Fos-immunoreactive neurons in the CeC was similar to that of BST-sham-operated and morphine-dependent rats (Fig. 4B, D). Quantitative analysis showed that there was no significant difference in the number of morphine withdrawal-induced c-Fos immunoreactive neurons in any subdivision of the Ce between the BST-sham-operated and BST-lesioned groups (Table 3).

**DISCUSSION**

The present studies using the place-conditioning paradigm and immunohistochemistry for c-Fos protein show the in-
volvement of activation of the BST mediated by the Ce in
the morphine withdrawal-induced CPA. This finding further
supports the recent notion that the extended amygdala,
including the Ce and the BST, plays a role in the negative
affective component of morphine withdrawal (Delfs et al.,

There is a body of evidence for the involvement of the
Ce in the negative affective component of morphine with-
drawal. Stinus et al. (1990) showed that microinjection of
methylnaloxonium, a hydrophilic opioid antagonist, into the
Ce elicited CPA in morphine-dependent rats. We previ-
ously showed that bilateral excitotoxic lesions of the Ce,
but not the basolateral nucleus of the amygdala, attenu-
ated the naloxone-precipitated morphine withdrawal-in-
duced CPA (Watanabe et al., 2002b), a finding consistent
with those of the present study. In addition, we have re-
ported that intra-Ce injection of glutamate receptor antag-
onists or β-adrenoceptor antagonists attenuated acquisi-
tion of morphine withdrawal-induced CPA, while they had
little effect on the expression of somatic signs (Watanabe et al.,
2002a, 2003). On the other hand, limited studies have
directly shown the role of the BST in the negative
affective component of morphine withdrawal (Delfs et al.,
2000), although there are numerous findings regarding
positive and negative affective components of other drugs
of abuse, such as cocaine (Epping-Jordan et al., 1998; Erb et al.,
2001; McFarland et al., 2004), heroin (Walker et al.,
2000) and ethanol (Eiler et al., 2003). Nevertheless, it was
reported that morphine withdrawal precipitated by low
doses of naloxone (7.5–30 μg/kg) induced CPA and c-Fos or
f-c-fos gene expression in the BST and the Ce, while it did
not induce somatic signs (Frenois et al., 2002; Gracy et al.,
2003).

Fig. 3. Brightfield light micrographs of the BST showing the distribution of naloxone-precipitated morphine withdrawal-induced c-Fos expression in the
Ce-sham-operated (A, C, D) and Ce-lesioned (B, E, F) morphine-dependent rats. The upper left panel represents the approximate area shown in light
micrographs A and B. (C–F) Light micrographs at higher magnification in the BSTLJ (C, E) and BSTMA (D, F) of the Ce-sham-operated (C, D) and
Ce-lesioned (E, F) morphine-dependent rats. ac, anterior commissure; ic, internal capsule; st, stria terminalis. Scale bar: 400 μm for A and B; 100 μm
for C–F.
Injections of some types of glutamate receptor antagonists, \(\beta\)-adrenoceptor antagonists or \(\alpha\)-adrenoceptor agonists into the Ce (Taylor et al., 1998; Watanabe et al., 2002a, 2003) or the BST (Delfs et al., 2000) significantly but partially attenuated the expression of somatic withdrawal signs, while other types of antagonists had no effects (Watanabe et al., 2002a, 2003). On the other hand, in the present study, excitotoxic lesions of the Ce or BST unexpectedly facilitated a somatic sign, paw shaking. Although we cannot fully explain this discrepancy, the Ce and the BST do not seem to play essential roles in the mediation of somatic withdrawal signs. At least, it is considered that the inhibitory effects of Ce- and BST-lesion on the morphine withdrawal-induced CPA and c-Fos expression are unlikely to be due to the reduction of the development of morphine physical dependence and expression of morphine withdrawal somatic signs.

The pattern of c-Fos expression induced by naloxone-precipitated morphine withdrawal in the present immunohistochemical study is in good agreement with the findings of previous studies (Frenois et al., 2002; Gracy et al., 2001; Hamlin et al., 2004; Le Guen et al., 2001; Veinante et al., 2003), although there are some discrepancies. In the present study, naloxone at a relatively low dose of 0.3 mg/kg was intraperitoneally injected in morphine-dependent rats, which were s.c. implanted with a 75-mg morphine pellet for 3 days. This procedure induced significant CPA and moderate somatic withdrawal signs. Under these conditions, we found that c-Fos immunoreactive neurons were observed in the CeC and throughout the lateral and medial BST, but there were few such neurons in the CeL and CeM. On the other hand, some other studies reported the induction of c-Fos immunoreactive neurons even in the CeL as well as in the CeC (Hamlin et al., 2004; Le Guen et al., 2001; Veinante et al., 2003). However, those previous studies used higher doses of naloxone (>2 mg/kg), which could precipitate strong morphine withdrawal-induced somatic signs (Higgins and Sellers, 1994; Le Guen et al., 2001), and induce c-Fos immunoreactive neurons in the some regions, including the Ce and BST even of naive rats. Thus, the discrepancies about the c-Fos induction in the CeL might have been due to differences in the degree of morphine withdrawal. The CeC appears to receive inputs directly from the several thalamic areas (Turner and Herkenham, 1991), the pontine parabrachial area (Bernard and Besson, 1990; Bernard et al., 1993), and the cortical areas (Mcdonald and Mascagni, 1996). Indeed, we and other groups have reported that a variety of aversive and/or stressful stimuli induced the expression of c-Fos or c-fos gene (Frenois et al., 2002; Nakagawa et al., 2003; Swank, 1999), and activated neurons, especially in the CeC (Bernard et al., 1992). These findings suggest that the CeC may be a key gate associating aversive information, including morphine withdrawal, with aversive emotion.

In the present study, we found that Ce-lesion significantly reduced not only morphine withdrawal-induced CPA, but also the number of c-Fos-immunoreactive neurons in the lateral and medial BST, including the BSTLJ,
BSTLP, BSTLV, and BSTMA, and tended to reduce the number in the BSTLD. These results suggest that the Ce, at least in part, contributes to activation of the BST neurons during morphine withdrawal. Previous retrograde and anterograde tracing studies indicated that that all subdivisions of the Ce have extensive projections to specific areas of the BST in rats, and that the neurons arising from the CeC project to the lateral BST, such as the BSTLP and BSTLV, and to some extent the BSTMA (Bourgeais et al., 2001; Dong and Swanson, 2004; Krettek and Price, 1978; Petrovich and Swanson, 1997; Sun et al., 1991), although they primarily and extensively project to the substantia innominata dorsalis (Bourgeais et al., 2001). Those findings taken together with the above finding that morphine withdrawal induced c-Fos immunoreactive neurons especially in the CeC among of the subdivisions of the Ce imply that it is likely that the neural pathway from the CeC could activate the BST neurons. Indeed, half of the morphine withdrawal-induced c-Fos immunoreactive neurons in the CeC were retrogradely labeled following injection of a retrograde tract tracer into the BST (our unpublished data). However, the number of morphine withdrawal-induced c-Fos immunoreactive neurons in the BSTMA, which receives little or no input from the CeC, was also decreased significantly by Ce-lesion. It has been reported that the neurons arising from the CeL extensively project to the BSTLP and BSTLV, and to some extent the BSTLD and BSTMA, and that the neurons arising from the CeL project mainly to the lateral BST, similarly to those from the CeC and CeL, and clearly to the BSTMA (Bourgeais et al., 2001; Dong and Swanson, 2004; Krettek and Price, 1978; Petrovich and Swanson, 1997; Sun et al., 1991). Taken together, these observations imply that it is conceivable that the decrease in the BSTMA and other BST subdivisions might be due to the loss of projections from the CeL and/or CeM due to the excitotoxic lesions, although these Ce subdivisions had few morphine withdrawal-induced c-Fos immunoreactive neurons. It was reported that some neurons projecting from the CeL and CeM to the medial BST, including the BSTMA, are GABAergic (Sun and Cassele, 1993). It seems to be of interest to examine the possibility of the disinhibition of the GABAergic neurons during morphine withdrawal. Another potent candidate is corticotropin-releasing factor (CRF), which is known to be

Fig. 4. Brightfield light micrographs of the Ce showing the distribution of naloxone-precipitated morphine withdrawal-induced c-Fos expression in the BST-sham-operated (A, C) and BST-lesioned (B, D) morphine-dependent rats. The lower left panel represents the approximate area shown in the light micrographs A and B. (C, D) Light micrographs at higher magnification in the CeC of the BST-sham-operated (C) and BST-lesioned (D) morphine-dependent rats. BLA, basolateral nucleus of the amygdala; Scale bar=400 μm for A and B; 100 μm for C–F.
involved in drug addiction (Koob, 1999). It has been reported that some neurons projecting from the CeL to the lateral and medial BST contain CRF (Sakanaka et al., 1986). Heinrichs et al. (1995) reported that suppression of CRF receptor by a CRF receptor antagonist α-helical CRF (9–41) or impairment of CRFergic neurons by immunotargeted toxins in the Ce reduced morphine withdrawal-induced CPA. Furthermore, it was shown that morphine dependence and withdrawal increased the expression of CRF mRNA in the Ce, but not the BST (McNally and Akil, 2002). Taken together, it is considered that the CRFergic neurons projecting from the CeL might contribute to the activation of the neurons in the medial and lateral BST, including the BSTMA (Erb et al., 2001). Indeed, our preliminary data showed that microinjection of α-helical CRF (9–41) into the BST reduced morphine withdrawal-induced CPA.

On the other hand, it is certain that the Ce receives inputs from the BST, and that the activity of the Ce is regulated by GABAergic neurons originating from the BST (Sun and Cassell, 1993; Veinante and Freund-Mercier, 1998). In the present study, we found that the BST-lesion had no effect on the number of c-Fos-immunoreactive neurons induced by morphine withdrawal in the CeC, nor in the CeL and CeM, although it significantly reduced the morphine withdrawal-induced CPA. These results suggest that the activation of the Ce neurons during morphine withdrawal is not affected through the BST.

Both the Ce and the BST are innervated by noradrenergic afferents mainly through the ventral noradrenergic bundle from the A1/A2 cell groups. It was shown that morphine withdrawal-induced CPA was reduced by impairment of the ventral noradrenergic bundle or microinjection of β-adrenoceptor antagonists into the Ce or the BST (Delfs et al., 2000; Watanabe et al., 2003). Furthermore, both the Ce and the BST are reciprocally connected with and regulated by the mesocorticolimbic dopaminergic neurons arising from the ventral tegmental area (Georges and Aston-Jones, 2002; Hasue and Shammah-Lagnado, 2002; Phillips et al., 2003), and the dopaminergic terminals have shown to contact CRFergic neurons in the Ce and the BST (Asan, 1998; Phelix et al., 1994). The dopaminergic system is known to be critical for not only drug addiction and reward, but also aversive emotional responses (Borowski and Kokkinidis, 1996; Shippenberg and Bals-Kubik, 1995). Taken together, it is likely that the neural pathway from the Ce to the BST might compose part of the neural circuit with noradrenergic and dopaminergic system, and the neural circuit might play an important role in the negative affective component of morphine withdrawal (Koob, 1999).

### Table 3. Effect of BST-lesions on the number of c-Fos immunoreactive cells in the subdivisions of the Ce induced by naloxone-precipitated morphine withdrawal

<table>
<thead>
<tr>
<th>Subdivision of Ce</th>
<th>Mean number of c-Fos immunoreactive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BST-sham</td>
</tr>
<tr>
<td>CeC</td>
<td>220.5±6.1</td>
</tr>
<tr>
<td>CeL</td>
<td>9.9±3.8</td>
</tr>
<tr>
<td>CeM</td>
<td>7.5±3.1</td>
</tr>
</tbody>
</table>

The mean total number of c-Fos immunoreactive cells was counted in each subdivision of the Ce from four BST-sham operated or BST-lesioned morphine-dependent rats 90 min after an injection of naloxone (0.3 mg/kg). Of note, the numbers of c-Fos immunoreactive cells in any subdivisions of the Ce from placebo pellet-implanted naive rats after naloxone challenge are 0–6.

### CONCLUSION

In summary, we found that neural activation in some subdivisions of the BST during morphine withdrawal is mediated, at least in part, through the neural pathway from the Ce, while the neural activation in the Ce is not affected by BST-lesions. These results suggest that activation of the neural pathway from the Ce to the BST during morphine withdrawal is crucial for stimulating neurons within the BST, which leads to the initiation of aversive behavior induced by morphine withdrawal.

Acknowledgments—This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Special Coordination Funds for Promoting Science and Technology Target-oriented Brain Science Research Program.

### REFERENCES


(Accepted 25 March 2005)