Total Neurochemical Lesion of Noradrenergic Neurons of the Locus Ceruleus Does Not Alter Either Naloxone-Precipitated or Spontaneous Opiate Withdrawal nor Does It Influence Ability of Clonidine To Reverse Opiate Withdrawal¹

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ABSTRACT

It has been suggested that an increase firing rate of noradrenergic neurons of the locus ceruleus is responsible for the opiate withdrawal syndrome. However, lesion studies have indicated that the noradrenergic neurons of the locus ceruleus are not essential for either the expression or suppression by clonidine of opiate withdrawal. The present study was designed to determine the effect of the almost complete 6-hydroxydopamine lesion of noradrenergic neurons (94%) of the locus ceruleus on various components of the opiate withdrawal syndrome and on its protection by clonidine. Morphine dependence was induced by s.c. implantation of morphine pellets (2 × 75 mg base). The following paradigms were used: 1) naloxone-induced conditioned place aversion, 2) naloxone-precipitated acute opiate withdrawal syndrome, 3) nycthemeral locomotor activity as a measure of spontaneous opiate withdrawal. The results showed that quasi-total lesion of noradrenergic neurons of the locus ceruleus did not modify opiate dependence as revealed by naloxone-induced conditioned place aversion and the expression of an acute morphine withdrawal syndrome. Moreover, clonidine prevented the opiate withdrawal syndrome in both lesioned and sham-operated rats, suggesting that the action of clonidine is certainly mediated through postsynaptic α₂-adrenoceptor stimulation. Finally, the nycthemeral locomotor activity during spontaneous morphine withdrawal did not differ between the lesioned and the sham-operated rats.

Opiate dependence is thought to involve counteradaptive processes, reformulated for the drug addiction domain (Koob and Bloom, 1988). In this view, adaptive processes triggered to counter the morphine effect are responsible for the dependence, and in the absence of drug, these processes are unopposed, giving rise to the opiate withdrawal syndrome. However, the neurobiologic bases of these opponent processes have yet to be elucidated. It is known that such neuroadaptations can involve either homologous regulation by modifying the endogenous opioid systems, or a heterologous regulation by modifying other neurotransmitter systems such as those involving noradrenaline (NA) (Maldonado, 1997). Noradrenergic neurons from the locus ceruleus (NA-LC) are thought to be an essential component of opiate withdrawal. Early studies in heroin addicts revealed the anti-withdrawal effect of clonidine, an α₂-adrenergic agonist. This drug was found to alleviate most of the somatic signs of opiate withdrawal (Gold et al., 1978), and platelet α₂-adrenoceptor densities in heroin addicts were found to correlate with the severity of the abstinence syndrome (Garcia-Sevilla et al., 1984). In addition, electrophysiological studies in rats have shown that naloxone-induced withdrawal triggers intense hyperactivity of NA-LC neurons, and that this increase in firing rate is prevented by clonidine treatment. It has thus been assumed that the therapeutic action of clonidine

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ABBREVIATIONS: NA, noradrenaline; LC, locus coeruleus; NA-LC neurons, noradrenergic neurons from locus coeruleus; 6-OHDA, 6-hydroxydopamine neurotoxin; G-H score, Gellert and Holtzman score.
stemmed from its ability to reduce NA-LC firing via presynaptic stimulation of \(\alpha_2\)-adrenoceptors (Aghajanian, 1978; Freedman and Aghajanian, 1985). Moreover, microdialysis studies in freely moving rats have shown an increase in the extraneuronal level of NA in both hippocampus and cortex during naloxone-induced withdrawal (Rossetti et al., 1993; Silverstone et al., 1993). Clonidine also appeared to reverse such biochemical changes in projection areas during acute opiate abstinence (Crawley et al., 1979). The expression of opiate withdrawal signs therefore appeared to be linked with behavioral, electrophysiological, and biochemical changes in NA-LC neurons (Rasmussen et al., 1990). This hypothesis was also supported by the detection of an increase in c-fos expression in the LC during opiate withdrawal (Hayward et al., 1990; Chieng et al., 1995).

Assuming a causal role of NA in opiate withdrawal, lesions to NA-LC neurons should provide more direct evidence for this hypothesis. Electrolytic lesions of the LC area have been found to attenuate some symptoms of the opiate withdrawal syndrome (Maldonado and Koob, 1993). Similarly, it has been shown that opiate withdrawal can be precipitated by intracerebral injections of the hydrophilic opiate antagonist methylnaloxonoxin in various brain structures in morphine-dependent rats, and the LC area appears to be the most sensitive brain site for such effects (Maldonado et al., 1992). However, several biochemical lesion studies have questioned the NA-LC hypothesis of opiate dependence. Neither N-2-chloroethyl-N-ethyl-2-bromobenzylamine (DSP4) nor 6-hydroxydopamine (6-OHDA) lesion of the NA neurons of the LC have been able to reduce significantly the opiate withdrawal syndrome (Thatcher-Britton et al., 1984; Chieng and Christie, 1995). Moreover, such 6-OHDA lesions failed to suppress the protective effects of clonidine on opiate withdrawal, supporting the hypothesis that clonidine acts on postsynaptic \(\alpha_2\)-adrenoceptors (Thatcher-Britton et al., 1984). The impact of these results has been to some extent lessened by the fact that only partial NA lesions (70% depletion) have been obtained by such procedures. To resolve the apparently contradictory results and to elucidate the role of NA on opiate dependence, a 6-OHDA lesion technique producing total (>90% depletion) and specific destruction of NA-LC neurons was used.

In the present study, the effects of total NA-LC lesion in morphine-dependent rats were examined on: 1) the affective component of opiate abstinence by evaluating naloxone-induced place aversion conditioning; 2) the somatic component of opiate withdrawal by quantifying naloxone-induced opiate withdrawal symptoms; 3) the protective action of clonidine on naloxone-induced place aversion conditioning and the naloxone-precipitated opiate withdrawal syndrome; and 4) the development of spontaneous opiate withdrawal by continuous evaluation of locomotor activity.

Materials and Methods

Animals

A total of 607 Sprague-Dawley male rats (IFFA-CREDO, Lyon, France) weighing 220 to 240 g at the beginning of the experiments were used. Animals were housed either collectively (four/cage) or singly in cages located in a thermoregulated room (22°C) with a 12:12 h light/dark cycle (light from 8:00 AM to 8:00 PM). Food and water were available ad libitum. These conditions were maintained constant throughout the experiments.

Drugs

The following compounds were used in this study: citalopram hydrobromide (a generous gift from Lundbeck, Copenhagen, Denmark), clonidine hydrochloride [B-001; Research Biochemicals Inc. (RBI), Natick, MA], 6-OHDA hydrochloride (H-4381; Sigma Chemical Co., St. Louis, MO), morphine sulfate (Sanofi-Francopia, France), naloxone hydrochloride (O-002; RBI), and nomifensine maleate (N-123; RBI). Naloxone hydrochloride was dissolved in isotonic saline and injected s.c. Naloxone doses were always expressed as free base (1 mg of naloxone base = 1.11 mg of naloxone hydrochloride). Clonidine was dissolved in isotonic saline and always injected i.p.

Surgery

Noradrenergic innervation arising from the LC was totally destroyed by multiple intracerebral injections of 6-OHDA hydrochloride aimed at several locations along the axons of the noradrenergic A6 neurons. The rats were anesthetized with chloral hydrate (270 mg/kg i.p.) and placed in a Kopf stereotaxic apparatus with the incisor bar 5.0 mm above the interaural line. The 6-OHDA solution contained 4 \(\mu\)g base in 1 \(\mu\)l (1 \(\mu\)g base = 1.12 \(\mu\)g 6-OHDA, HCl). Vehicle was made with NaCl isotonic solution (0.9% in water) containing 0.1 mg/ml of ascorbic acid. The rate of injection was 1 \(\mu\)l in 100 s. The following coordinates are expressed in millimeters: L for lateral from the midline, AP for anteroposterior from the bregma, and V for vertical from the skull surface at the trepanation site. Three bilateral targets were chosen according to the distributional map of tyrosine-hydroxylase-immunoreactive neurons in the rat brain: 1) the first site located just beneath the LC and the nucleus of the mesencephalic tract of the trigeminal nerve (1.5 \(\mu\)l, 6 \(\mu\)l 6-OHDA), L \(\pm\) 1.5, AP \(-7.6, V \sim 7.7\); 2) the second site located above the decussation of the brachium conjunctivum or superior cerebellar tubercule (2 \(\mu\)l, 8 \(\mu\)l 6-OHDA), L \(\pm\) 1.5, AP \(-5.6, V \sim 6.4\); and 3) the third site located beneath the decussation of the brachium conjunctivum or superior cerebellar tubercule (2 \(\mu\)l, 8 \(\mu\)l 6-OHDA), L \(\pm\) 2.0, AP \(-5.6, V \sim 8.3\). Sham-operated rats received vehicle injections. Thirty minutes before the 6-OHDA or vehicle injections, rats were pretreated with citalopram (1 mg/kg i.p.), an inhibitor of serotonin reuptake (Invernizzi et al., 1992) and with nomifensine (10 mg/kg i.p.), an inhibitor of dopamine (DA) reuptake (Dugast et al., 1994); to protect serotonergic and dopaminergic neurons from the neurotoxin.

Induction of Opiate Dependence

Two pharmacological approaches were used to induce opiate dependence. For opiate antagonist-precipitated withdrawal, dependence was produced by s.c. implantation of morphine pellets, whereas for the spontaneous withdrawal paradigm, opiate dependence was induced by injection of escalating doses of morphine.

Induction of Morphine Dependence by Implantation of Slow-Release Morphine Pellets. Morphine dependence was induced by s.c. implantation (lower back) under deep anesthesia (halothane/air; induction 4/100, i/v, for 10 s followed by 1.5/100, i/v, for 30 s) of two slow-release, morphine-containing pellets (each morphine pellet contained 75 mg of morphine base (N = 1165; National Institute on Drug Abuse, Baltimore, MD). Full dependence to morphine is achieved 24 h after implantation of the morphine pellet and remains constant for 15 days. Dependence on opiates decreases thereafter as the remaining morphine is depleted (Gold et al., 1994).

Induction of Morphine Dependence by Injection of Escalating Doses of Morphine. Morphine dependence was induced by i.p. injections of morphine sulfate twice a day for 10 days according to the following schedule: 5 mg/kg the first day, 10 mg/kg the second day, and subsequent increases of 10 mg/kg/day to reach a dose of 90 mg/kg per injection on the 10th day. Injections were delivered at 8:00 AM

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Activity Recording

Locomotor activity was measured in activity cages (35 × 25 × 25 cm) whose floor was formed of wire mesh and whose side walls were made of 10 mm Plexiglas (Imetronic, Pessac, France). Two infrared photoelectric cells were placed 14 cm apart and 3 cm above the floor so that each passage of the animal interrupted the beam and was recorded by a computer. A computer program (Imetronic) analyzed the total number of activity counts (total beam interruptions on both cells), which represented the total motor activity of the animal.

Conditioned Place Aversion Paradigm

When dependence was established by s.c. implantation of morphine pellets, pharmacologically precipitated opiate withdrawal induces conditioned place aversion. This can be observed at doses of opiate antagonists that produce few if any overt somatic signs of withdrawal (Stinus et al., 1990; Schulteis et al., 1994). Briefly, the conditioned place aversion apparatus used to produce a reliable conditioned aversion consisted of three (A, B, and C) rectangular boxes (40 × 33 × 34 cm) arranged radially (120° to each other) and accessible from a triangular central compartment. Distinctive visual and tactile cues distinguished the three compartments: the walls and floor coloring (either black dots, black stripes, or white), and the floor texture (either smooth, medium rough, or rough). The sensory cue combinations were for walls, floor coloring, and floor texture, respectively: A) black dots, smooth; B) black stripes, medium rough; and C) white, rough. Each compartment was equipped with five infrared photocells spaced 8.5 cm apart along the longer wall, 3.5 cm above the floor. This allows automatic detection and recording with a computer of an animal’s position at all times. Four of these setups were placed in a sound-attenuated testing room, with white noise (75 dB) to further mask external noise, and were illuminated by three 15 W red lights located 1.5 m above each compartment. The experimental protocol comprised three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase.

In the preconditioning phase (4 days after implantation of two pellets of morphine), animals were placed in the central triangular compartment and allowed to freely explore the apparatus for 20 min (1200 s). Animals showing strong unconditioned aversion (less than 17% of the session time, i.e., 200 s) or preference (more than 44%, i.e., 530 s) for any compartment were discarded. For each rat, the two compartments with the closest time allotments were chosen. One was randomly chosen to be paired to naloxone and the other to vehicle. The unassigned compartment could be either the most or the least preferred of the three. After assigning the compartments, there were no significant differences between time spent in the naloxone-paired and the vehicle-paired compartments during the preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias before conditioning. The preconditioning test phase provided three variables which were D0, the time spent in the drug-paired compartment; S0, the time spent in the saline-paired compartment; and N0, the time in the neutral compartment.

In the second phase (conditioning), rats received an injection of vehicle on days 5, 7, and 9 postpellet implantation before being confined to their preselected, vehicle-paired compartment for 20 min. On days 6, 8, and 10 postpellet implantation, rats received one of the doses of naloxone immediately before confinement in the naloxone-paired compartment for 20 min.

The testing phase consisted of 20 min free exploration of the entire apparatus on days 11 and 12 postpellet implantation (i.e., 24 and 48 h after the final conditioning session). The postconditioning test phase provided three variables which were D, the mean time spent in the drug-paired compartment; S, the mean time spent in the saline-paired compartment and N, the mean time spent in the neutral compartment. A place aversion score was calculated as the difference between postconditioning (mean of the 24 and 48 h postconditioning test) and the time spent in the same compartment during the preconditioning phase (D-D0).

Opiate Withdrawal Syndrome Recording

Opiate withdrawal syndrome was quantified in a circular enclosure (in diameter 31 cm and 40 cm high), which was covered to prevent the rat from escaping. The fourth day after implantation of the morphine pellets, rats received a naloxone injection, and 7 min later they were enclosed and videotaped (Sony camera and TV monitor, Panasonic videorecorder) for 10 min.

To study the effect of the complete lesion of the NA-LC neurons on opiate withdrawal, each session was analyzed by an ethopharmacological approach (Espejo et al., 1995) in which the following parameters were quantified: salivation, vocalization, rhinorrhea, diarrhea, chromatodacryorrhea, attenuated gait, body shake, jumping, leaning, mastication, weight loss and the duration of immobile sniffing, freezing, body grooming, genital grooming, writhing and lying. Two different scores indicating the overall intensity of the opiate withdrawal syndrome were determined. The first score was calculated using the scale described by Gellert and Holtzman (1978) in which two classes of signs were distinguished: graded signs (weight loss, escape attempts, abdominal contractions, and wet-dog shakes), which were quantified numerically, and checked signs (diarrhea, teeth chattering, swallowing, salivation, chromatodacryorrhea, ptosis, abnormal posture, genital grooming, and irritability), for which only its presence or absence was evaluated. The second score, etho-score (Espejo et al., 1995), is a linear representation of the intensity of the withdrawal syndrome as a function of naloxone dose.

Etho-score = (MF/10) + WL

where MF represents the mastication frequency and WL the weight loss during the hour following naloxone injection as a percentage of the initial body weight.

The antitreatment effect of clonidine in NA-LC-lesioned rats was tested in the same enclosure. On the fourth day following implantation of the morphine pellets, each rat received one of the following treatments: saline + saline, saline + naloxone, clonidine + naloxone, and saline + clonidine. These four treatments were applied in a random order; rats were tested on days 5, 7, 9, and 11 postpellet implantation. The behavioral analysis was based on the Gellert and Holtzman scale (Gellert and Holtzman, 1978) in which points are attributed to each behavior. The sum of these behaviors produces a score reflecting the withdrawal state. The presence of rhinorrhea was also checked.

In both opiate withdrawal recording experiments, the person who recorded opiate withdrawal was different from the one who scored the behaviors who was blind to the treatment received by rats.

Measurement of Tissue Levels of Biogenic Amines

At the end of the experiments, when the effects of morphine had ceased (i.e., 1 month postpellet implantation), lesion and sham-operated rats were sacrificed by decapitation. Because it has been shown that inhibition of decapitation-induced motor discharges is an excellent indication of central NA lesions (Asin et al., 1982), particular note was taken of the presence or absence of motor discharges. Brains were rapidly removed and the cortex, striatum, hippocampus, and medulla were dissected bilaterally at 4°C. Dissected structures were immediately frozen on dry ice and stored at −80°C until biochemical assays. DA, NA, and 5-hydroxytryptamine (5-HT) contents were measured in the dissected brain regions by HPLC coupled with electrochemical detection. Tissues were homogenized in 200 μl of 0.1 N HClO4 and centrifuged at 11,000 rpm for 30 min at 4°C. Aliquots
(10–20 μl) of the supernatants were injected into the HPLC system after dilution with appropriate volumes of mobile phase. The mobile phase was as follows: 60 mM NaH₂PO₄, 0.1 mM disodium EDTA, 0.2 mM octane sulfonic acid, and 7% methanol, adjusted to pH 3.9 with orthophosphoric acid and filtered through a 0.22-μm Millipore filter. This mobile phase was delivered at 1.2 ml/min (Pump 116, System Gold; Beckman, Paris) through a Chromasyl column (C8, 150 × 4.6 mm, 5 μm; Touzard & Matignon, Paris) protected by a Brownlee-Newgard precolumn (RP-8, 15 × 3.2 mm, 7 μm). A refrigerated injector (Injector 507, System Gold, Beckman) was used. Detection of NA, DA, and 5-HT was carried out with a coulometric detector (CoulochromeI, ESA, Paris) coupled to a dual electrode analytic cell (model 5011) and a conditioning cell (model 5021). The conditioning cell was set at +100 mV, the first electrode at +350 mV, and the second at −270 mV. Results were expressed as nanograms per milligram of tissue and each value was the mean ± S.E.M.

Experimental Design

Experiment 1: Effects of Quasi-Total Lesion of NA-LC Neurons on Naloxone-Induced Conditioned Place Aversion in Morphine-Dependent Rats. Seventy-six lesioned rats and 58 sham-operated rats were used. Following the postoperative recovery period, dependence was induced by s.c. implantation of morphine pellets. On the fourth day following implantation, the naloxone-induced place aversion conditioning paradigm was initiated. Naloxone doses used were 1.8, 3.7, 7.5, or 15 μg/kg s.c. (Nal-1.8, Nal-3.7, Nal-7.5, and Nal-15, respectively).

Experiment 2: Clonidine-Induced Attenuation of Naloxone-Induced Place Aversion Conditioning in Morphine-Dependent Rats as a Function of Interval between Clonidine and Naloxone Injections. Place aversion conditioning was induced by naloxone (Nal-15) in nonlesioned morphine-dependent rats (s.c. implantation of two morphine pellets). The schedule used in this experiment was the same as in experiment 1, except that clonidine (200 μg/kg s.c., Clo-200) was injected before the naloxone. The interval between these injections was 0.5, 1, 3, 6, 9, or 12 h. A total of 78 rats were used (10–14 rats/group).

Experiment 3: Effects of Quasi-Total Lesion of NA-LC Neurons on Ability of Clonidine to Block Naloxone-Induced Conditioned Place Aversion in Morphine-Dependent Rats. Place aversion conditioning was induced by naloxone in morphine-dependent rats (s.c. implantation of two morphine pellets). We used a dose of 15 μg/kg s.c. naloxone (Nal-15), which was found to induce a robust and reliable conditioned place aversion in both lesioned and sham-operated rats in the first experiment.

This experiment was performed on 98 lesioned and 151 sham-operated rats. The same schedule as experiment 1 was used except that the rats received a clonidine injection 1 h before the naloxone injection. For both groups, the doses of clonidine tested were: 0, 25, 50, 100, and 200 μg/kg i.p. (Clo-0, Clo-25, Clo-50, Clo-100, and Clo-200, respectively), and in addition, for the sham group a higher dose was required to reverse completely the place aversion (400 μg/kg i.p., Clo-400).

Experiment 4: Effect of Quasi-Total Lesion of NA-LC Neurons on Expression of Acute Opiate Withdrawal Syndrome. Naloxone-induced opiate withdrawal syndrome was evaluated on 65 NA-lesioned and 42 sham-operated morphine-dependent rats (s.c. implantation of two morphine pellets). Both groups were allocated into five subgroups, which received the following doses of naloxone: 0, 10, 50, 100, and 1000 μg/kg s.c. (Nal-0, Nal-10, Nal-50, Nal-100, and Nal-1000, respectively). On the fourth day following morphine pellet implantation, each rat received its respective naloxone injection, and 7 min later, its behavior was videotaped for 10 min. for both groups.

Experiment 5: Effect of Quasi-Total Lesion of NA-LC Neurons on Ability of Clonidine to Block Naloxone-Induced Opiate Withdrawal Syndrome. Nine NA-lesioned and eight sham-operated morphine-dependent rats were used (s.c. implantation of two morphine pellets). The experiment started on the fourth day following the morphine pellets implantation. Each animal received the following pharmacological treatments in random order: saline + saline, saline + Nal-1000, Clo-200 + Nal-1000, and Clo-200 + saline. Clonidine and naloxone were injected 60 and 7 min, respectively, before the videotape recording. The clonidine injection time was chosen on the basis of the results of experiment 2. The experiment began 4 days postpellet implantation, and the rats were tested every 2 days.

Experiment 6: Effects of Quasi-Total Lesion of NA-LC Neurons on Spontaneous Morphine Withdrawal: a Locomotor Activity Study. Ten 6-OHDA lesioned and 12 sham-operated rats were used. The experiment lasted 25 days. After the postoperative recovery period, animals were permanently housed in individual cages that allowed continuous recording of locomotor activity. After a 2-day habituation period, locomotor activity was recorded during 4 days to provide the baseline activity of each rat. During the following 10 days, morphine dependence was induced by repeated injection of the drug. The following day (11th), the last dose was administered, and locomotor activity was continuously recorded during abstinence (9 days). Temperature, dark-light cycle, and food and water availability were identical with the animal colony housing conditions.

Statistical Analysis

Tissue Levels of Biogenic Amines. Differences between NA-lesioned and sham-operated groups were tested using a two-tailed Student’s t test.

Place Aversion Conditioning. Data were analyzed with the nonparametric Wilcoxon signed ranks test in which the subgroup variables were the pharmacological treatments (naloxone doses in experiment 1, clonidine delay in experiment 2, and clonidine doses in experiment 3).

Withdrawal Syndrome. Frequency or the duration of each withdrawal syndrome pattern in experiment 4 was analyzed using the Kruskal-Wallis nonparametric rank test with the naloxone dose as the independent variable. Post hoc comparisons were based on nonparametric pairwise comparison versus the group Nal-0.

In experiment 5, the nonparametric Wilcoxon signed ranks test was employed to compare the pharmacological treatment regarding the withdrawal parameters (pharmacological treatment as dependent variable); and lesion effects on these withdrawal parameters were analyzed with the Kruskal-Wallis nonparametric rank test (6-OHDA lesion as independent variable).

Locomotor Activity. Data were analyzed with a three-way ANOVA with one “between factor” (pharmacological treatment) and two “within factors” (day postwithdrawal and time of day) followed by post hoc ANOVA for interaction and Dunnett’s test for two by two comparisons.

Results

Effect of 6-OHDA-Induced Lesion on Monoamines Levels in Various Rat Brain Areas. After decapitation, intense convulsions were observed in all the sham-operated rats for about 20 s. In contrast, NA-lesioned rats did not move at all.

As shown in Table 1, multiple intracerebral injections of 6-OHDA induced an almost complete depletion of NA in cortex (−92%, p < .001) and hippocampus (−94%, p < .001; median 6% and interquartile values, 5 and 8%), and a marked depletion in the medulla (−64%, p < .01). Although inhibitors of DA and 5-HT reuptake were employed, DA levels were reduced in striatum (−33%, p < .001), whereas 5-HT levels were increased in cortex (+23%, p < .05). These results could indicate long-term adaptive processes that were triggered by the lesion of NA-LC.
Verification of Model of Three-Compartment Place Aversion Test. As expected, during the preconditioning phase, the preference for the three compartments (A, B, and C) was uneven (ANOVA, F(2,910) = 38.16, p < .001). The rank order of preference was for black-dotted walls and smooth floor (A, 422 ± 7 s), to black-striped walls and medium rough floor (B, 353 ± 6 s), to white walls and rough floor (C, 331 ± 5 s). The post hoc analysis showed that the preferences for the three compartments were statistically significant (A/B and A/C, Newman-Keuls, p < .001; and B/C, Newman-Keuls, p < .05). After the allocation of the compartments (D0, S0, and N0), post hoc analysis showed that D0 (389 ± 4 s) and S0 (380 ± 6 s) were identical, but were both different from N0 (429 ± 9 s) (ANOVA, F(2,910) = 9.31, p < .001; Newman-Keuls, p < .01 in both cases). Thus, the compartments chosen for the conditioning phase were balanced and there was no bias before the conditioning phase.

Experiment 1: Effects of Quasi-Total Lesion of NA-LC Neurons on Naloxone-Induced Conditioned Place Aversion in Morphine-Dependent Rats. Results are presented in Fig. 1. For the sham-operated rats, the changes in time spent in each compartment are summarized in Fig. 1a. Wilcoxon paired tests where D0 was compared with D, S0 with S, and N0 with N, showed that in sham-operated rats, conditioned place aversion appeared at the lower dose (Nal-3.7) and persisted for higher doses (Nal-7.5, T = −1.95, p < .05; Nal-7.5, N.S.; Nal-15, T = −3.04, p < .001); whereas they did not alter the time spent in the neutral compartment (N0/N, N.S. for all doses except Nal-15, T = 2.32, p < .05).

The results show that after an almost complete lesion of NA-LC neurons, morphine-dependent rats still express a conditioned place aversion. Moreover, NA-lesioned rats exhibit a strategy of avoidance stronger than the one developed by sham rats, as shown by the quasi-systematic increase of the time spent in the saline-paired compartment.

Experiment 2: Clonidine-Induced Attenuation of Naloxone-Induced Place Aversion Conditioning in Morphine-Dependent Rats, as a Function of Interval between Clonidine and Naloxone Injections. As indicated in Fig. 2, naloxone-induced place aversion conditioning (Nal-15) was attenuated by a Clo-200 pretreatment if it was injected at least 3 h before Nal-15. Naloxone alone induced a conditioned place aversion (T = −3.29, p < .001), which was reversed by Clo-200 for the following intervals: 0.5 h (T = −1.35, N.S.), 1 h (T = −1.07, N.S.), 3 h (T = −1.58, N.S.). When the interval exceeded 3 h, clonidine was no longer effective.

Experiment 3: Effects of Quasi-Total Lesion of NA-LC Neurons, on Ability of Clonidine to Reverse Naloxone-Induced Conditioned Place Aversion in Morphine-Dependent Rats. Results are presented in Fig. 3. For each clonidine dose, D was compared with D0, S with S0, and N with N0 using the Wilcoxon paired test.

In sham-operated rats (Fig. 3a), doses up to Clo-100 were ineffective in reverting the naloxone effect, because statistically significant place aversion was observed at this dose (D/D0: Clo-0 and Clo-25, p < .001; Clo-50, Clo-100, p < .05). Only the higher dose (Clo-200 and Clo-400) was able to reverse completely place aversion conditioning (Wilcoxon, N.S.). As observed before (experiment 1) the avoidance of the naloxone-paired compartment was associated with a preference for the saline-paired compartment (Clo-0, T = −2.46, p < .05; Clo-25, T = −2.53, p < .05 and Clo-100, T = −2.76, p < .01). The time spent in the neutral compartment was not altered in any of the rats.

In NA-lesioned rats (Fig. 3b), the conditioned place aversion was reversed at all doses used (D/D0, Clo-25, and higher doses, Wilcoxon, N.S.). However, even when the aversion was reversed, NA-lesioned rats still preferred the saline-paired compartment up to the dose of Clo-50 (Clo-0, Clo-25, and Clo-50, Wilcoxon, p < .05 in each case); after this dose, the time spent

### Table 1

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### Table 2

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<td>hippocampus</td>
<td>325</td>
<td>21.4</td>
</tr>
<tr>
<td>medulla</td>
<td>192.8</td>
<td>69.9</td>
</tr>
<tr>
<td>striatum</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* p < .05, *p < .01, and *p < .001, versus sham-operated animals (two-tailed Student’s T test); N.D., not determined.
in the saline-paired compartment returned to the preconditioning level. Rats did not alter the time spent in the neutral compartment except for the dose Clo-0 ($T = 2.32, p < .05$).

These results suggest that clonidine can reverse naloxone-induced place aversion conditioning in both lesioned and sham-operated rats. Both groups allocated their times in the compartments in a similar way. The NA-LC-lesioned rats appeared, however, to be more sensitive than the sham-operated rats to clonidine.

**Experiment 4: Effect of Quasi-Total Lesion of NA-LC Neurons on Expression of Acute Opiate Withdrawal Syndrome in Morphine-Dependent Rats.** The significant changes in frequency and duration of the main patterns of opiate withdrawal syndrome are shown in Tables 2 and 3. In sham-operated rats, Kruskal-Wallis analysis showed that the following patterns were dose dependently modified in response to naloxone treatment: salivation, vocalization, rhinorhea, diarrhea, leaning, attenuated gait, body shake, jumping, mastication, weight loss, immobile sniffing, freezing, genital grooming, writhing, lying, Gellert and Holtzman score, and etho-score (for statistical significance see Table 2).

In NA-lesioned rats, Kruskal-Wallis analysis showed a dose-dependent naloxone-induced modifications of several opiate withdrawal responses: salivation, vocalization, rhinorhea, diarrhea, chromodacryorrhea, attenuated gait, body shake, mastication, weight loss, immobile sniffing, freezing,
body grooming, writhing, lying, Gellert and Holtzman score (G-H score), and etho-score (for statistical significance see Table 3).

In common with sham-operated rats, NA-lesioned animals were able to develop an acute dose-dependent naloxone-induced opiate withdrawal syndrome. Qualitative comparison of sham-operated with NA-lesioned rats showed subtle differences in some behavioral patterns. Chromodacryorrhea and body grooming were unchanged by naloxone in sham-operated rats, whereas leaning, jumping, and genital grooming were unaffected in the withdrawal response of the NA-lesioned rats. Moreover, immobile sniffing and writhing duration were differently affected by opiate withdrawal in sham and NA-lesioned rats. Surprisingly, NA-lesioned rats exhibited mastication frequency stronger than the sham for Nal-0, whereas it was identically increased in both groups for higher doses of naloxone.

When global scores were considered (G-H score and etho-score), maximal values were recorded for Nal-100; except for NA-lesioned rats G-H score for Nal-100, which was statistically lower than Nal-1000 (Kruskal-Wallis, $p < .05$). However, there was no difference between groups at both Nal-100 and Nal-1000 doses (Mann-Whitney, N.S.). Then, Nal-100 and Nal-1000 scores were similar for sham and NA-lesioned rats.

**Experiment 5: Effect of Quasi-Total Lesion of NA-LC Neurons on Reversal by Clonidine of Naloxone-Induced Acute Opiate Withdrawal Syndrome in Morphine-Dependent Rats.** The data are presented in Table 4. The Gellert and Holtzman opiate withdrawal rating scale showed again that, under Nal-1000, there were no differences between the NA-lesioned and the sham-operated groups for withdrawal scores (Kruskal-Wallis, N.S.) or for weight loss (Kruskal-Wallis, N.S.). NA-LC-lesioned rats exhibited rhinorrhea more frequently than did the sham-operated rats (Kruskal-Wallis, $H(1) = 7.5, p < .01$).

Although the results suggest that Clo-200 alone had an effect because for both groups the saline score was different from the Clo-200 score (Wilcoxon, sham, $T = -2.55, p < .01$; lesioned, $T = -2.70, p < .01$), the withdrawal score and weight loss under Clo-200 were significantly smaller than under Nal-1000 in both lesioned and sham-operated groups (sham score, $T = 2.53, p < .05$; lesioned score, $T = 2.67, p < .01$; sham weight loss, $T = 2.52, p < .05$; lesioned weight loss, $T = 2.07, p < .05$).

Lastly, Clo-200 treatment attenuated the opiate withdrawal syndrome in both groups because the withdrawal scores recorded under Nal-1000 + Clo-200 were lower than those under saline + Nal-1000 (Wilcoxon, sham, $T = 2.53, p < .05$; lesioned, $T = 2.66, p < .01$). Moreover, the results show that total lesion of NA-LC neurons had no effect on the antiw ithdrawal properties of Clo-200 because under Nal-1000 + Clo-200 there are no significant differences between NA-lesioned and sham-operated rats either in withdrawal score or weight loss (Kruskal-Wallis, N.S.). As shown previously, lesioned rats exhibited more rhinorrhea than did sham-operated rats (Kruskal-Wallis, $H(1) = 3.86, p < .05$).

**Experiment 6: Effects of Quasi-Total Lesion of NA-LC Neurons on Spontaneous Withdrawal to Morphine.** The results are presented in Fig. 4. To clarify the data presentation in Fig. 4, results were plotted in 6-h segments. The first day represents the mean activity recorded during a 4-day period (baseline activity) after habituation to the experimental condi-
tions. Sham-operated and NA-lesioned rats exhibited similar biphasic patterns of locomotor activity, with a high level at night and a low level during the day. ANOVA showed no difference either in overall activity (group effect, F(1,20) = 3.19, N.S.) or in rhythmicity (group × time interaction, F(3,60) = 0.41, N.S.).

The following 9 days represent the locomotor activity of rats immediately after abstinence from morphine. Statistical differences between two groups emerged from overall ANOVA of the 9-day abstinence period (group × day × time interaction, F(24,480) = 8.26, p < .001; lesioned, F(24,216) = 7.82, p < .001). When compared with baseline activity levels, sham-operated as well as NA-lesioned rats exhibited a disruption of circadian activity rhythm during the first 3 days of abstinence (day × time interaction, p < .001 for both groups). During this 3-day period, overall motor activity stabilized at a high level, comparable with the activity displayed during the dark phase of the baseline recording.

Rats of both groups slowly resumed a normal circadian cycle...
of activity after the fourth day postinjection. However, whereas activity of sham-operated rats rose until day 9 (days 7, 8, and 9, p < .05), overall locomotor activity was reduced in the 6-OHDA-treated rats (days 8 and 9, p < .05).

### Discussion

The aim of this study was to examine the effect of an almost total lesion of the NA-LC neurons on various components of the opiate withdrawal syndrome. The neurochemical analysis showed that 92 to 94% of the ascending noradrenergic innervation arising from LC had been destroyed in lesioned rats. In addition, 64% of the descending innervation had been destroyed at the level of the medulla. Quasi-total lesion of NA-LC neurons did not alter opiate dependence as revealed by naloxone-induced conditioned place aversion and the expression of an acute morphine withdrawal syndrome. Moreover, clonidine alleviated the aversive and physical components of the opiate withdrawal syndrome in both sham-operated and NA-LC-lesioned rats. Finally, the results indicated that morphine-dependent NA-lesioned rats also exhibited a disruption of circadian activity rhythm, as did the sham-operated rats during the spontaneous withdrawal phase.
The place aversion conditioning and the somatic syndrome rating have been shown to be reliable paradigms for studying the aversive and the somatic components of opiate withdrawal. Low doses of naloxone (3.7–15 µg/kg s.c.) elicit none or few somatic signs, whereas higher doses elicit most of the somatic withdrawal signs (Schulteis et al., 1994). Moreover, the place aversion paradigm allows the conditioning of a robust and long-lasting naloxone-induced place aversion in morphine-dependent rats that lasts for up to 8 weeks (Koob et al., 1997).

Total lesion of NA-LC neurons had the following effects on the behavioral and the somatic symptoms of opiate withdrawal. First, a strong place aversion in morphine-dependent rats was shown at the naloxone dose of 7.5 µg/kg without eliciting somatic signs. Results at 1.8 µg/kg could suggest an increased sensitivity to the opiate antagonist in NA-lesioned rats, however this was not confirmed by results obtained at 3.7 µg/kg. The same place aversion was observed in the NA-LC-lesioned morphine-dependent rats with the same dose of naloxone (7.5 µg/kg), without producing any somatic signs. This supports the hypothesis that the aversive component of the opiate dependence is mediated by limbic structures (Stinus et al., 1990) rather than by the LC activation. Moreover, the total lesion did not modify the strategy of avoidance chosen by morphine-dependent rats. Indeed, to avoid the naloxone-paired compartment, both groups of rats chose to go to the saline-paired compartment, which appeared safer to the rats than the previously once-visited neutral compartment. Finally, even with total lesion of NA-LC neurons, rats responded to conditioned stimuli.

In sham-operated rats, a dose-response naloxone-induced somatic withdrawal syndrome was observed, with a maximal intensity at the Nal-100 dose. Total lesion of NA-LC neurons did not block the acute somatic opiate withdrawal syndrome. In NA-LC-lesioned rats, withdrawal signs appeared gradually and the full syndrome was also observed at the Nal-100 dose.
dose. Indeed, in both groups, the global withdrawal scores (Gellert and Holtzman score and Etho-score) reached its maximal intensity at Nal-100. The physical opiate withdrawal was quantitatively comparable in both groups of rats, in agreement with other 6-OHDA-lesion (Thatcher-Britton et al., 1984) and DSP4-lesion studies (Christie et al., 1995) showing that LC lesion did not modify the symptomatology of opiate withdrawal. In recent studies, the occurrence of opiate withdrawal syndrome was also reported in rats bearing complete lesions of NA-LC neurons (DSP4; Delfs et al., 1997). Because both naloxone-induced place aversion conditioning and somatic signs of withdrawal still persist in NA-LC-lesioned rats, these results do not support the hypothesis of a direct functional relationship between opiate withdrawal and NA-LC neurons, and would tend to rule out the notion that the increased firing rate NA-LC neurons is responsible for the opiate withdrawal syndrome.

Another important objective of this study was to investigate the persistence of the antiwithdrawal properties of clonidine on naloxone-induced opiate withdrawal in NA-LC-lesioned rats. Clonidine, an $\alpha_2$-adrenergic agonist, is still used successfully in the treatment of opiate dependence in humans (Gold et al., 1978). Clonidine is able to suppress naloxone-induced increases in firing in noradrenergic neurons in morphine-dependent rats (Aghajanian, 1978). Because clonidine attenuated naloxone-induced place aversion conditioning in both morphine-dependent NA-LC-lesioned and sham-operated rats, the present results also do not support the current hypothesis that clonidine alleviation of opiate withdrawal is mediated through stimulation of $\alpha_2$-adrenergic autoreceptors. Moreover, these observations rather indicate that the protective effects of clonidine may be mediated via stimulation of postsynaptic $\alpha_2$-adrenoceptors because all forebrain NA neurons were destroyed. This is supported by the fact that NA-LC-lesioned rats were more sensitive to the protective properties of clonidine, because naloxone-induced place aversion conditioning was blocked at 25 mg/kg and above, whereas for the sham-operated rats, complete inhibition was only observed at the 200 mg/kg dose of clonidine. This difference in efficiency of clonidine could be explained by a denervation-induced supersensitivity (U'Prichard et al., 1980) of postsynaptic structures.

Similarly, the naloxone-induced acute opiate withdrawal syndrome was also attenuated by clonidine in both morphine-dependent NA-LC-lesioned and sham-operated rats. Indeed, the Gellert and Holtzman score was decreased to the same extent in both groups after treatment with clonidine. This confirms that the NA-LC neurons are not required for the action of clonidine and suggests that clonidine exerts antiwithdrawal effects via an action on postsynaptic $\alpha_2$-adrenoceptors (Thatcher-Britton et al., 1984). Interestingly, the aggressiveness associated with the opiate withdrawal syndrome was enhanced by clonidine in both NA-lesioned and sham-operated rats (Gianutsos et al., 1976).

Finally, our experiments showed that total lesion of NA-LC neurons did not protect rats from nycthemeral locomotor activity disruption induced by spontaneous opiate withdrawal. However, the NA lesion does appear to prevent the protracted effects of the withdrawal syndrome on locomotor activity, because the NA-lesioned rats did not exhibit the increase in activity on days 7, 8, and 9 that was observed in the sham-operated rats.

The purpose of this study was to verify the involvement of NA-CL neurons in the opiate withdrawal syndrome by a specific and complete neurochemical lesion. The biochemical analysis demonstrated a 92 to 94% depletion of ascending NA-LC and a total depletion of descending NA-LC. The 36% NA level remaining in the spinal cord derived from other NA nuclei that were not destroyed intentionally, such as the A5 ventral nucleus (Hökfelt et al., 1984) or the A1 and A2 medullary nuclei (Hökfelt et al., 1984), which have numerous local and descending connections. The absence of effects of this selective and total NA-LC neuron lesion argues against an important role in the expression of opiate withdrawal (Aghajanian, 1978; Maldonado, 1997).

Although the NA-LC do not appear to be responsible for the expression of opiate withdrawal syndrome, several studies point to a crucial role for the region surrounding the LC. A specific and marked increase in proto-oncogene $c$-fos immunoreactivity has been observed in this region during the opiate withdrawal syndrome (Hayward et al., 1990). Injection of methyl naloxonium into the LC region triggered a dramatic syndrome (Maldonado et al., 1992), and electrolytic lesion of this region was found to reduce certain withdrawal signs (Maldonado and Koob, 1993). However, such lesions damage other neuronal systems and fibers of passage, and these results could be interpreted in terms of a more direct involvement of the LC region than of the NA-LC neurons themselves. In view of these observations and the present findings, manifestations of element of the opiate withdrawal syndrome may hinge more on non-NA-LC projections such as NA from the region of the bed nucleus of the stria terminalis (Delfs et al., 1997) or on nonnoradrenergic neurons in the vicinity of the LC (Christie et al., 1997). For example, Barrington's nucleus is close to the LC (Koob et al., 1976). Divergent projections to NA-LC system and the spinal parasympathetic system from Barrington's nucleus have been identified and suggest a parallel activation of these structures (Valentino et al., 1996). Such a coregulation points to a role for Barrington's nucleus in coordinating autonomic system functions and forebrain activity. Other nonnoradrenergic structures that seem to be important are the spinal cholinergic neurons (Marshall and Buccafusco, 1987); periaqueductal gray neurons, which also exhibit specific $c$-fos activity during opiate withdrawal (Chieng et al., 1995) and which display a local opiate withdrawal effect in vitro (Chieng and Christie, 1996). Another important structure is the medullary nucleus paragigantocellularis, an excitatory acid afferent input to the LC (Akaoka and Aston-Jones, 1991; Christie et al., 1997), as well as to peripheral sympathetic nervous system (Loewy and McKellar, 1980) and is responsible for the coactivation of these systems (Svensson, 1987), which could be another pathway for coordination of autonomic functions and forebrain activity. In addition, certain non-NA-LC neurons may be a substrate for the opiate withdrawal syndrome, such as the NA neurons from the bed nucleus of the stria terminalis (Delfs et al., 1997), or the A2 noradrenergic solitary tract nucleus in view of its projections to the central amygdala nucleus (Zardetto-Smith and Gray, 1990), as well as several parts of the medulla (Halsell et al., 1996). This pathway may be involved in the regulation of cardiovascular, respiratory, and gastrointestinal activity during opiate withdrawal syndrome. In summary, neuroanatomical studies provide evidence that the paragigantocellu-
laris, Barrington’s nucleus, the noradrenergic solitary tract nucleus, and the rostral medulla (Van Bockstaele and Aston-Jones, 1992) coactivate the LC and the peripheral nervous system. This could explain why the NA-LC neurons are hyperactive during the opiate withdrawal syndrome (Aghajanian, 1978) and why the structure is not essential for the overt manifestations of withdrawal, because its absence did not modify the syndrome. In fact, the observed activation of the NA-LC neurons may be secondary to the activation of one or several of the above-mentioned afferent structures, which could trigger opiate withdrawal symptoms via their descending projections to the peripheral nervous system. This is consistent with findings on the contribution of the spinal cord to the opiate withdrawal syndrome (Rhode et al., 1997) and a spinal antiwithdrawal action of clonidine (Buccafusco, 1990). However, the exact role of each of these systems in the opiate withdrawal syndrome remains to be elucidated.

To summarize: 1) almost complete lesion of NA-LC neurons did not prevent morphine-dependent rats from developing a naloxone-induced conditioned place aversion and a naloxone-induced acute opiate withdrawal syndrome; 2) clonidine had antiwithdrawal activity in morphine-dependent NA-lesion rats, suggesting that clonidine blocks the withdrawal syndrome via an action on postsynaptic α2-adrenoceptors; and 3) the NA-lesioned rats showed the same disturbances in nycthemeral locomotor activity as did the sham-operated rats during spontaneous withdrawal. In conclusion, noradrenergic transmission from the LC does not appear as an essential component for the expression of opiate abstinence syndrome.

References