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Focal κ-opioid receptor-mediated dependence and withdrawal in the nucleus paragigantocellularis

S. Sinchaisuk, I.K. Ho, R.W. Rockhold*

Department of Pharmacology and Toxicology, The University of Mississippi Medical Center, 2500 N. State Street, Jackson, MS 39216-4505, USA

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Abstract

The nucleus paragigantocellularis (PGi) has been hypothesized to play an important role in the development of physical dependence on opioids, including the prototype μ-opioid receptor agonist, morphine, and the mixed agonist/antagonist, butorphanol, which shows selective κ-opioid receptor agonist activity, in rats. In confirmation of previous work, electrical stimulation of the PGi in opioid-naïve rats induced stimulus-intensity-related, withdrawal-like behaviors similar to those observed during naloxone-precipitated withdrawal from dependence upon butorphanol. Novel findings were made in rats surgically implanted with cannulae aimed at the lateral ventricle and the right PGi and made physically dependent by intracerebroventricular infusion of either morphine (26 nmol/μl/h) or butorphanol (26 nmol/μl/h) through an osmotic minipump for 3 days. Two hours following termination of the opioid infusion, microinjections of naloxone (11 nmol/400 nl), a nonselective opioid receptor antagonist, or nor-binaltorphimine (nor-BNI) (3.84 nmol/400 nl), a selective κ-opioid receptor antagonist, were made into the PGi of morphine-dependent and butorphanol-dependent rats. Discrete PGi injections precipitated withdrawal behaviors, with significant ($P < .05$) increases noted in the incidence of teeth chattering, wet-dog shakes, and scratching. Composite scores for behavioral withdrawal were significantly higher in nor-BNI-precipitated, butorphanol-dependent rats (score = 6.8 ± 0.6), in naloxone-precipitated, butorphanol-dependent rats (8.9 ± 0.8), and in naloxone-precipitated, morphine-dependent rats (11.5 ± 0.9) than in all other groups. Both κ- and μ-opioid receptor mediated dependence can be demonstrated at the level of a discrete medullary site, the PGi, which further supports a specific role for this nucleus in elicitation of behavioral responses during opioid withdrawal.

Keywords: Butorphanol; κ-Opioid receptor; Nor-binaltorphimine; Nucleus paragigantocellularis

1. Introduction

At present, it is well established that the pontine locus coeruleus plays a pivotal role in opioid dependence. Increased firing of locus coeruleus neurons is strongly correlated with many of the signs and symptoms of opioid withdrawal (Aghajanian, 1978; Koob et al., 1992; Nestler and Aghajanian, 1997). However, this increase in locus coeruleus neuronal activity during withdrawal from opioid dependence is not consistently seen in vitro in brain slices taken from opioid-dependent animals (Andrade et al., 1983; Kogan et al., 1992; Ivanov and Aston-Jones, 2001), suggesting that neuronal connections outside the locus coeruleus play an important role in the withdrawal-induced activation of these neurons. One major afferent projection to the locus coeruleus that has been implicated in opioid withdrawal behaviors arises from glutamatergic neurons in the nucleus paragigantocellularis (PGi). Evidence in favor of this hypothesis includes the fact that lesions of the PGi greatly attenuate the activation of locus coeruleus neurons during withdrawal from morphine dependence (Rasmussen, 1991). Pretreatment with kynurenic acid, a nonspecific antagonist at glutamate receptors, also greatly attenuated the activation of locus coeruleus neurons during withdrawal from morphine dependence (Rasmussen and Aghajanian, 1989; Tung et al., 1990). Electrical stimulation of the PGi has been demonstrated to induce opioid withdrawal-like behaviors in the rat (Liu et al., 1999a). Furthermore, using in vivo microdialysis, significant increases in extracellular glutamate within the locus coeruleus have been observed during withdrawal from dependence on either morphine or

* Corresponding author. Tel.: +1-601-984-1634; fax: +1-601-984-1637.
E-mail address: rrockhold@pharmacology.umsmed.edu (R.W. Rockhold).

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butorphanol (Aghajanian et al., 1994; Zhang et al., 1994; Feng et al., 1995). In previous studies from our laboratory, electrical stimulation of the PGi in opioid-naïve rats induced voltage- and frequency-dependent, opioid withdrawal-like behaviors similar to those seen during naloxone-precipitated withdrawal from dependence upon butorphanol. Increases in extracellular glutamate within the locus coeruleus were also observed during the electrical stimulation (Liu et al., 1999a,b). Recently, Saiepour et al. (2001) have shown that microinjection of naloxone into the PGi of morphine-dependent rats significantly increased neuronal activity of PGi neurons, indicating the occurrence of dependence on morphine in the PGi neurons and the probable role of μ-opioid receptors in such dependence. Butorphanol differs meaningfully from morphine in part because dependence upon butorphanol is uniquely a function of its agonist activity at the κ-opioid receptor (Jaw et al., 1993b, 1994; Feng et al., 1997). To better determine whether the κ-opioid receptor plays a significant role in opioid dependence elicited at the level of the PGi, behavioral responses were observed following microinjection of nor-binaltorphimine (nor-BNI, a specific κ-opioid receptor antagonist) or naloxone (a nonselective opioid receptor antagonist) into the PGi of morphine- and butorphanol-dependent rats.

2. General methods and procedures

2.1. Acquisition, housing and care

Adult male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) weighing 300 to 350 g were used in all experiments. Aseptic techniques were employed during all surgical procedures. Animals were housed prior to surgical intervention in plastic group cages (three rats per cage). Following surgery, each rat was housed individually in a plastic cage. All rats were kept in IACUC-approved animal facilities and maintained under controlled conditions of temperature (22–24 °C), light cycle (light period of 0800–2000 h), and humidity (50–55%). Surgical procedures were performed under halothane (2.5% halothane in medical-grade oxygen) anesthesia. Postoperative analgesia was routinely provided to each animal by infiltration of the wound site with Sensorcaine (0.5% bupivacaine with 1:200,000 epinephrine). Animals were weighed, and wound sites inspected and cleaned on a regular basis. Experiments were performed 6–10 days following surgical intervention. Subsequently, rats were sacrificed by decapitation and their brains were examined using histological methods. All experimental protocols were reviewed and approved by the University of Mississippi Medical Center IACUC.

2.2. Stereotaxic manipulations

Animals were anesthetized with halothane (2.5% halothane in medical grade oxygen). The dorsal surface of the head of each rat was shaved and scrubbed with 10% povidone iodine solution. Rats were then placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). A midline dorsal skull incision was made and the soft tissue overlying the skull was removed. Three stainless screws were placed in the skull. The skull landmarks, bregma and lambda, were identified and the skull was oriented such that both landmarks were positioned at the same horizontal level. A burr hole was then made through the skull over coordinates corresponding to the site of interest. The coordinates were estimated from the rat brain atlas of Paxinos and Watson (1998). Coordinates for implantation of a bipolar, concentric electrode (SNEX-100 × 20 mm, David Kopf) unilaterally into the PGi were: posterior 14.0 mm, lateral 1.6 mm from bregma and 9.5 mm ventral to the skull surface. Coordinates for implantation of a guide cannula (21-gauge, stainless steel, 10 mm in length) for intracerebroventricular (icv) infusion were: posterior −0.5 mm, lateral 1.3 mm from bregma and 4.5 mm ventral to the skull surface. Coordinates for implantation of a guide cannula (26-gauge, stainless steel, 10 mm in length) for unilateral injection of chemicals into the PGi were: posterior 14.0 mm, lateral 1.6 mm from bregma and 5.5 mm ventral to the skull surface. The actual microinjection cannula (32-gauge, stainless steel, 13.5 mm in length) was inserted into each guide cannula to a depth of 9.5 mm ventral to the skull surface. A stylet was inserted to seal each cannula until used. After implantation, all guide cannulae and electrodes were firmly fixed to the skull with dental acrylic cement (Lang Dental, Wheeling, IL).

2.3. Histology

Verification of the placement of cannulae and electrodes was performed following sacrifice of animals upon completion of the experiments. Each brain was carefully removed, frozen in liquid nitrogen, and mounted in a cryostat. Serial 40-μm sections were cut and mounted on glass slides. Sections were stained with cresyl violet and examined under the light microscope. The locations of cannulae and electrodes were plotted using a microprojector (Bausch & Lomb) and normalized to representative tracings from a standard brain atlas (Paxinos and Watson, 1998).

2.4. Osmotic minipump preparation and implantation

Opioid dependence was induced in each animal by icv infusion of either morphine sulfate (26 nmol/μl/h) or butorphanol tartrate (26 nmol/μl/h) for 3 days through an osmotic minipump (Alzet 2001, Alza, Palo Alto, CA). Both the infusion period and dose paradigm were determined according to previous studies (Horan and Ho, 1991; Jaw et al., 1993a,b). Before introduction into the pump, the solution was passed through 0.2-μm sterile Acrodisk filters (Gelman Science, Ann Arbor, MI). The minipump was primed overnight at room temperature by immersion in sterile normal

saline so that the nominal pumping rat (1 μl/h) was achieved prior to implantation.

Animals were re-anesthetized with halothane (2.5% halothane in medical-grade oxygen) 6–10 days following implantation of guide cannulae. An osmotic minipump was inserted into a subcutaneous pocket through a midline skin incision between the scapulae. A 4-cm piece of Tygon tubing (0.38-mm inner diameter, Cole-Palmer, Chicago, IL) was used to connect the outlet of the minipump to a piece of L-shape stainless steel injector tubing (26-gauge, 30 mm long) equal to length of the guide cannula.

2.5. Intracerebral infusion

All infusions were made unilaterally into the right PGi. Each rat was gently restrained by hand and the stylet removed from the PGi guide cannula. The 32-gauge injection cannula, prefilled with the drug of interest, was then inserted into the guide cannulae. The tip of the microinjection cannula was directed to terminate in the PGi (4.0 mm beyond the tip of guide cannula, 9.5 mm from the skull surface). The microinjection cannula was attached to a 10-μl glass Hamilton syringe by PE-20 tubing filled with the drug or vehicle solution. The volume infused was 400 nl delivered over 2-min period. After the infusion, the microinjection cannula was left in place for an additional 3 min to reduce leakage of the drug up the microinjection cannula track.

2.6. Diffusion pattern of naloxone

To estimate the extent of diffusion of naloxone after PGi microinjection, a radioisotope method was used. A working solution was made by mixing tritium-labeled naloxone (*N*-allyl-2,3-^{3}H naloxone, 53.1 Ci/mmol, New England Nuclear, Boston, MA) with naloxone (Sigma Chemical, St. Louis, MO) to a concentration of 10 mg/ml, which is identical to the working concentration of unlabeled naloxone used in all other PGi injection experiments. Microinjection of the solution into the PGi was carried out the same way as previously described. Four hundred nanoliters of the solution was microinjected unilaterally into the PGi over a 2-min period and an additional minute was allowed to elapse before the microinjection cannula was removed. Rats were then sacrificed by decapitation; the brain was removed and frozen in liquid nitrogen immediately (requiring approximately 7 min). Frozen 200-μm sections were cut in either the sagittal, coronal, or horizontal planes. The sections were solubilized in 1 ml Protosol (New England Nuclear) by shaking overnight in plastic vials. Ten milliliters of scintillation cocktail was added and contents of the vials were equilibrated by shaking for 48 h. The radioactivity then was

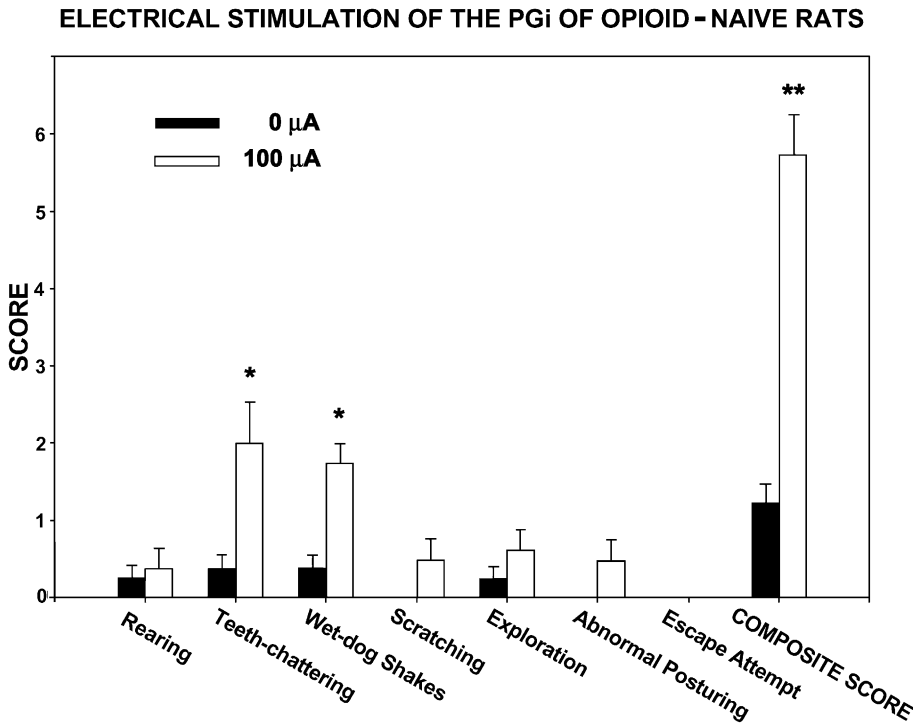

Fig. 1. Electrical stimulation of the PGi (0.5 Hz, 1 ms) was performed on a single group of animals ($n=8$). Each animal received two separate trials during which the PGi was stimulated with either 0 (control) or 100 μA (stimulated). The behavioral responses were observed for 30 min. Data are expressed as mean values of opioid withdrawal behaviors ± S.E.M. The Wilcoxon test was used for comparison between the two groups. * $P<.05$ and * * $P<.01$. (The asterisks denote statistical differences between control and PGi-stimulated groups.)

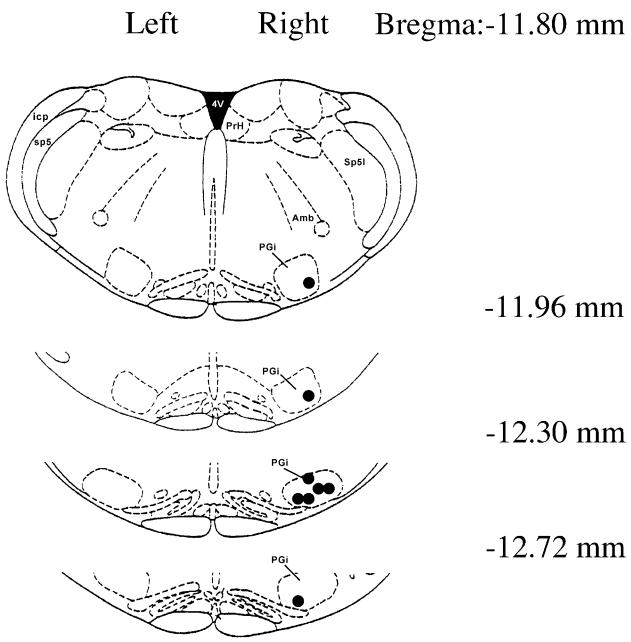

Fig. 2. Diagrams of coronal sections of the medulla showing the sites of electrical stimulation of the PGi of opioid-naïve rats. The figures are redrawn after Paxinos and Watson (1998). The anterior–posterior level of each figure, in millimeters relative to skull landmark, bregma, is noted to the upper right of each line drawing. Each dot in the PGi represents one stimulation site.

measured by a liquid scintillation analyzer (model 2500TR, Packard Instrument, Meriden, CT).

2.7. Assessment of stereotyped behaviors

Animals were placed individually in a clean plastic observation bowl (35 cm in diameter and 35 cm in height, Bioanalytical Systems, West Lafayette, IN) and acclimated for at least 1 h prior to behavioral assessment. Ten distinct behaviors (rearing, exploration, teeth chattering, wet-dog shakes, scratching, escape attempts, abnormal posturing, ptosis, diarrhea, and penis licking) were scored during a 30-min period of PGi stimulation. The withdrawal signs to be measured were defined as follows: rearing, an animal stands on its hindpaws with the forepaws off the bedding; exploration, an animal circles around the cage, thrusting its head in several directions and examining its surroundings; teeth chattering, an animal vigorously moves its jaws together in chewing movement and chatters his teeth together audibly; wet-dog shakes, an animal shakes his head, neck and body vigorously; scratching, an animal puts his forepaws up and scratches the back of its neck or the top of the head; abnormal posturing, an animal presses his abdomen and lower jaw against the floor of the cage; escape attempts, an animal looks apprehensive and attempts to escape from the cage by climbing the walls; ptosis, an animal drops his upper eye lid(s), but the animal

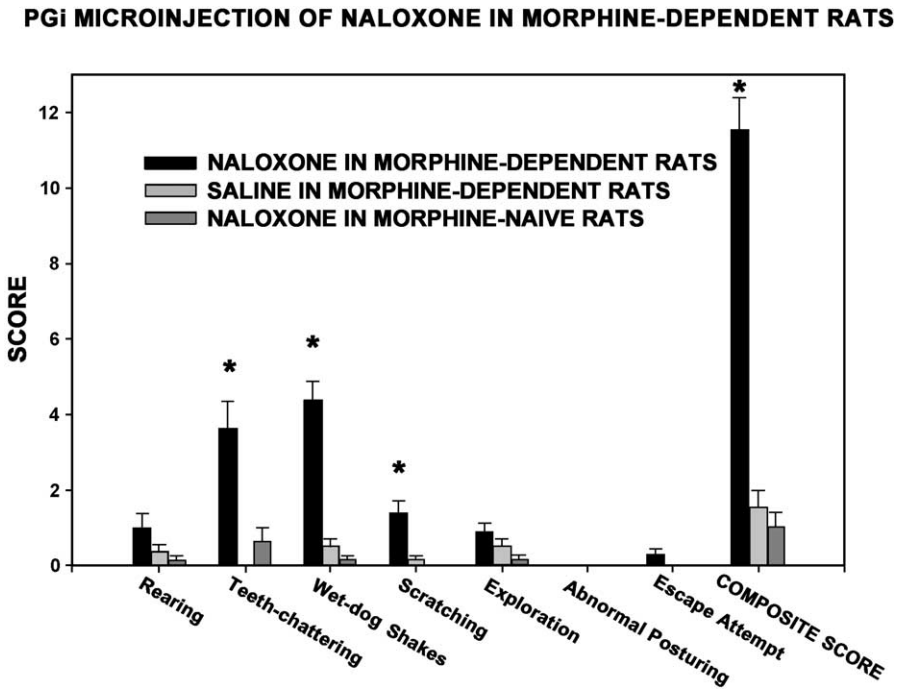

Fig. 3. Animals received 3 days of icv infusion of either morphine (26 nmol/μl/h; $n=8$) or saline (1 μl/h; $n=8$). Two hours following termination of the opioid infusion, unilateral microinjection of naloxone (11 nmol/400 nl) or saline (400 nl) into the PGi was carried out over a 2-min period and behavioral responses were observed for 30 min. Data are expressed as mean values of opioid withdrawal behaviors ± S.E.M. The Kruskal–Wallis test followed by a nonparametric Student–Newman–Keuls test was used for comparisons of opioid withdrawal behaviors among groups. * $P<.05$. (The asterisks denote statistical differences between control and naloxone-precipitated, morphine-dependent groups.)

Table 1
Site specificity for naloxone injection

Behaviors	Microinjection of naloxone in morphine-dependent rats	
	Within the PGi ($n=8$)	Outside the PGi ($n=5$)
Rearing	1.00 ± 0.378	0.000 ± 0.000
Teeth chattering	3.625 ± 0.730 **	0.000 ± 0.000
Wet-dog shakes	4.375 ± 0.498 **	0.000 ± 0.000
Scratching	1.375 ± 0.324 *	0.200 ± 0.200
Exploration	0.875 ± 0.227	0.400 ± 0.245
Abnormal posturing	0.000 ± 0.000	0.400 ± 0.400
Escape attempt	0.250 ± 0.164	0.000 ± 0.000
Composite score	11.500 ± 0.866 **	1.000 ± 0.775

Animals received 3 days of icv infusion of morphine (26 nmol/μl/h). Two hours following termination of the opioid infusion, unilateral microinjections of naloxone (11 nmol/400 nl) *into* the PGi or *outside* the PGi were made over a 2-min period and behavioral responses were observed for 30 min. Data are expressed as mean values of opioid withdrawal behaviors ± S.E.M. The Mann–Whitney rank sum test was used for comparison of opioid withdrawal behaviors between these two groups. The asterisks denote statistical differences at the indicated levels between these two groups.

* $P<.05$.

** $P<.01$.

does not sleep; diarrhea, an animal passes soft and wet fecal pellets onto the bedding; penis licking, an animal licks its penis.

The following rating scales were used to assess the incidence of each of the first seven types of stereotyped behavior; 0 = *not displayed*, 1 = *1–5 episodes of a behavior*, 2 = *6–10 episodes of a behavior*, 3 = *11–15 episodes of a behavior*, 4 = *16–20 episodes of a behavior*, 5 = *21 or more episodes of a behavior*. In the case of quantal (all or none) behavioral signs (ptosis, diarrhea, and penis licking), only a single episode of the behaviors had to be displayed. The composite score for each animal was calculated as the sum of scores for each behavior in that rat. Behavioral responses were evaluated by an observer without knowledge of the nature of the treatment received by each animal.

2.8. Electrical stimulation procedures

On the evening prior to an experiment, each animal was anesthetized with halothane (2.5% halothane in medical-grade oxygen). Two male connectors were soldered to the leads of each stimulation electrode. Animals were then allowed to recover from anesthesia overnight. The next morning, animals were connected to an electrical stimulator (model A365, World Precision Instruments, Sarasota, FL) through two female connectors. Each animal was placed individually in an observation bowl and acclimated for at least 1 h prior to behavioral measurement.

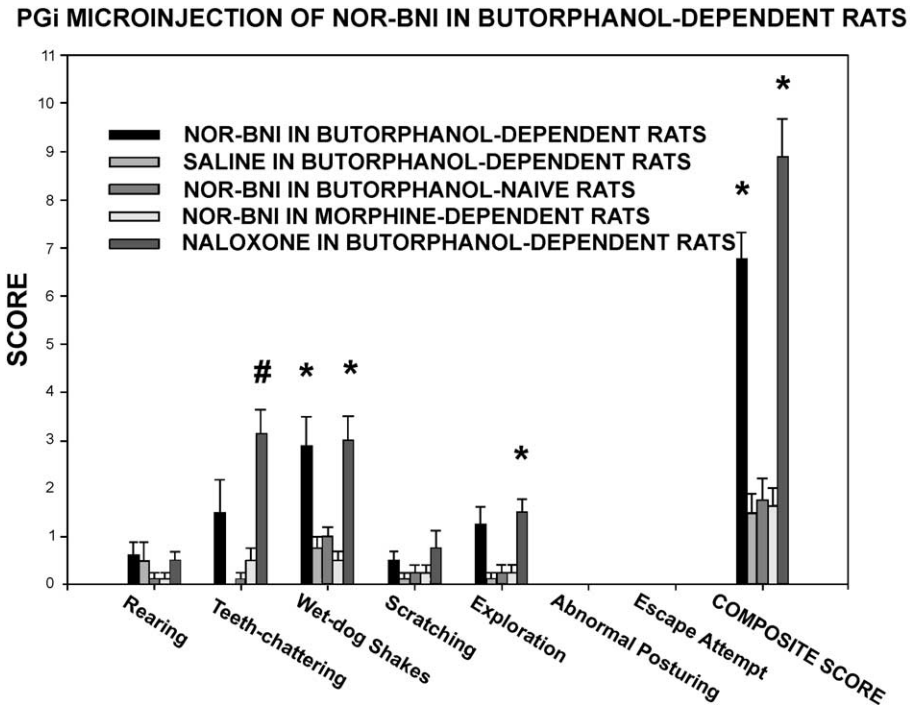

Fig. 4. Animals received 3 days of icv infusion of either butorphanol (26 nmol/μl/h; $n=8$), morphine (26 nmol/μl/h; $n=8$), or saline (1 μl/h; $n=8$). Two hours following termination of the opioid infusion, unilateral microinjection of nor-BNI (3.84 nmol/400 nl) or saline (400 nl) into the PGi was carried out over a 2-min period and behavioral responses were observed for 30 min. Data are expressed as mean values of opioid withdrawal behaviors ± S.E.M. The Kruskal–Wallis test followed by a nonparametric Student–Newman–Keuls test was used for comparisons of opioid withdrawal behaviors among groups. * $P<.05$ (The asterisks denote statistical differences between the control group and either the nor-BNI-precipitated, butorphanol-dependent or the naloxone-precipitated, butorphanol-dependent). # $P<.05$. (The asterisks denote statistical differences between the naloxone-precipitated, butorphanol-dependent group and all other groups including the nor-BNI-precipitated, butorphanol-dependent group.)

2.9. Protocols

Electrical stimulation of the PGi (0.5 Hz, 1 ms) was performed on a single group of eight opioid-naïve rats. Each animal received two separate trials during which the PGi was stimulated with either 0 (control) or 100 μA (stimulated). The animals were given a 30-min run of unilateral PGi stimulation, during which behavioral responses were observed and scored. A minimum of 24 h elapsed between consecutive stimulation runs in any animal.

A second set of animals was randomly divided into three groups (eight animals per group) for determination of the effect of microinjection of naloxone into the PGi of morphine-dependent and morphine-naïve rats. All animals were treated chronically with morphine (26 nmol/μl/h) or saline (1 μl/h) via osmotic minipump for 3 days. On the test day, 2 h following termination of opioid infusion, a microinjection of naloxone (11 nmol/400 nl, a nonselective opioid receptor antagonist), or saline (400 nl) was made into the PGi. Behavioral responses were observed for 30 min.

A third set of animals was randomly divided into five groups (eight animals per group) for determination of the effects of microinjection of naloxone and nor-BNI into the PGi of butorphanol- and morphine-dependent rats. All animals were treated chronically with morphine (26 nmol/μl/h), butorphanol (26 nmol/μl/h), or saline (1 μl/h) via osmotic minipump for 3 days. On the test day, 2 h following termination of opioid infusion, microinjection of naloxone (11 nmol/400 nl), nor-BNI (3.84 nmol/400 nl, a selective κ-opioid receptor antagonist), or saline was made into the PGi. The dose of naloxone used in this study was similar to doses used in a study done by Saiepour et al. (2001). The dose of nor-BNI was the same as that used in several studies done by our laboratory (Feng et al., 1997; Jaw et al., 1994). Behavioral responses were observed for 30 min.

2.10. Statistical analyses

Rating scale scores for behavioral assessments were subjected to nonparametric analyses. The Kruskal–Wallis

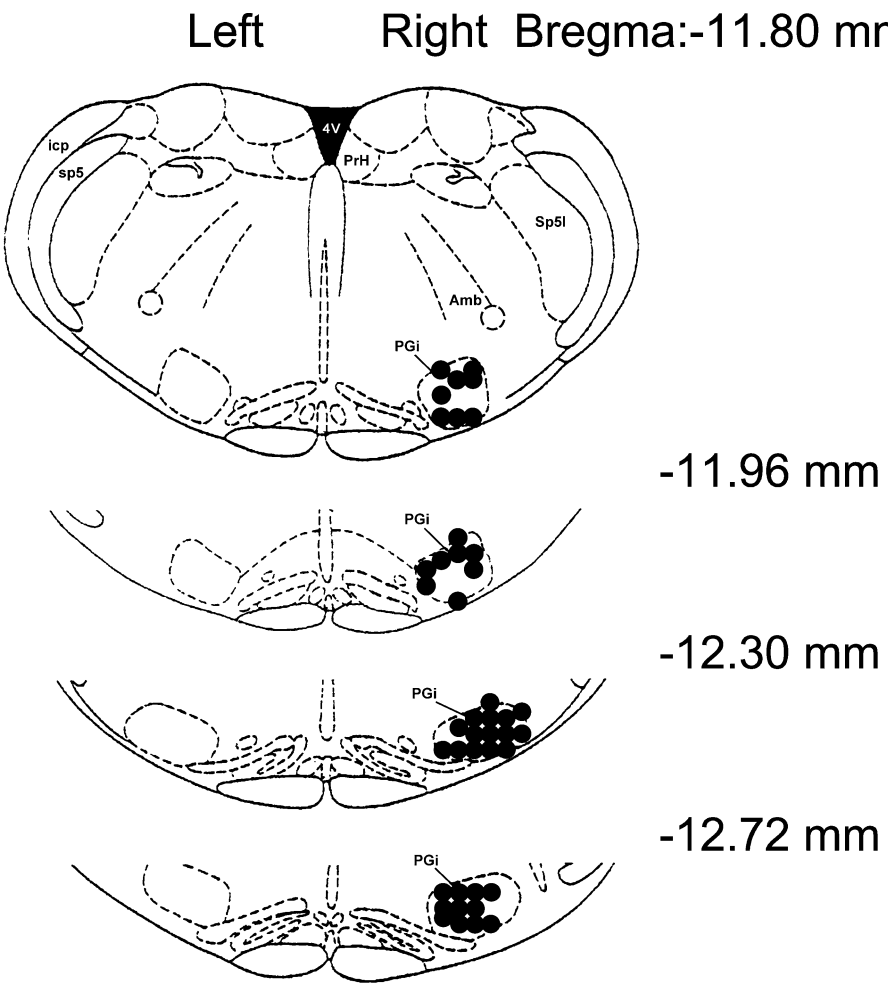

Fig. 5. Diagrams of coronal sections of the medulla showing the sites of microinjection of nor-BNI, naloxone, or saline into the PGi of butorphanol-dependent, morphine-dependent, or opioid-naïve rats. The figures are redrawn after Paxinos and Watson (1998). The anterior–posterior level of each figure, in millimeters relative to the skull landmark, bregma, is noted to the upper right of each line drawing. Each dot in the PGi represents one microinjection site. Some dots cannot be seen due to overlay of one by another.

test, followed by a nonparametric Student–Newman–Keuls test, was used for multiple comparisons. Comparisons between two groups were made using either the Wilcoxon signed rank test (for pairwise tests) or the Mann–Whitney rank sum test (for unpairedwise tests). Mean values ± 1 S.E.M. are presented. Calculated values of $P < .05$ were considered statistically significant.

3. Results

3.1. Effect of electrical stimulation of the PGi of opioid-naive rats

Unilateral constant current electrical stimulation of the PGi (100 μA) of opioid-naïve rats was found to induce several withdrawal-like behaviors when compared with 30-min observation periods during which no electrical stimulation (0 μA) was applied, as shown in Fig. 1. Statistically significant ($P < .05$) increases in the incidence of teeth chattering and wet-dog shakes were observed. The composite score was also significantly increased ($P < .01$) when compared to that obtained during the no-stimulation period. No incidents of ptosis, diarrhea, and penis licking were observed. The anatomical location of effective stimulation sites is provided as Fig. 2. Only data from those animals in which the electrode tip was found to reside within the anatomical borders of the PGi were used for analysis. Previous studies have demonstrated the anatomical specificity of this behavioral response (Liu et al., 1999a).

3.2. Effect of microinjection of naloxone into the PGi of morphine-dependent rats

To follow up the observation that electrical stimulation of the PGi can induce withdrawal-like behaviors and to examine the involvement of local opioid receptors within the PGi in such behaviors, discrete microinjections of naloxone (11 nmol/400 nl) were made into the PGi of three groups of rats. As shown in Fig. 3, unilateral microinjection of naloxone into the PGi of morphine-dependent rats precipitated withdrawal symptoms. Statistically significant increases were observed in three individual signs (teeth chattering, wet-dog shakes, and scratching) as well as in the composite score ($P < .05$) when compared with morphine-dependent rats that received equivalent volumes of saline or morphine-naïve rats that received identical naloxone injections. No incidents of ptosis, diarrhea, and penis licking were observed. Injection sites from these experiments are depicted graphically in Fig. 5. Table 1 specifically compares the behavioral scores in eight morphine-dependent rats in which the injection cannulae were found to terminate within the PGi and five in which the cannulae terminated outside the borders of the PGi. No opioid-withdrawal-like behaviors were noted in those animals whose cannulae terminated outside the PGi.

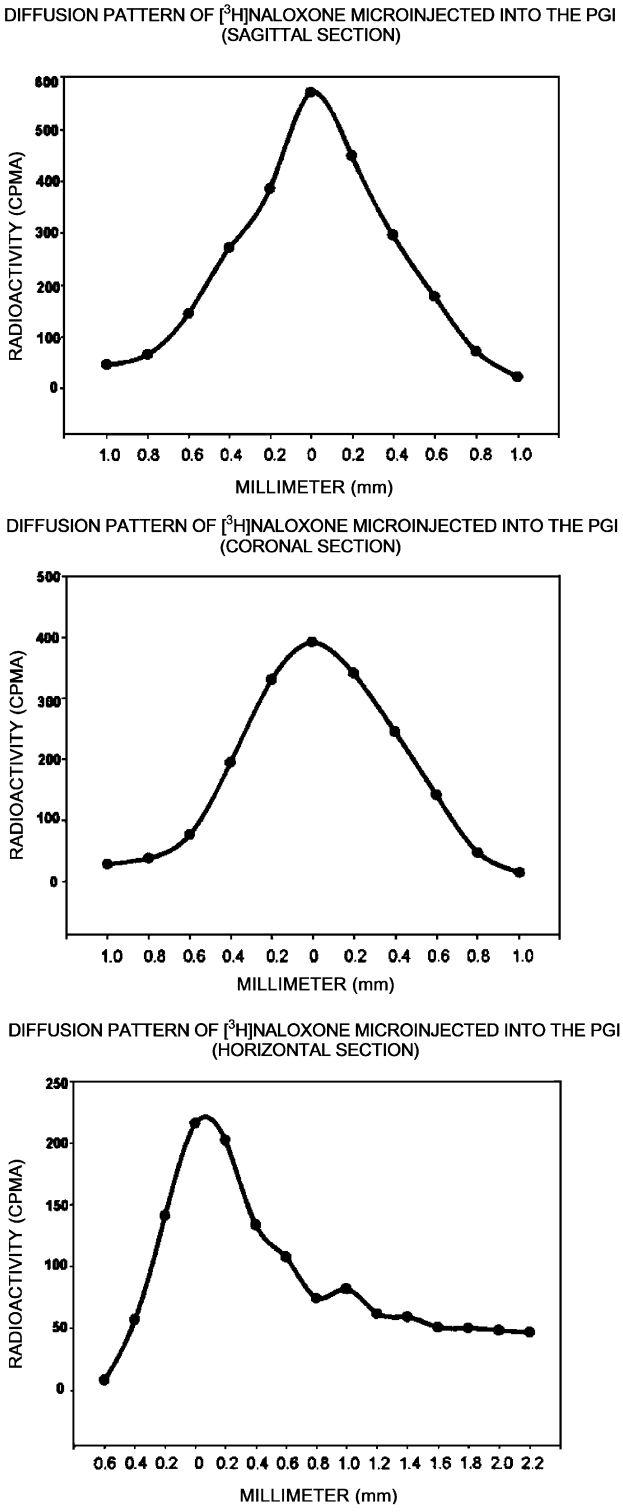

Fig. 6. Diffusion pattern of [^{3}H]naloxone 10 min after injection into the PGi in the sagittal, coronal, and horizontal planes. The *x*-axis represents distances (in millimeters) away from the injection site (0 millimeters). The *y*-axis represents detected radioactivity in brain sections.

3.3. Effect of microinjection of naloxone and nor-BNI into the PGi of butorphanol-dependent rats

To selectively examine the involvement of local κ-opioid receptors within the PGi in opioid withdrawal, microinjections of naloxone (11 nmol/400 nl) and nor-BNI (3.84 nmol/400 nl), a selective κ-opioid receptor antagonist, were made into the PGi in five experimental groups: butorphanol-dependent/saline; butorphanol-dependent/naloxone; butorphanol-dependent/nor-BNI; butorphanol-naïve/nor-BNI; and morphine-dependent/nor-BNI. As shown in Fig. 4 and similarly to naloxone-elicited responses in morphine-dependent rats, unilateral microinjection of both naloxone and nor-BNI into the PGi of butorphanol-dependent rats precipitated withdrawal symptoms. Naloxone injection increased the incidence of teeth chattering, wet-dog shakes, and exploration only in butorphanol-dependent rats, while nor-BNI injection elicited increases in wet-dog shakes only in the butorphanol-dependent group. In both naloxone- and nor-BNI-injected butorphanol-dependent rats, the composite score was significantly increased ($P < .05$). No increases in the incidence of any sign or in the composite score was noted in any other group. No incidents of ptosis, diarrhea, and penis licking were observed. Fig. 5 shows the location of cannula tips in this experiment.

3.4. Diffusion pattern of naloxone

To better identify the anatomical specificity of drugs applied into the PGi by microinjection, the diffusion pattern of naloxone was examined. As shown in Fig. 6, naloxone diffused away from the injection site in a symmetrical, roughly bell-shaped fashion in both the sagittal and coronal planes. In the horizontal plane, there was additional diffusion back up the cannula track in the dorsal direction. Totals of 79%, 60%, and 32% of the ^{3}H label were found to be contained within ± 0.6 mm from the injection site in the sagittal, coronal, and horizontal planes, respectively.

3.5. Behavioral response to microinjection of naloxone outside the PGi of morphine-dependent rats

To further rule out possible false-positive results that might occur in microinjection studies because drugs injected into a desired brain region diffused to neighboring regions, the results of microinjections that were misdirected into regions outside the anatomical boundaries of the PGi were evaluated. The tips of microinjection cannulae of five animals were found to be placed outside the PGi (Fig. 7). As shown in Table 1, animals in which microinjections were made outside the PGi showed a significantly lower incid-

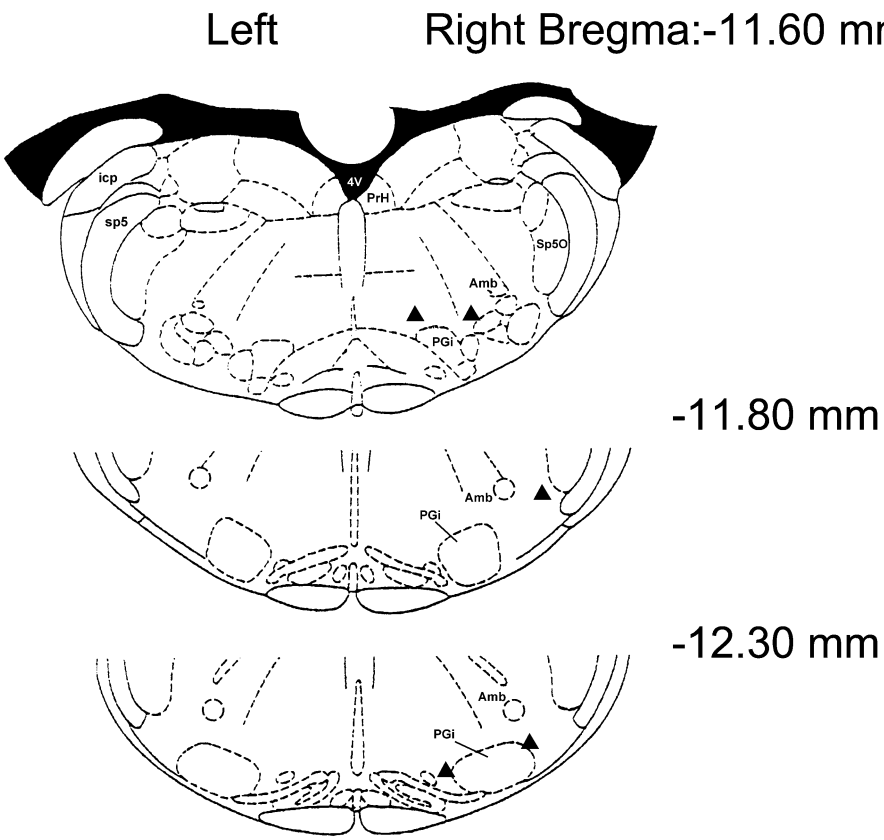

Fig. 7. Diagrams of coronal sections of the medulla showing the sites of microinjection of naloxone *outside* the PGi of morphine-dependent rats. The figures are redrawn after Paxinos and Watson (1998). The anterior–posterior level of each figure, in millimeters relative to the skull landmark, bregma, is noted to the upper right of each line drawing. Each dot represents one microinjection site.

ence of behaviors (composite score of 1.0 ± 0.8) when compared with animals in which microinjections were placed within the PGi (composite score of 11.5 ± 0.9). There were also significant differences in the incidences of teeth chattering, wet-dog shakes, and scratching between these two groups.

4. Discussion

The concept that dependence upon and withdrawal from morphine can occur in discrete neurons (Chieng and Christie, 1996) and small groups of neurons within the central nervous system (Haghparast et al., 1998; Kimes et al., 1998; Maldonado et al., 1992; Saiepour et al., 2001) is well established. Studies from this laboratory have provided evidence that, at the level of the locus coeruleus, such dependence upon morphine is, not surprisingly, mediated primarily through the μ- and, to a lesser extent, δ-opioid receptors (Feng et al., 1996). In contrast, dependence upon the mixed agonist/antagonist, butorphanol (Stadol) is uniquely dependent upon interactions with κ-opioid receptors (Feng et al., 1997). The results of the present study provide direct support for a discrete involvement of κ-opioid receptors, at the level of the medullary PGi, in the physical dependence produced by administration of butorphanol. Specifically, the results have shown that discrete tissue application of the κ-opioid receptor antagonist, nor-BNI, into the PGi can precipitate opioid withdrawal behaviors in conscious, freely moving butorphanol-, but not morphine-dependent, rats.

Earlier reports had shown that discrete electrical stimulation of the PGi could, in opioid-naïve rats, evoke a series of opioid withdrawal-like behaviors similar to those observed during naloxone-precipitated withdrawal from dependence upon butorphanol (Liu et al., 1999a). In confirmation and extension of that body of evidence, the present results demonstrated a current-intensity-dependent evocation of withdrawal-like behaviors following unilateral electrical stimulation with constant current from 0 to 125 μA (data not shown). The maximal effect (composite behavioral score of 5.8) was observed at 100–125 μA. The threshold for the stimulation was 6.25 μA (composite score of 4.8, $P < .05$ from no stimulation with composite score of 1.2). As noted in the previous study (Liu et al., 1999a) and consistent with the known parallel, ipsilateral projections from PGi to the locus coeruleus (Aston-Jones et al., 1991), a much higher incidence of withdrawal-like behavior can be elicited following bilateral stimulation of the PGi than with unilateral stimulation. Thus, the relatively low maximal incidence of evoked behaviors following unilateral stimulation is not surprising.

A unilateral approach to stimulation of, or injection into, the PGi was used primarily to reduce the technical complexity of experimentation in these conscious, freely moving animals. Each animal had received an indwelling icv cannula, as well as a PGi implant. Implantation and use of a second PGi implant increases stress on the animal, increases the potential for postsurgical morbidity, and increases the likelihood of experimental error in placement of cannulae equally into both sides of the PGi. As noted above, previously published data from bilateral versus unilateral electrical stimulation support the viability of such a unilateral approach.

To more clearly define the role played by different opioid receptors within the PGi, discrete microinjections of non-selective (naloxone) and κ-opioid receptor selective (nor-BNI) antagonists were made both in opioid-naïve rats and in rats dependent upon either morphine or butorphanol. Administration of naloxone into the PGi of rats made dependent upon the prototypical opioid agonist, morphine, were noted to elicit significant increases in withdrawal-like behaviors. This effect was not seen following injection of naloxone into morphine-naïve rats or following saline vehicle injection into morphine-dependent rats. Both Saiepour et al. (2001) and Haghparast et al. (1998), using single unit recording techniques in urethane-anesthetized rats, noted the development of tolerance to, dependence upon, and naloxone-precipitated withdrawal from morphine in individual PGi neurons.

Butorphanol is a mixed agonist/antagonist that binds to μ-, δ-, and κ-opioid receptors with an affinity ratio of 1:4:25 (Chang et al., 1983). Whereas the principal activity at μ-opioid receptors is as a partial agonist, butorphanol is a very efficacious κ-opioid receptor agonist, and much of the dependence upon butorphanol is mediated through the κ-opioid receptor (Hoshi et al., 1997; Jaw et al., 1994; Wongchanapai et al., 1999). A series of previous studies from this laboratory have demonstrated a unique κ-opioid receptor component to dependence upon butorphanol, as opposed to morphine. In those investigations, rats were made dependent upon either morphine or butorphanol and both extracellular glutamate concentrations in the locus coeruleus and behavioral signs of withdrawal were monitored following precipitation of withdrawal by icv administration of antagonists selective for the μ-(the peptide, CTOP), δ-(naltrindole), or κ-(nor-BNI) opioid receptors. The greatest degree of withdrawal (increased glutamate levels within the locus coeruleus and behavioral signs) could be elicited in morphine-dependent rats by CTOP, the μ-opioid receptor antagonist, and to a lesser degree by naltrindole, the δ-opioid receptor antagonist. No withdrawal phenomena were evident following administration of nor-BNI to morphine-dependent rats. In contrast, the degree of withdrawal precipitated by either CTOP or naltrindole was less in butorphanol- than in morphine-dependent rats. Importantly, nor-BNI administration precipitated robust withdrawal phenomena in butorphanol-dependent rats while eliciting no withdrawal phenomena from morphine-dependent animals (Feng et al., 1996, 1997). Since a majority of extracellular glutamate within the locus coeruleus is believed to originate from terminal projections from the

PGi (Liu et al., 1999b; Rasmussen, 1991), these data at least partially implicate opioid receptors at the level of the PGi in mediation of withdrawal phenomena and suggest a unique involvement of κ-opioid receptors in the withdrawal seen in butorphanol-dependent rats.

Data from the present study demonstrate even more clearly that behavioral withdrawal from dependence upon either morphine or butorphanol can be precipitated locally from within the PGi. Moreover, local PGi injection of a κ-opioid receptor antagonist can precipitate behavioral withdrawal uniquely in butorphanol-dependent, as opposed to morphine-dependent, rats. These results are also consistent with findings that microinjection of nor-BNI into the locus coeruleus precipitated withdrawal in butorphanol- but not in morphine-dependent rats (Hoshi et al., 1997) and that icv injection of nor-BNI precipitated withdrawal behaviors similar to those precipitated by naloxone in butorphanol-dependent rats (Jaw et al., 1994). However, the results with naloxone-precipitated withdrawal in butorphanol-dependent rats are in contrast with a study done by Cowan et al. (1988), in which little evidence for naloxone-precipitated withdrawal in rats chronically treated with the selective κ-opioid receptor agonist, U50,488H, was observed. The discrepancy between our findings and those of Cowan et al. (1988) may be explained by the fact that although a relatively high dose of naloxone (3 mg/kg sc) was used by Cowan et al. (1988), the dose of naloxone at focal sites within the CNS where interaction between naloxone and opioid receptors takes place can be expected to be lower. Thus, the local tissue concentration of naloxone is likely to have been much lower than that achieved in our study. Naloxone is known to have a 10-fold greater affinity for the μ-opioid receptor than other opioid receptors, especially the κ-opioid receptor (Reisine and Pasternak, 1996). In the study by Cowan et al. (1988) the dose of naloxone used may have been high enough to trigger withdrawal signs mediated by μ-opioid receptors, but not those mediated by actions at κ-opioid receptors. In contrast, the dose of naloxone used in our study was great enough to trigger responses mediated by both μ- and κ-opioid receptors within the PGi, as evidenced by the fact that microinjection of naloxone into the PGi of both morphine and butorphanol-dependent animals produced withdrawal signs.

It should also be noted that whereas Cowan et al. (1988) were unable to demonstrate significant abstinence responses to infusion of U50,488H into the sylvian aqueduct of rats, several publications from this laboratory have demonstrated the development of dependence upon, and the expression of withdrawal from, icv administration of both butorphanol (Horan and Ho, 1991; Jaw et al., 1994; Feng et al., 1996, 1997) and the more selective κ-opioid receptor agonist, U69,593 (Hoshi et al., 1996, 1997).

In the case of butorphanol, both μ- and δ-opioid receptors contribute to dependence/withdrawal (Feng et al., 1996), although the unique involvement of κ-opioid receptors has been repeatedly established (Jaw et al., 1993b, 1994; Feng et al., 1997). Recently, evidence for the existence of functional heterodimers of δ- and κ-opioid receptors has been reported (Jaw et al., 1993a; Jordan and Devi, 1999). The properties of such a dimeric receptor differ from those of the individual parent homeric receptors and the dimer has characteristics of a κ_2-opioid receptor (Jordan and Devi, 1999). The potential involvement of this phenomenon should also be given consideration with respect to the present results.

Thus, clear evidence exists for the PGi as a site within which neuronal opioid dependence can be produced and which can mediate at least some of the behavioral signs of opioid withdrawal. Typically, the behaviors that are most commonly evoked by electrical stimulation of the PGi and by focal antagonist-precipitated withdrawal are teeth chattering, wet-dog shakes and, to a lesser degree, stereotyped scratching. As reported by Maldonado et al. (1992), certain behavioral signs of withdrawal (diarrhea, salivation, lacrimation, and rhinorrhea) could only be observed when methylnaloxonium was administered systemically, rather than discretely into distinct brain regions, in morphine-dependent rats. Furthermore, different patterns of behavior were noted to be elicited in morphine-dependent rats following injection of this nonselective opioid receptor antagonist into anatomically distinct brain regions. The present data do differ slightly from those presented by Kimes et al. (1998) and Maldonado et al. (1992) in terms of the relative incidences of different signs elicited from injection into the locus coeruleus. However, these discrepancies appear minor, given the normal variability in the spectrum and incidence of withdrawal signs reported by different investigators, in the method of inducing opioid dependence and in the location and parameters of local tissue injection of opioid receptor antagonists. It seems probable that the efferent connections of the PGi mediate a distinct subset of the signs and symptoms normally observed in the rodent during withdrawal from opioid dependence.

In the present study, microinjections of nor-BNI or of naloxone into the PGi were unable to evoke behavioral evidence of withdrawal in opioid-naïve animals. This is consistent with previous findings in which direct injection of naloxone into the locus coeruleus of saline-treated (opioid-naïve) rats did not precipitate withdrawal phenomena (Tokuyama et al., 1998). Moreover, at the level of single neurons, neither systemic nor discrete application of naloxone into the PGi had any effect on the rate of firing of spontaneously active PGi neurons (Haghparast et al., 1998; Saiepour et al., 2001). In contrast, discrete electrical stimulation of the PGi can elicit withdrawal signs and increases in extracellular glutamate within the locus coeruleus in opioid-naïve animals (Liu et al., 1999a,b; Rockhold et al., 2000). These results suggest an absence or a paucity of resting opioidergic tone in PGi neurons. Other studies have indicated that opioid receptors desensitize to agonists and become supersensitive to opioid receptor antagonists during the development of opioid dependence. This is specifically

true for the κ-opioid receptor during the development of dependence upon butorphanol (Jaw et al., 1993b; Wongchanapai et al., 1998, 1999). This supersensitivity to antagonists may help explain the pronounced withdrawal phenomena that are associated with antagonist-precipitated withdrawal.

The present studies utilized discrete tissue microinjections to elicit responses selectively from the region of the PGi. Volumes of 400 nL were employed. These volumes are greater than those normally considered as optimal for discrete anatomical mapping of tissue responsiveness in the central nervous system, where volumes of 25–50 nL are more typically used. However, several factors suggest that the volumes used did provide anatomical selectivity that was sufficient for the purposes of these experiments. First, the studies employed antagonists and it can be expected that precipitation of withdrawal behaviors would require that virtually the entire volume of any given brain region would have to see effective concentrations of an antagonist for an effect to become manifest. Furthermore, the PGi is a relatively poorly defined region and neurons that project discretely to the locus coeruleus are found diffusely throughout much of its volume (Aston-Jones et al., 1991; Pieribone and Aston-Jones, 1991; Van Bockstaele et al., 1989). Therefore, relatively larger volumes are needed to ensure blockade of receptor sites throughout an active area. For example, Maldonado et al. (1992), in a seminal mapping study of methylnaloxonium-sensitive brain areas, utilized injection volumes of 500 nL. In the present investigation, evidence for site selectivity includes the fact that injections of naloxone that were found to occur outside the generally accepted anatomical borders of the PGi were ineffective in eliciting withdrawal behaviors. In addition, identification of the pattern of diffusion of radiolabeled naloxone indicated a functionally restricted distribution of the compound in the coronal and saggital planes. It should be pointed out that the labeled drug was prepared so that the absolute concentration of naloxone in the diffusion studies was identical to that employed in the behavioral experiments. Some limited spread of the injected drug was noted to occur dorsally along the cannula tract, but there is no evidence that a functionally active set of neurons, relevant to opioid related behaviors, is present immediately dorsal to the PGi. Only the diffusion of naloxone was tested, since no appropriately labeled compound was available to test diffusion of nor-BNI. Thus, it seems appropriate to consider that the data reflect participation of opioid receptors confined to the immediate region of the PGi.

For the past several years and through numerous publications, our laboratory has adopted a standardized paradigm for evaluation of narcotic antagonist-precipitated opioid withdrawal that follows behavioral responses for a 30-min period following administration of an antagonist, whether that antagonists is given peripherally into the lateral cerebral ventricles or discretely into brain tissue sites. This time period has become routine, despite the fact that withdrawal and withdrawal-like (in the case of discrete brain electrical stimulation; Liu et al., 1999a,b) behaviors are not generally distributed evenly throughout the observation period. Under the conditions of the present study, symptoms clustered overwhelmingly within the initial 15 min following initiation of injection, with an apparent peak of responses occurring near the 10th min. Symptoms were rarely observed in the final 15 min of observation. The analysis of diffusion of naloxone at 10 min following injection seems to provide a more appropriate correlation to the behavioral response pattern.

In summary, the result of electrical stimulation of neurons within the PGi does elicit behaviors consistent with those evoked during withdrawal from opioid dependence. Similarly, the application of opioid receptor antagonists locally within the PGi can precipitate withdrawal behaviors in rats made dependent upon either morphine or butorphanol. Finally, dependence upon butorphanol is at least partly the result of interactions with κ-opioid receptors that are present within the anatomically discrete PGi.

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