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Contribution of Glutamatergic Systems in Locus Coeruleus to Nucleus Paragigantocellularis Stimulation-Evoked Behavior

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LIU, N., I. K. HO AND R. W. ROCKHOLD. *Contribution of glutamatergic systems in locus coeruleus to nucleus paragigantocellularis stimulation-evoked behavior*. PHARMACOL BIOCHEM BEHAV **63**(4) 555–567, 1999.—The role of extracellular glutamate, within the locus coeruleus, in mediation of the behavioral signs elicited by electrical stimulation of the nucleus paragigantocellularis (PGi) was investigated in conscious, opioid-naïve rats. Each rat was prepared with a chronically implanted unilateral electrode within the PGi and a microdialysis guide cannula directed at the ipsilateral locus coeruleus. Opioid withdrawal-like behaviors (rearing, teeth-chattering, wet-dog shakes, etc.) and increases in extracellular glutamate concentrations within the locus coeruleus were evoked, in a frequency-dependent (0.5–50 Hz) manner, during PGi stimulation. Reverse dialysis perfusion of the locus coeruleus with the nonspecific glutamate receptor antagonist, kynurenic acid (0.1, 1 mM), reduced the intensity of stimulation-induced behaviors by roughly 50%, but had no effect on the corresponding increases in glutamate concentrations. Perfusion of the locus coeruleus with the glutamate transporter inhibitor, L-*trans*-pyrrolidine dicarboxylic acid, at 1, but not at 0.1, mM significantly increased glutamate levels in dialysates. Neither concentration of the transporter inhibitor altered the behavioral score. The results indicate that the opioid withdrawal-like behaviors elicited by electrical stimulation of the brainstem at the site of the PGi are positively correlated with locus coeruleus levels of glutamate, and suggest further that the behaviors are partially mediated by release of glutamate within the locus coeruleus or its immediate vicinity. © 1999 Elsevier Science Inc.

Microdialysis Glutamate Nucleus paragigantocellularis Locus coeruleus Opioid withdrawal Kynurenic acid L-*trans*-pyrrolidine-2,4-dicarboxylic acid

THE etiology of the behavioral signs and symptoms that define withdrawal from opioid dependence in the intact organism has yet to be fully understood. Withdrawal behaviors have been associated with hyperactivity of noradrenergic neurons within the pontine locus coeruleus, induced by acute administration of opioid antagonists in opioid-dependent animals (1,41). Hyperactivity of this type results, in part, from opioid-initiated alterations in μ- and δ-opioid receptors upon, and/or second messengers within, the noradrenergic cells themselves (2). However, recent arguments have cogently summarized data that indicate that the locus coeruleus does not initiate withdrawal behaviors in isolation (15). Multiple brain regions, some of which directly regulate neuron firing within the locus coeruleus, act in concert to generate the full behavioral expression of opioid withdrawal. Thus, withdrawal, or more properly, withdrawal-like, symptoms in opioid-dependent rats have been elicited following discrete tissue injection of opioid antagonists into a number of brain sites other than the locus coeruleus. These sites particularly include the periaqueductal gray, with lesser degrees of withdrawal-like behaviors being elicited from injections into the ventral tegmental area, central nucleus of the amygdala, nucleus raphe magnus, nucleus accumbens, and regions of the hypothalamus and spinal cord (12,30,35,39). Moreover, the increase in firing rate observed in neurons of the locus coeruleus during opioid withdrawal in intact organisms is absent, or significantly reduced, when recordings are made from this region in brain slices, suggesting that excitatory afferent input

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from distant brain regions, such as the nucleus paragigantocellularis (PGi) of the rostral ventrolateral medulla, is important for locus coeruleus hyperactivity (5,14,29).

Extracellular fluid levels of the excitatory amino acid, glutamate, are known to increase within the locus coeruleus, in parallel with the appearance of withdrawal symptoms during opioid antagonist-precipitated withdrawal from both morphine and butorphanol dependence (3,21,23,52). Currently, it is hypothesized that glutamatergic projections, from the PGi to the locus coeruleus, are responsible for this phenomenon, and participate significantly in mediation of withdrawal-induced behavioral symptoms (25,42,53). Double-labelling studies combining retrograde tract tracing and immunohistochemistry for glutaminase confirm the presence of putative glutamatergic neurons in the PGi that project to the locus coeruleus (7). This input has been verified electrophysiologically, and has been shown to mediate the well-characterized activation of locus coeruleus neurons by footshock, as well as that observed during opioid withdrawal (49). Glutamatergic neurotransmission in the locus coeruleus has been vigorously investigated using single-unit recording and application of various pharmacological agents. One such study revealed that focal electrical stimulation of the PGi monosynaptically activated 73% of locus coeruleus neurons, and that ICV injections of each of two nonselective glutamate receptor antagonists, kynurenic acid or γ-D-glutamylglycine, completely blocked the excitation of locus coeruleus neurons induced by PGi stimulation (20). Thus, a glutamatergic pathway from the PGi subserves a variety of sensory inputs to the locus coeruleus, and acts as an important regulator of neuronal activation during withdrawal from opioid dependence. However, evidence in support of this hypothesis, while strong, has been derived almost exclusively from studies in anesthetized animals (4,8,20,40). For example, electrolytic lesions of the PGi have been shown to greatly attenuate withdrawal-induced excitation of locus coeruleus neurons in anesthetized rats (40). Recently, bilateral electrical stimulation of the PGi has been reported to evoke a pattern of withdrawal-like behaviors in conscious, opioid-naïve rats (27,32,45). Such results are consistent with a major role for the PGi in determination of the signs and symptoms of opioid withdrawal. The objective of the present study was to determine the degree to which withdrawal-like symptoms elicited by PGi stimulation were associated with alterations in glutamatergic neurotransmission within the locus coeruleus.

METHOD

Animals

Male Sprague–Dawley rats, weighing 300 to 350 g (Harlan–Sprague–Dawley Inc., Indianapolis, IN), were used in these experiments. Aseptic techniques were employed during all surgical procedures, which were approved by the University of Mississippi Medical Center IACUC. Animals were housed, prior to surgical intervention, in plastic group cages (four/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%). Survival surgical procedures were performed under halothane (2.5% halothane in medical-grade oxygen) anesthesia. Preoperative and postoperative analgesia were 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 in conscious animals. Rats were sacrificed with an acute overdose of Nembutal®, and their brains were examined using histological methods.

Stereotaxic Manipulations

Animals were anesthetized and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). A midline dorsal skull incision was made and the soft tissues overlying the skull were removed. The skull landmarks, bregma and lambda, were identified, and the skull was oriented such that both points were positioned at the same horizontal level. A burr hole was made in the skull over coordinates corresponding to the site of interest. The coordinates were estimated from the rat brain atlas of Paxinos and Watson (38), and are presented as millimeters relative to bregma, except in the case of vertical coordinates. Coordinates for implantation of CMA/11 guide cannulae (Bioanalytical Systems Inc., Lafayette, IN) for in vivo microdialysis of the locus coeruleus were: posterior −9.8 mm, lateral 1.1 mm, and 6.8 mm ventral to the skull surface; coordinates for implantation of bipolar, concentric electrodes (SNEX-100 × 20 mm; David Kopf Instruments) for PGi stimulation were: posterior −12.3 mm, lateral 1.6 mm, and 8.5 mm ventral to the skull surface. A stylet was inserted to seal each microdialysis guide cannula until use. After implantation, all guide cannulae and electrodes were fixed firmly to the skull with stainless steel anchor screws and dental cement.

Assessment of Stereotyped Behaviors

Animals were placed individually in a stainless steel cage (25 × 20 × 20 cm) and acclimated therein for at least 1 h prior to behavioral measurement. Ten distinct behaviors (rearing, sniffing, exploration, teeth chattering, wet-dog shakes, scratching, escape attempts, abnormal posturing, ptosis, and diarrhea) were scored during two consecutive 15-min periods of PGi stimulation. The withdrawal signs measured in the present study were defined as follows: rearing—elevation of the body with the forepaws off the bedding; sniffing—short audible inhalations, with elevation of the muzzle and movement of the nares and nasal vibrissae; exploration—circling of the cage with questing movements of the head; teeth chattering—vigorous chewing movements of the jaw and audible chattering of the teeth; wet-dog shakes—vigorous shaking of the head, neck, and body; scratching—scratching of the back of neck or the top of head with both forepaws; abnormal posturing—pressing of the ventral surface of the head, thorax, and abdomen against the floor of the cage; escape attempts—abrupt, vigorous attempts to scale the walls of the cage; ptosis—drooping of the upper eye lid(s); diarrhea—deposition of poorly formed, wet fecal pellets on the bedding. The quantal signs of diarrhea and ptosis were scored if the event was noted at any point during the observation period. The remaining signs were scored using the following rating scale to assess the incidence of each type of behavior during an observation period: 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, and 5 = 21 or more episodes of a behavior. The mean value of the rating scale for each behavior was used to indicate the average incidence of each behavior. The composite score for each animal was calculated as the sum of scores for each behavior in that rat. Behavioral responses were evaluated by a single observer

without knowledge of the nature of the treatment received by each animal. The similarities between the profile of responses following electrical stimulation of the PGi and that noted following opioid antagonist-precipitated withdrawal from opioid dependence has prompted the use of the term, opioid withdrawal-like, to define this pattern (32,45).

Microdialysis Sampling

Microdialysis probes (CMA/11, 2 mm tip, 240-μm outer diameter, 210-μm inner diameter, molecular weight cutoff of 20,000 Daltons) were inserted into the locus coeruleus through guide cannulae 12 h prior to initiation of an experimental sequence. Microdialysis probes were perfused with artificial cerebrospinal fluid [CSF; (47)], initiated at a rate of 0.2 μl/min (Carnegie Medizin CMA/100 nanoliter infusion pump) and continued for the 12-h period prior to an experiment. During this time, rats were housed individually, with chest harnesses and microdialysis tubing connected to a standard microdialysis counterweight arm. Upon completion of the stabilization period, the infusion rate was increased to 2.0 μl/min, and samples were collected into 300-μl polyethylene vials at 15-min intervals. Each experimental sequence was begun with a series of at least three 15-min sample collections for baseline determination. Baseline was considered to be established when three consecutive samples showed less than 10% variability in peak heights for glutamate (52). Probe calibration was conducted in vitro, prior to insertion of a probe into the locus coeruleus. The in vitro recovery of glutamate was determined by immersing a probe in CSF containing 10 μM of glutamate at room temperature. Probes were perfused with CSF, samples collected, and dialysate samples subsequently analyzed by HPLC-EC. The values from three to four consecutive samples were averaged to determine the average recovery value for each probe. Due to the variability of probe recovery, the extracellular levels of glutamate were individually calculated for each animal (21).

Analysis of Glutamate Levels

For measurement of glutamate, the method of Ellison et al. (19) was used with minor modification. Briefly, the measurements were performed on an HPLC (BAS 200B; Bioanalytical Systems, Inc.) with electrochemical detection by using a BAS Unijet C_{18} column (100 × 1 mm; i.d., 3 μm. The mobile phase consisted of 0.1 M sodium phosphate (mono and dibasic)/methanol buffer (80/20, v/v; pH 5.1). The derivatizing reagent consisted of *o*-phthaldialdehyde (50 mg), 2-mercaptoethanol (40 μl), absolute ethanol (1 ml), and 0.1 M borate buffer to make the final volume of 10 ml. The glutamate peak was verified by retention time, peak shape, and comparison of samples with a standard consisting of eight amino acids (aspartate, glutamate, glutamine, histidine, arginine, glycine, taurine, and alanine, each of 5 μM concentration).

Electrical Stimulation Procedures

On the evening prior to experiment, animals were anesthetized with halothane (2.5% halothane in medical-grade oxygen). Two male connectors were soldered to the leads of stimulation electrode. Animals were allowed to recover from anesthesia overnight. The next morning, animals were connected to an electrical stimulator (S-90, Medical Systems Corp., Greenvale, NY) through two female connectors. Animals were placed individually in an observation cage and acclimated for at least 1 h prior to behavioral measurement. The average electrical impedance of the implanted stimulation electrodes was approximately 50,000 ohms.

Histology

Verification of the placement of cannulae and electrodes was performed following sacrifice of animals. Brain tissue was fixed in situ by retrograde aortic perfusion with saline, followed by 10% phosphate-buffered formalin. Brains were removed, frozen in liquid nitrogen, and mounted in a cryostat. Frozen 40-μm sections were cut and mounted on glass slides. Sections were then stained with cresyl violet and examined under the light microscope. The locations of cannulae and electrodes were plotted onto drawings of the sections, using a microprojector (28). Figure 1 demonstrates the placements of stimulation electrodes (Fig. 1A) and microdialysis probes (Fig. 1B) in individual experiments.

Protocols

A group of six animals was used to evaluate the frequency dependence of the responses to PGi stimulation. After an initial 15-min recording of spontaneous behaviors (control, no voltage applied) and taking a dialysate sample from the locus coeruleus by in vivo microdialysis, animals were randomly assigned to receive a series of PGi stimulations (1 ms, 1 V) at different frequencies (0.5, 5, and 50 Hz). Behavioral responses to PGi stimulation were recorded and locus coeruleus glutamate concentrations were sampled during each 15-min session of PGi stimulation. There was at least a minimum of a 1-h interval between any two consecutive PGi stimulation sessions.

A second set of animals was randomly divided into two groups (eight animals per group) for determination of the effects of PGi stimulation on the levels of locus coeruleus glutamate, as determined by microdialysis perfusion. Both groups received perfusion of the locus coeruleus with ACSF for a period of 105 min. One group was also given two consecutive 15-min periods of PGi stimulation (0.5 H, 1 ms, 1 V), beginning 45 min after the initiation of perfusion and collection of dialysates for determination of glutamate levels. The second group, in which PGi electrodes were implanted, did not receive any electrical stimulation at any time. Behavioral responses, were recorded, and locus coeruleus glutamate concentrations were sampled throughout the period of locus coeruleus perfusion.

A third set of animals was randomly divided into two groups (six animals per group) for determination of the effects of locus coeruleus reverse microdialysis perfusion with the nonselective glutamate receptor antagonist, kynurenic acid, on the responses to PGi stimulation. Extracellular concentrations of glutamate in the ipsilateral locus coeruleus were sampled by in vivo microdialysis at 15-min intervals. After collection of a minimum of three 15-min samples of dialysate for determination of basal glutamate concentrations, the perfusion medium was switched to a solution of CSF containing kynurenic acid (0.1 mM for the first group; 1 mM for the second group) and maintained for a period of 105 min. A 30-min run of unilateral PGi stimulation (0.5 Hz, 1 ms, 1 V) was commenced 45 min after initiation of perfusion of the locus coeruleus with a kynurenic acid-containing medium. Behavioral responses to PGi stimulation and kynurenic acid perfusion were recorded and evaluated during the course of the experiment.

A fourth set of animals was prepared for determination of the effects of locus coeruleus reverse microdialysis perfusion

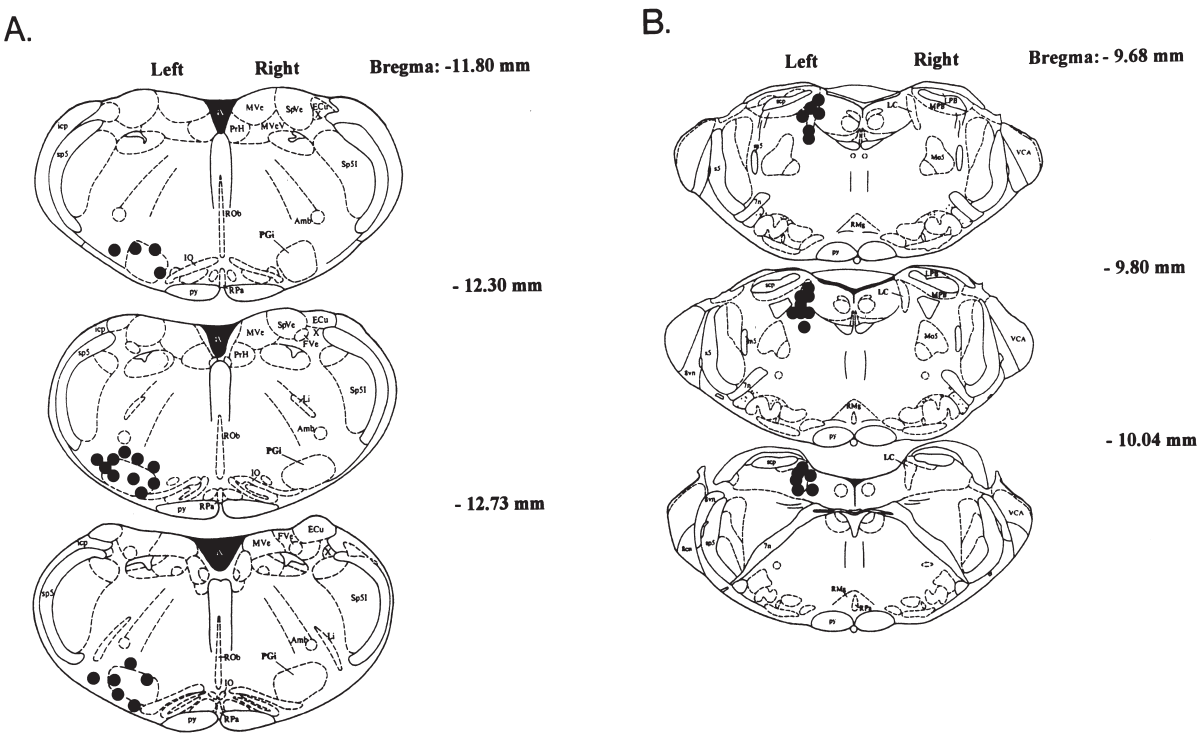

FIG. 1. Location of electrode tip placements (A, left panel) within, or in the immediate vicinity of, the PGi and location of microdialysis probe placements (B, right panel) within the locus coeruleus from rats in which unilateral electrical stimulation of the PGi and ipsilateral microdialysis sampling from the locus coeruleus was performed. Figures are redrawn after Paxinos and Watson (38) and represent cross-sections of the medulla and pons, respectively. The anterior–posterior level of each figure, in millimeters relative to the skull landmark, bregma, are noted to the upper right of each line drawing. Electrode and probe tracks were identified from cresyl violet-stained, 40-μm cross-sections of formalin-fixed, frozen rat brain. Abbreviations include PGi (nucleus paragigantocellularis), Li (linear nucleus of medulla), X (nucleus X), 4V (fourth cerebral ventricle), PrH (nucleus prepositus hypoglossi), MVe (medial vestibular nucleus), MVeV (medial vestibular nucleus, ventral), SpVe (spinal vestibular nucleus), ECu (external cuneate nucleus), Sp51 (spinal trigeminal nucleus, interpolar), sp5 (spinal trigeminal tract), icp (inferior cerebellar peduncle), ROb (raphe obscurus nucleus), IO (inferior olive), py (pyramidal tract), RPa (raphe pallidus nucleus), Amb (ambiguus nucleus), locus coeruleus (locus coeruleus), MPB (medial parabrachial nucleus), LPB (lateral parabrachial nucleus), VCA (ventral cochlear nucleus, anterior), Mo5 (motor trigeminal nucleus), RMg (raphe magnus nucleus), scp (superior cerebellar peduncle), m5 (motor root trigeminal nerve), s5 (sensory root, trigeminal nerve), and 7n (facial nerve).

with the glutamate transporter inhibitor, L-*trans*-pyrrolidine dicarboxylic acid (L-*trans*-PDC), on the responses to PGi stimulation. Rats were randomly divided into two groups (six rats per group) and given a 30-min run of unilateral PGi stimulation (0.5 Hz, 1 ms, 1 V). Extracellular concentrations of glutamate in the ipsilateral locus coeruleus were sampled by in vivo microdialysis at 15-min intervals. After collection of three samples at 15-min intervals, the perfusion medium was switched to medium containing L-*trans*-PDC (0.1 mM for the first group; 1 mM for the second group) and maintained for a period of 105 min. The 30-min run of unilateral PGi stimulation (0.5 Hz, 1 ms, 1 V) was commenced 45 min after initiation of perfusion of the locus coeruleus with L-*trans*-PDC. Behavioral responses to PGi stimulation and PDC perfusion were recorded and evaluated during the course of the experiment.

Statistical Analyses

Values for extracellular fluid levels of glutamate that were expressed as percentage change from basal values were analyzed using one-way analysis of variance and the Newman–Keul's test. Rating scale scores for behavioral assessments were subjected to nonparametric analyses. In this case, the Kruskal–Wallis test, followed by a Dunn's test, was used for multiple comparisons. Comparisons between two groups were made using the Mann–Whitney rank-sum test (for non-pairwise tests) or the Wilcoxon signed-rank test (for pairwise tests). Quantal (all or none) behavioral data were analyzed by the chi-square test and the Bonferroni inequality to adjust the *p*-values. Correlation between behavioral responses to PGi stimulation and locus coeruleus glutamate concentrations was measured by Pearson correlation. Calculated values of $p < 0.05$ were considered statistically significant.

RESULTS

Behavioral Responses, and Glutamate Concentrations Within the Locus Coeruleus, Following Electrical Stimulation of the PGi

Bilateral electrical stimulation of the PGi has been shown to elicit a characteristic series of behaviors that are graded in intensity, anatomically specific to the immediate vicinity of the PGi, and frequency dependent (32,45). The present study

has examined rats in which an electrical stimulation electrode was implanted unilaterally in the PGi and a microdialysis cannula was directed towards the locus coeruleus on the ipsilateral side, for the purpose of determining the participation of locus coeruleus glutamatergic systems in PGi stimulation-induced behavior. Results from such experiments demonstrate that unilateral electrical stimulation of the PGi (0–50 Hz, 1 ms, 1 V) increased the incidence of opioid withdrawal-like behaviors, such as rearing, sniffing, exploration, teeth chattering, wet-dog shakes, scratching, escape attempts, and abnormal posturing. Ptosis and diarrhea were not seen in the animals given unilateral PGi stimulation, although in previous studies in which bilateral electrical stimulation of the PGi was performed both of these two signs were positive (32,45). The composite score of overall behavioral responses to unilateral PGi stimulation at a frequency of 0.5 Hz was 4.83 ± 0.48 (n = 6) during a 15-min period of stimulation. The composite score of overall behaviors was significantly higher ($p < 0.001$) in animals given PGi stimulation unilaterally than that observed during a control period of observation, during which time no current was passed through the electrodes (composite score = 1.33 ± 0.21) (n = 6). A previous study, in which electrical stimulation of the PGi was performed using methods identical to those reported here, except for placement of electrodes bilaterally within the PGi, noted that such bilateral stimulation evoked a significantly greater incidence of behavioral signs. In that study, an average score of 13.0 ± 0.8 was noted with bilateral stimulation for 30 min (0.5 Hz, 1 ms, 1 V; n = 12). Ptosis and diarrhea were noted in 7 of 12 and 8 of 12 rats, respectively (32,45).

During perfusion of the locus coeruleus with CSF vehicle, the behavioral responses observed in these rats were found to be frequency dependent over the range of 0 to 50 Hz. The composite scores of overall behaviors during each 15-min session of PGi stimulation were significantly increased from control ($p < 0.05$; Fig. 2A). The composite score of overall behaviors elicited by 5-Hz PGi stimulation was not significantly different from that elicited by 0.5-Hz PGi stimulation. The composite score of overall behaviors during stimulation at 50 Hz was greater ($p < 0.05$) compared with that elicited by 0.5-Hz PGi stimulation.

Extracellular glutamate concentrations in the locus coeruleus were sampled in the same animals by in vivo microdialysis during the entire course of the experiment. The basal glutamate concentration within the locus coeruleus was 5.15 ± 0.74 μM in this group of rats prior to stimulation. Unilateral PGi stimulation (0.5–50 Hz, 1 ms, 1 V), when applied as trains of impulses, significantly increased locus coeruleus glutamate concentrations ($p < 0.05$) when compared with this control value (Fig. 2B). However, a single pulse (1 ms, 1 V) applied to the locus coeruleus had no effect on glutamate levels. Stimulation of the PGi at 50 Hz increased locus coeruleus glutamate concentrations further, to approximately 325% of control values ($p < 0.05$ vs. values during stimulation at 0.5 Hz; NS vs. 5 Hz).

Effect of Locus Coeruleus Perfusion With the Nonselective Glutamate Receptor Antagonist, Kynurenic Acid, During PGi Stimulation

Figure 3 depicts responses of behavior (Fig. 3A) and extracellular glutamate concentrations (Fig. 3B) in rats during perfusion of the locus coeruleus with CSF vehicle (CSF) without stimulation of the PGi, and in rats during perfusion of the locus coeruleus with CSF vehicle (CSF + PGi stimulation) or kynurenic acid (0.1, 1.0 μM) for 60 min prior to, during and for 30 min following two consecutive 15-min periods of ipsilateral PGi stimulation. The composite scores for behavior were 5.8 ± 0.8 during the initial period of PGi stimulation, and 5.1 ± 0.5 during a second, consecutive period of stimulation, in rats in which the locus coeruleus was perfused with CSF. These scores were significantly greater than those noted in rats during perfusion of the locus coeruleus with CSF in the absence of PGi stimulation. Kynurenic acid perfusion itself did not significantly change the incidence of spontaneous behaviors. The composite scores of overall behaviors during two 15-min sessions of unilateral PGi stimulation were both significantly higher ($p<0.001$) in animals given unlateral PGi stimulation during perfusion of the locus coeruleus with CSF than in animals in which no stimulation of the locus coeruleus was performed. The composite scores for behavioral response to PGi stimulation were reduced to approximately 50% of the CSF + PGi stimulation values ($p < 0.05$) during locus coeruleus perfusion with both the low concentration (0.1 mM) and the high concentration (1 mM) of kynurenic acid. There was no significant difference between low dose and high dose kynurenic acid-treated groups in terms of the composite behavioral responses to unilateral PGi stimulation. Behavioral scores in both the low dose and the high dose kynurenic acid-treated groups, while reduced compared to the PGi/CSF values, did not return to baseline and remained significantly elevated above CSF-perfused rats throughout the period of PGi stimulation.

The basal glutamate concentrations in the locus coeruleus of kynurenic acid-treated animals were 5.45 ± 0.37 μM in animals perfused with 0.1 mM kynurenic acid and 5.24 ± 0.36 μM in the animals perfused with 1 mM kynurenic acid. These basal locus coeruleus glutamate concentrations were comparable to those observed in CSF-perfused (5.38 ± 1.95 μM) and CSF-perfused + PGi-stimulated (6.19 ± 1.84 μM) animals.

Unilateral PGi stimulation (0.5 Hz, 1 ms, 1 V) increased ($p < 0.001$) locus coeruleus glutamate concentrations during the first (217 ± 32% of basal value) and the second (231 ± 29% of basal value) 15-min periods of PGi stimulation in animals perfused with 0.1 mM kynurenic acid, while in animals perfused with 1 mM kynurenic acid, locus coeruleus glutamate concentrations were also significantly increased (268 ± 53% of basal value during the first 15-min period of stimulation and 240 ± 38% of basal value during the second 15-min period of stimulation, Fig. 3B). The changes in locus coeruleus glutamate levels in kynurenic acid-perfused animals were not significantly different from those measured in CSF-perfused animals during PGi stimulation (Fig. 3B). Extracellular glutamate levels returned to levels not different from basal levels within the first 15 min following cessation of PGi stimulation in all groups.

Effect of Perfusion of the Locus Coeruleus With the Glutamate Transporter Inhibitor, L-trans-*PDC, on PGi Stimulation-Induced Opioid Withdrawal-Like Behaviors and Locus Coeruleus Glutamate Concentrations*

Perfusion of the locus coeruleus with the glutamate transporter inhibitor, L-*trans*-PDC (0.1 mM or 1 mM), did not alter spontaneous behavior prior to electrical stimulation of the PGi. Unilateral stimulation of the PGi significantly ($p <$ 0.001) increased the composite behavioral scores in rats treated with either low (0.1 mM) or high (1 mM) concentration of L-*trans*-PDC, when compared with scores from rats in

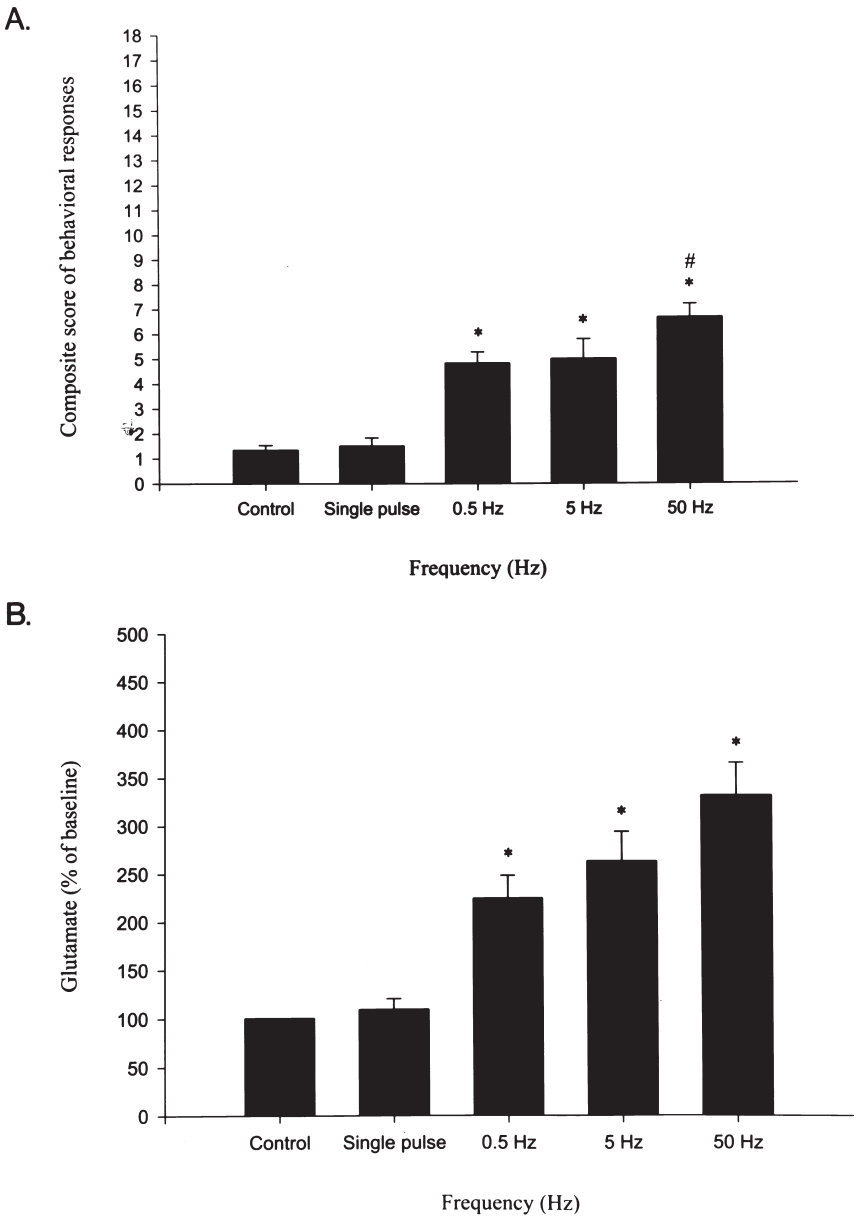

FIG. 2. Frequency dependence of behavioral responses (A, top panel) and glutamate concentrations measured by microdialysis within the ipsilateral locus coeruleus (B, bottom panel) following unilateral electrical stimulation of the PGi (0–50 Hz, 1 ms, 1 V). Single pulse refers to application of a single 1-ms, 1-V shock to the PGi. All other stimulations were performed in trains of 1-ms duration. Data represent the mean values ± SEM from six rats. The Kruskal–Wallis test and the Dunn's test were used for comparisons of behavioral responses among the designated groups. One-way analysis of variance and the Newman–Keul's test were used for comparisons of glutamate concentrations among the designated groups. $^{*}p < 0.05$ (the asterisks denote statistical differences between control and PGi-stimulated groups). $^{\#}p < 0.05$ (the pound sign denotes a statistical difference between animals given 0.5 Hz PGi stimulation and 50 Hz PGi stimulation). Basal glutamate concentrations within the locus coeruleus were 5.15 ± 0.74 μM.

which the locus coeruleus was perfused with CSF in the absence of PGi stimulation (Fig. 4A). However, no significant differences were noted between these scores and the behavioral activation observed in rats given during unilateral PGi stimulation and CSF perfusion of the locus coeruleus. There were also no significant differences between low concentration and high concentration PDC-treated groups in terms of overall behavioral responses to unilateral PGi stimulation.

The basal glutamate concentrations in the locus coeruleus of L-*trans*-PDC–treated animals were 4.96 ± 1.59 μM in animals perfused with 0.1 mM L-*trans*-PDC and 4.24 ± 1.78 μM in the animals perfused with 1 mM L-*trans*-PDC. These basal locus co-

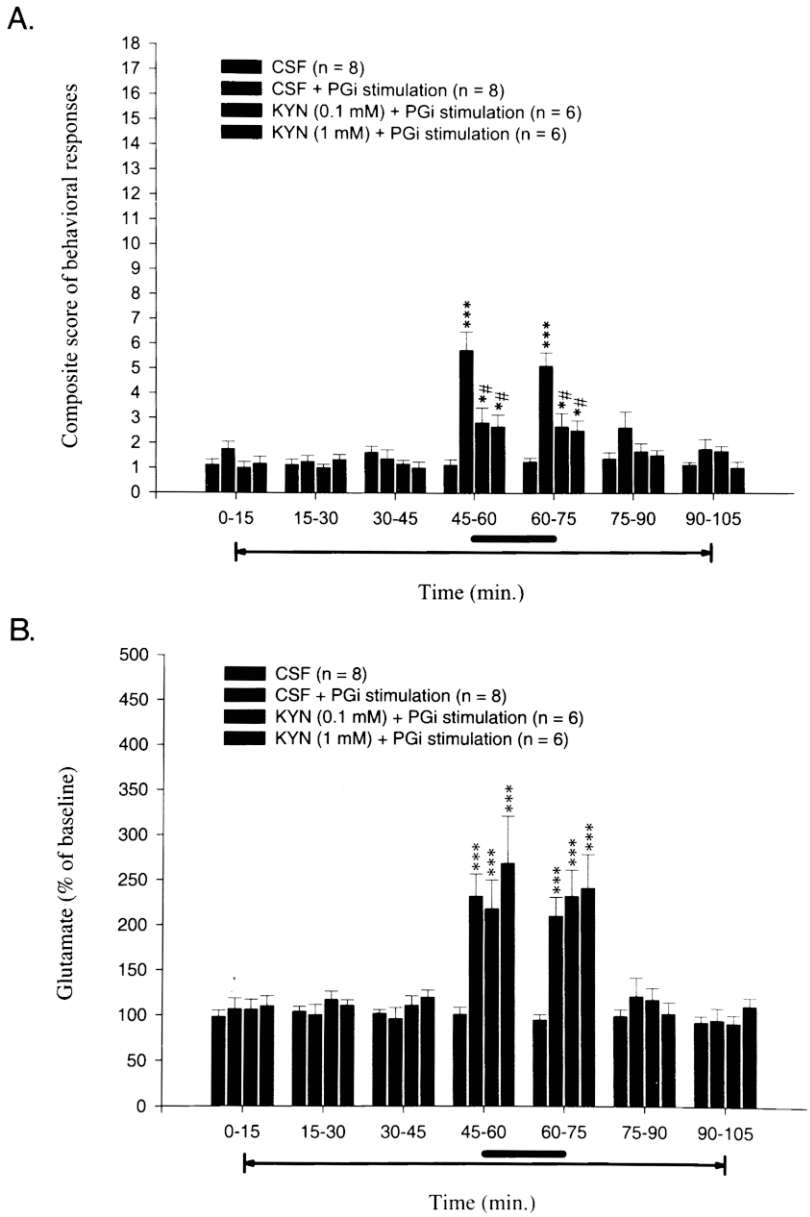

FIG. 3. Effect of reverse dialysis perfusion of the locus coeruleus with kynurenic acid (KYN; 0.1, 1 mM) on the behavioral responses (A, top panel) and the percentage changes in glutamate concentrations (B, bottom panel) prior to, during, and following two consecutive 15-min periods of ipsilateral PGi stimulation (0.5 Hz, 1 ms, 1 V). The black line with double-headed arrows, denotes the period of locus coeruleus perfusion with the indicated solution, either artificial cerebrospinal fluid (CSF) or kynurenic acid. The heavy black bar underneath the X-axis denotes the period of unilateral PGi stimulation (0.5 Hz, 1 ms, 1 V, 30 min) starting at 45-min after initiation of locus coeruleus perfusion. Data represent the mean values ± SEM. The Mann–Whitney rank-sum test was used for comparison of behavioral responses between the indicated groups. The asterisks ($*p < 0.05$; $***p < 0.001$) denote statistical differences between the CSF-perfused and the PGi-stimulated groups. One-way analysis of variance and the Newman–Keul's test were used for comparison of glutamate concentrations between the indicated groups. The pound signs denote statistical differences ($p < 0.05$) between the CSF + PGi-stimulation group and the KYN+ PGi-stimulation groups). Basal glutamate concentrations within the locus coeruleus were: CSF: 5.38 ± 1.95 μM; CSF + PGi stimulation: 6.19 ± 1.84 μM; KYN (0.1 mM) + PGi stimulation: 5.45 ± 0.3 μM; KYN (1 mM) + PGi stimulation: 5.24 ± 0.36 μM.

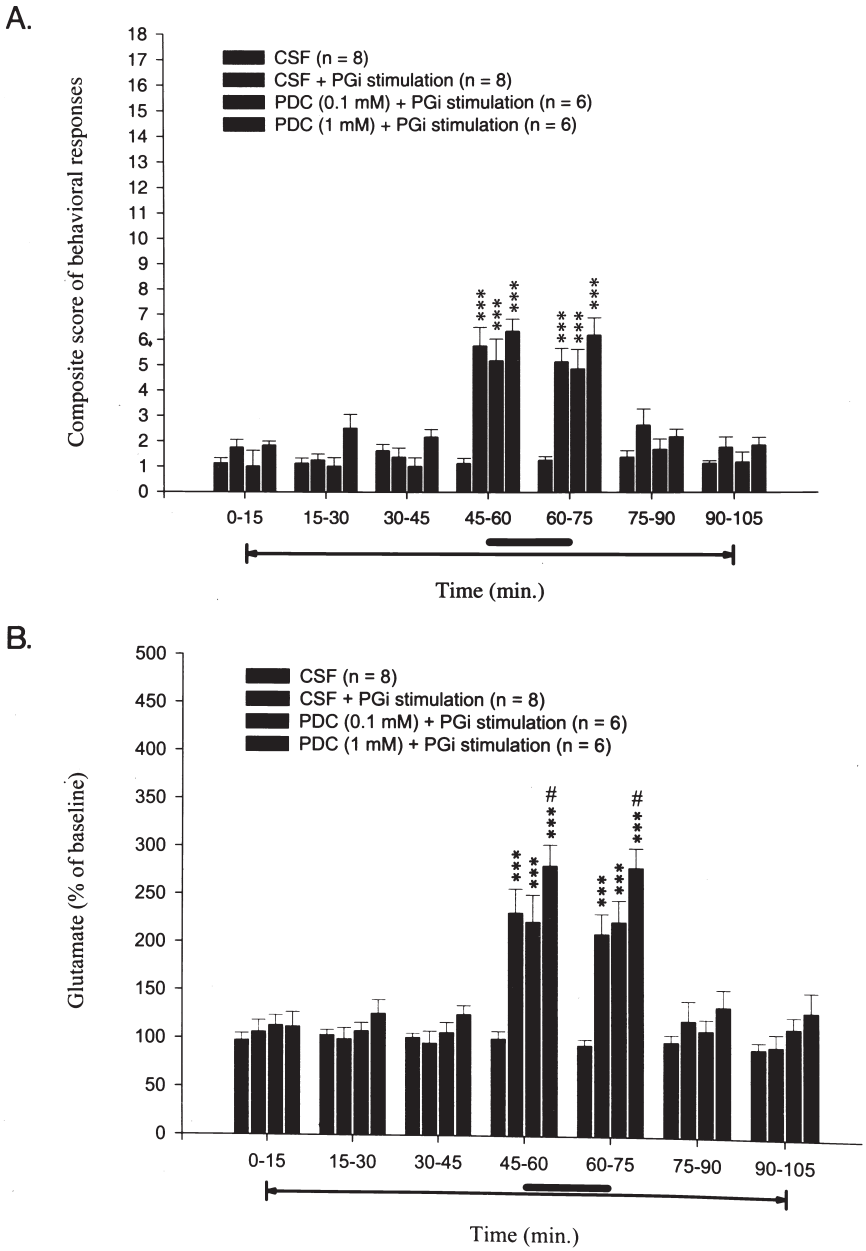

FIG. 4. Effect of reverse dialysis perfusion of the locus coeruleus with L-*trans*-PDC (PDC; 0.1, 1 mM) on the behavioral responses (A, top panel) and the percentage changes in glutamate concentrations (B, bottom panel) prior to, during, and following two consecutive 15-min periods of ipsilateral PGi stimulation (0.5 Hz, 1 ms, 1 V). The black line with double-headed arrows denotes the period of locus coeruleus perfusion with the indicated solution, either artificial cerebrospinal fluid (CSF) or L-*trans*-PDC. The heavy black bar underneath the X-axis denotes the period of unilateral PGi stimulation (0.5 Hz, 1 ms, 1 V, 30 min) starting at 45 min after initiation of locus coeruleus perfusion. Data represent the mean values ± SEM. The Mann–Whitney rank-sum test was used for comparison of behavioral responses between the indicated groups. The asterisks ($*p < 0.05$; $***p < 0.001$) denote statistical differences between the CSF-perfused and the PGi-stimulated groups. One-way analysis of variance and the Newman–Keul's test were used for comparison of glutamate concentrations between the indicated groups. The pound signs denote statistical differences ($p < 0.05$) between the CSF + PGi stimulation group and the PDC + PGi stimulation groups). Basal glutamate concentrations within the locus coeruleus were: CSF: 5.38 ± 1.95 μM; CSF + PGi stimulation: 6.19 ± 1.84 μM; PDC (0.1 mM) + PGi stimulation: 4.96 ± 1.59 μM; PDC (1 nM) + PGi stimulation: 4.24 ± 1.78 μM.

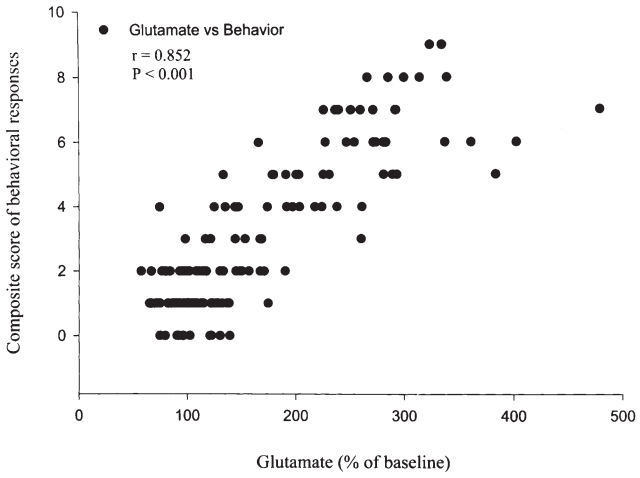

FIG. 5. Correlation between the percentage changes in locus coeruleus glutamate concentrations and the behavioral responses to unilateral PGi stimulation in 34 rats given different treatments. The correlation coefficient (r) value was 0.852, $^*p < 0.001$. Data are pooled from animals in which the locus coeruleus was perfused with artificial cerebrospinal fluid and L-*trans*-PDC. Data from rats perfused with kynurenic acid are excluded.

eruleus glutamate concentrations were comparable to those observed in CSF-perfused (5.38 ± 1.95 μM) and CSF-perfused + PGi-stimulated (6.19 ± 1.84 μM) animals. Perfusion with L-*trans*-PDC had no effect on glutamate concentrations in the locus coeruleus prior to initiation of electrical stimulation.

During the 60-min period of locus coeruleus perfusion prior to PGi stimulation, locus coeruleus levels of glutamate averaged 4.96 ± 1.59 μM in rats perfused with 0.1 mM L-*trans*-PDC and 4.24 ± 1.78 μM in rats perfused with 1 mM L-*trans*-PDC. These glutamate concentrations were comparable to those of CSF-perfused rats. Unilateral PGi stimulation significantly increased ($p < 0.001$) glutamate concentrations during the first (222 ± 28% of basal value) and the second (222 ± 23% of basal value) 15-min periods of PGi stimulation in rats perfused with 0.1 mM L-*trans*-PDC (Fig. 4B). The changes in locus coeruleus glutamate concentrations during unilateral PGi stimulation with this low dose of L-*trans*-PDC did not differ from those in rats perfused with CSF. However, in rats perfused with 1 mM L-*trans*-PDC, glutamate concentrations were significantly increased (280 ± 22% of basal value during the first 15-min period of stimulation and 278 ± 20% of basal value during the second 15-min period of stimulation, $p < 0.05$ above those noted with locus coeruleus perfusion of CSF). The concentrations of glutamate in locus coeruleus dialysates returned to control levels in all groups immediately upon cessation of PGi stimulation.

Correlation Between the Percentage Change in Locus Coeruleus Glutamate Concentrations and the Composite Behavioral Score in response to Unilateral PGi Stimulation

Figure 5 shows the correlation between the composite scores for behavioral responses and the maximal percentage changes in locus coeruleus glutamate concentrations in 34 rats give unilateral PGi stimulation (0.5–50 Hz, 1 ms, 1 V) and perfusion of the locus coeruleus with CSF or L-*trans*-PDC (0.1 or 1 mM). A significant correlation (Pearson correlation of $r = 0.852$, $p < 0.001$) was found between the composite score of behavioral responses to PGi stimulation and the percentage change in locus coeruleus glutamate concentrations.

DISCUSSION

The results of the present study confirm an earlier report that electrical stimulation of the PGi evokes a series of opioid withdrawal-like behaviors and demonstrate that such stimulation also yields increased extracellular fluid levels of glutamate within the immediate vicinity of the locus coeruleus. Furthermore, interpretation of these results indicates that PGi stimulation-induced increases in glutamate concentrations within the locus coeruleus are partially, but not completely, responsible for the associated behavioral responses in intact, conscious rats.

Glutamatergic neurotransmission within the locus coeruleus clearly participates in mediation of behavioral symptoms that accompany precipitated withdrawal from opioid dependence (3,21–23,52). Intracerebroventricular (4,40,42) and intracoerulear (4) infusion of kynurenic acid, a nonspecific antagonist of glutamate receptors, has been shown to block activation of locus coeruleus firing and behavioral symptoms during opioid antagonist-precipitated withdrawal from dependence on morphine. Additional investigations by Akaoka and Aston-Jones (4) determined that locus coeruleus neuron hyperactivity could be suppressed, although not totally abolished, by intracoerulear administration of kynurenic acid or antagonists selective for either NMDA or non-NMDA glutamate receptor subtypes. Of these, the NMDA-selective antagonist, AP5, was less effective than either kynurenic acid or the non-NMDA receptor antagonist, CNQX (4). The role of glutamate in opioid withdrawal is further supported by the results of microdialysis studies. Increased extracellular fluid levels of excitatory amino acid neurotransmitters within the locus coeruleus have been shown to occur contemporaneously with precipitated withdrawal from morphine dependence (3,52). Other studies have confirmed that result, and demonstrated further that naloxone-precipitated withdrawal from dependence on butorphanol (21) and on the selective κ-opioid receptor agonist, U-69,593 (26), elicit an increase in extracellular levels of glutamate in the locus coeruleus similar to that seen in morphine withdrawal. In addition, an increase in glutamate levels in the locus coeruleus was observed during butorphanol withdrawal induced by the κ-selective antagonist, nor-BNI (23,26). These results indicate that increases in the levels of glutamate in the locus coeruleus may be a common mechanism of opioid antagonist-precipitated withdrawal from opioid dependence.

Aston-Jones et al. (6) demonstrated that glutamatergic projections to the locus coeruleus originate primarily from the PGi of the rostral medulla oblongata. The importance of the PGi projections to the locus coeruleus in mediation of opioid withdrawal phenomena is emphasized by the fact that electrolytic lesions of the PGi, in anesthetized rats, abolish increased locus coeruleus firing during morphine withdrawal (40). The anatomical and electrophysiological data, demonstrating the neuronal link between the PGi and the locus coeruleus, coupled with the substantial body of evidence suggesting the involvement of locus coeruleus glutamate in activation of locus coeruleus neurons and generation of opioid withdrawal behaviors strengthen the hypothesis that PGi stimulation-induced opioid withdrawal-like behaviors

are at least partially mediated by locus coeruleus glutamate. In the present study, focal electrical stimulation of the PGi produced a pattern of behaviors, including rearing, sniffing, exploration, teeth chattering, wet-dog shakes, scratching, and abnormal posturing in rats that had not been treated previously with opioids. Such characteristic behaviors are typical of those elicited during withdrawal from dependence on systemically administered morphine (51). They also bear similarity to behaviors that can be elicited following discrete brain tissue injections of narcotic antagonists in opioid-dependent rats (12,30,35,39). The intensity of behavioral activation produced by unilateral PGi stimulation was less than that observed in an earlier study, in which bilateral PGi stimulation was performed (32,45). This might be expected from recognition that projections from the PGi to the locus coeruleus are almost exclusively ipsilateral (50). Significantly, the onset, maximal expression, and duration of unilateral PGi stimulation-induced behaviors were each paralleled by elevations in extracellular fluid levels of glutamate within the ipsilateral locus coeruleus. This pattern of neurotransmitter response supports the idea that glutamatergic projections from the PGi to the locus coeruleus are involved in the generation of behavioral responses to opioid withdrawal.

The response of neurons to an electrical stimulation is determined by three factors, intensity, frequency, and duration. Higher intensity, frequency, or longer duration would be expected to produce a stronger response. In the present study, we tested the effect of intensity and frequency on the behavioral response to PGi stimulation and locus coeruleus glutamate concentration. Our data indicated a clear voltage dependency in the behavioral response to PGi stimulation. However, the effect of frequency on behavioral responses to PGi stimulation was not so clear cut. When animals were given bilateral PGi stimulation at low frequencies (from 0.125 to 2 Hz), an increase in frequency did not change composite scores of overall behaviors significantly. In contrast, when animals were given unilateral PGi stimulation at high frequency (50 Hz), both the composite scores of overall behaviors and the locus coeruleus glutamate concentrations were increased significantly compared to those in animals given a standard (0.5 Hz, 1 ms, 1 V) PGi stimulation. These results suggest that at low frequency range (from 0.125 to 5 Hz), changes in frequency do not significantly change the incidence of behavior or locus coeruleus glutamate concentration, and that a higher frequency is required to elicit a more robust behavioral syndrome and higher increases in locus coeruleus glutamate concentrations.

To further verify the role of locus coeruleus glutamate in mediation of PGi stimulation-induced behaviors, glutamate receptors in the locus coeruleus were blocked by reverse microdialysis using the nonselective glutamate receptor antagonist, kynurenic acid. Perfusion of the locus coeruleus with both a low (0.1 mM) or a high concentration (1 mM) of kynurenic acid attenuated the composite scores of overall behaviors during two 15-min sessions of PGi stimulation by approximately 50%. The percentage changes in locus coeruleus glutamate concentrations in animals given kynurenic acid perfusion and unilateral PGi stimulation were equivalent to those of animals in which locus coeruleus perfusion with CSF was coupled with unilateral PGi stimulation. This is in agreement with results from Akaoka and Aston-Jones (4), who demonstrated partial blockade of locus coeruleus hyperactivity with intracoerulear injections of kynurenic acid.

In a separate experiment, the contributing role of glutamate transporters in mediating opioid withdrawal-like behaviors produced by PGi stimulation was investigated. In this experiment, animals given unilateral PGi stimulation and perfused with L-*trans*-PDC (0.1 mM or 1 mM) displayed a characteristic behavioral syndrome similar to that observed in CSF-perfused and PGi-stimulated animals. The composite scores of overall behaviors during two 15-min sessions of PGi stimulation were higher than those of sham-operated animals, but comparable to those of CSF-perfused and PGi-stimulated animals. The changes in locus coeruleus glutamate concentrations in these animals were somewhat paradoxical. First, no increases in basal levels of glutamate were noted in rats during perfusion with L-*trans*-PDC. Other studies in which L-*trans*-PDC has been used in connection with microdialysis have noted substantial elevations in basal glutamate levels in striatum (9,36,43,44), hippocampus (36), and prefrontal cortex (31). No specific explanation for the lack of increase in basal glutamate within the locus coeruleus is evident. In addition, it would appear that relatively large elevations in locus coeruleus glutamate levels may be necessary to evoke increases in opioid withdrawal-like behavior. When animals were perfused with 0.1 mM L-*trans*-PDC, unilateral PGi stimulation significantly increased locus coeruleus glutamate concentrations compared to those of sham-operated animals, and these changes were not significantly different from those of CSF-perfused and PGi-stimulated animals. However, when animals were given locus coeruleus perfusion with L-*trans*-PDC at 1 Mm, unilateral PGi stimulation produced a significantly higher increased in glutamate concentrations than did in CSF-perfused and PGi-stimulated animals. According to our hypothesis, changes in locus coeruleus glutamate concentrations should parallel the intensity of behavioral response to PGi stimulation. In this experiment, locus coeruleus perfusion with 1 Mm L-*trans*-PDC, the selective glutamate transporter inhibitor, significantly enhanced locus coeruleus glutamate concentrations, but not the intensity of behavioral responses to PGi stimulation, when compared to those of CSF-perfused and PGi-stimulated animals. This may be explained by the findings that multiple neurotransmitters, such as excitatory amino acids, epinephrine, enkephalins, serotonin, and others, have been found in the nerve terminals originating from the PGi (50). The release of glutamate from PGi terminals within the locus coeruleus may mediate only certain components or intensities of the behaviors elicited by PGi stimulation. Therefore, a minor change in locus coeruleus glutamate concentrations alone is not sufficient to enhance behavioral responses to PGi stimulation. In another experiment in which animals were given unilateral PGi stimulation at different frequencies (0.5, 5, or 50 Hz), the percentage change of locus, coeruleus glutamate concentration was 40% higher in animals given PGi stimulation at 5 Hz than in animals give PGi stimulation at 0.5 Hz. The composite score of overall behaviors were comparable in both groups. When animal were given PGi stimulation at high frequency (50 Hz), the percentage change of locus coeruleus glutamate concentration was about 100% higher than that in animals given PGi stimulation at 0.5 Hz. The composite score of overall behaviors was also significantly higher in animals given PGi stimulation at 50 Hz than in animals given PGi stimulation at 0.5 Hz. This suggests that a larger increase in locus coeruleus glutamate concentration or a higher frequency will be necessary to enhance the behavioral response to PGi stimulation.

To further confirm the role of locus coeruleus glutamate in mediating opioid withdrawal-like behaviors elicited by PGi stimulation, the strength of association between the composite score of behavioral responses and the percentage change

in locus coeruleus glutamate concentrations was measured by Pearson correlation. A significant correlation was found between the composite score of behavioral responses to unilateral PGi stimulation and the percentage change in locus coeruleus glutamate concentrations in 34 rats given unilateral PGi stimulation and CSF or L-*trans*-PDC perfusion. This result clearly indicates that changes in locus coeruleus glutamate concentrations parallel the intensity of behavioral syndrome produced by PGi stimulation and strongly supports our hypothesis that locus coeruleus glutamate plays a role in mediating opioid withdrawal-like behaviors produced by PGi stimulation.

Several caveats must be considered in interpretation of these data. The techniques used do not entirely rule out the possibility of anatomically nonspecific responses. Previous studies from this laboratory (32,45) have demonstrated that no withdrawal-like behaviors are elicited when stimulation electrodes are located either at distances of 1–2 mm outside the boundaries of the PGi, or within the nucleus prepositus hypoglossi which, like the PGi, sends a major afferent projection to the locus coeruleus. However, current leakage to areas immediately surrounding the PGi (i.e., at distances of less than 1–2 mm from the PGi) could activate systems other than those projecting from the PGi to the locus coeruleus, and might thus elicit behavioral responses that are not dependent on glutamatergic projections to the locus coeruleus. In addition, PGi stimulation has the potential to activate fibers of passage through the PGi that again do not terminate in the locus coeruleus. Similarly, interpretation of in vivo microdialysis results must recognize the relative proportions of the microdialysis probe, in relation to the size of the locus coeruleus. Almost certainly, the probe samples from brain tissue within, as well as outside, the borders of the locus coeruleus. Thus, glutamatergic projections that terminate on cells in areas adjacent to the locus coeruleus may be involved in the behavioral responses evoked by PGi stimulation. In fact, electrical stimulation of the PGi can be expected to cause elevations in glutamate at several brain sites to which the PGi projects. Because selective administration of a glutamatergic receptor antagonist to the vicinity of the locus coeruleus did not completely abolish PGi stimulation-induced behaviors, it is possible that the behavioral response is mediated by release of glutamate (or as discussed below, other neurotransmitters) at brain sites in addition to the locus coeruleus. Maldonado and Koob (34) demonstrated reductions in the behavioral responses of opioid withdrawal following electrolytic lesions of the locus coeruleus, further implicating a role for synaptic interactions at this level of the neuroaxis. However, other investigators have been unable to demonstrate reductions in opioid withdrawal behaviors following either 6-OHDA lesions of noradrenergic neurons within the locus coeruleus (48) or 6-OHDA (11) and DSP-4 (13,18)-induced lesions of noradrenergic locus coeruleus terminal fields. The present data argue for a contribution by glutamatergic systems projecting from the vicinity of the PGi to the locus coeruleus and its immediate vicinity. Additional studies will be necessary to resolve remaining ambiguities.

It is of interest to note that locus coeruleus perfusion with the nonselective glutamate receptor antagonist, kynurenic acid, did not completely abolish PGi stimulation-induced behaviors. Although it is currently believed that locus coeruleus glutamate plays an important role in mediating opioid withdrawal behaviors, several other neurotransmitters found in the locus coeruleus may contribute to regulation of some components of the opioid withdrawal syndrome. For example, adrenergic afferents originating in the PGi have been identified in the locus coeruleus using immunohistochemical techniques (7), and clear inhibitory effects of PGi stimulation on locus coeruleus neurons were observed when the glutamate-mediated activation of locus coeruleus from the same stimulation sites in the PGi was eliminated (20). Under kynurenic acid blockade, an underlying inhibition was observed in 88% of locus coeruleus neurons following PGi stimulation (20). Aston-Jones et al. (7) reported that systemic administration of the α_2 antagonist, idazoxan, attenuated this inhibition in 80% of neurons tested. A similar effect was seen for locally infused idazoxan. Serotonin, another neurotransmitter found in the locus coeruleus, has also been implicated in mediation of the opioid withdrawal syndrome. It has long been known that the locus coeruleus is densely innervated by serotonergic fibers. Electrophysiological studies revealed that iontophoretic application of serotonin had no consistent effect on spontaneous locus coeruleus activity, decreasing the activity of many neurons while the discharge of other neurons was unchanged or increased in the presence of this agent (7). However, similar iontophoretic application of serotonin consistently and potently attenuated responses of locus coeruleus neurons to iontophoretic glutamate (7). Because multiple neurotransmitters have been identified in the locus coeruleus, it is unlikely that a single neurotransmitter, glutamate, regulates all aspects of opioid withdrawal syndrome.

In a previous study, the effects of opioid receptor antagonists on PGi stimulation-induced opioid withdrawal-like behaviors were tested (34,45). The results indicated that the nonselective opioid receptor antagonist, naloxone, attenuated the intensity of opioid withdrawal-like behaviors induced by PGi stimulation by approximately 50%, suggesting partial involvement of opioid receptors and/or endogenous opioids in mediation of such behaviors. To further define this interaction, animals were given ICV injections of selective μ-, δ-, and κ-opioid receptor antagonists prior to PGi stimulation. The μ-selective antagonist, β-funaltrexamine, and the δ-selective antagonist, naltrindole, but not the κ-selective antagonist, nor-binaltorphimine, significantly reduced opioid withdrawal-like behaviors induced by PGi stimulation. However, no antagonist reduced behavioral responses by more than 50%. These results confirm that endogenous opioids are partially involved in mediating opioid withdrawal-like behaviors induced by PGi stimulation, and indicate further that PGi stimulation induces behaviors selectively through μ- and δ-, but not κ-opioid receptors. It is possible that PGi stimulation activates direct projections from the PGi to higher brain centers (33) wherein release of opioid peptides can elicit behaviors that resemble those observed during opioid withdrawal.

The anatomical locus or loci at which this opioidergic link operates is (are) uncertain at the present time. Certain data would lead one to question whether this link occurs in the locus coeruleus. Local application of opioid agonists, such as morphine, inhibit, rather than excite, the firing rate of locus coeruleus neurons (1,50). This inhibition is mediated by a membrane hyperpolarization triggered by μ-opioid receptors (37). Furthermore, a substantial body of evidence indicates that excitatory amino acids are a principal mediator of withdrawal-induced neuronal hyperactivity in the locus coeruleus (4,6,52). Therefore, it is unlikely that the locus coeruleus is the site in which endogenous opioids participate in the generation of opioid withdrawal-like behaviors induced by PGi stimulation. Several brain regions, such as the VTA and nucleus accumbens, have been implicated as potential sites where endogenous opioids mediate locomotor behaviors

(10,16,17,46). Immunohistochemical studies have also confirmed direct connections between the locus coeruleus and these brain regions (24). It is possible that ICV administration of opioid antagonists attenuated PGi stimulation-induced opioid withdrawal-like behaviors by blockade of the action of endogenous opioids, released from secondary or tertiary order locus coeruleus neurons, located in the VTA and/or nucleus accumbens. Thus, the opioid withdrawal-like behaviors elicited by PGi stimulation may well be mediated by multiple neurotransmitters, acting at the locus coeruleus as well as other, as yet undefined, brain sites.

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