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Opiate Withdrawal–induced Hyperactivity of Locus Coeruleus Neurons Is Substantially Mediated by Augmented Excitatory Amino Acid Input

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Single-cell activity was recorded in the locus coeruleus (LC) of morphine-dependent, halothane-anesthetized rats. Systemic administration of the opiate antagonist naloxone (0.1 mg/kg, i.v.) robustly increased the activity of LC neurons. Local microinjection of naloxone or of its hydrophilic derivative, naloxone methiodide, into LC (10 mM, 20–40 nl) did not activate LC neurons in dependent rats. Intracerebroventricular or intracoerulear injection of kynurenate, a broad-spectrum antagonist of excitatory amino acids (EAAs), substantially but incompletely attenuated the activation of LC cells induced by intravenous naloxone-precipitated withdrawal (more than 50% blockade). Intracoerulear microinjections of the non-NMDA-receptor antagonist 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX) or the selective NMDA-receptor antagonist AP5 significantly reduced the withdrawal-induced excitation. AP5 was the least effective among all antagonists tested. Similar microinjections of kynurenate or CNQX almost completely suppressed the excitation of LC neurons induced by electrical stimulation of a rear footpad. LC responses to footpad stimulation (mediated by endogenous EAAs) or iontophoretically applied glutamate were not modified by the chronic morphine treatment. These results indicate that a substantial part of LC hyperactivity during opiate withdrawal is mediated by an augmented EAA input to LC.

Dependence on abused substances constitutes one of the major problems in modern society. Such dependence originates from long-term adaptative processes in the nervous system due to its repeated exposure to drugs. However, the cellular mechanisms of such changes in the CNS or PNS are poorly understood.

Neurochemical changes have been found for chronic exposure to opiates in animals, including an upregulation of G-protein transduction systems (Nestler et al., 1989), increased adenylate cyclase activity (Duman et al., 1988), increased cAMP-dependent protein kinase activity (Nestler and Tallman, 1988), and an induction of phosphorylated proteins (Guitart and Nestler, 1989, 1990), one of which has been identified as tyrosine hydroxylase (Guitart et al., 1990). Interestingly, all these neurochemical changes occur simultaneously in the major noradrenergic (NA) cell group of the brain, the nucleus locus coeruleus (LC). Moreover, these NA neurons are well known to markedly increase their electrical impulse (Aghajanian, 1978; Rasmussen and Aghajanian, 1989b; Valentino and Wehby, 1989; Rasmussen et al., 1990) as well as metabolic (Kimes et al., 1990) activities during opiate withdrawal. Evidence indicates that the opiate abstinence syndrome involves several brain areas in addition to the LC system (such as the periaqueductal gray, mesolimbic dopamine system, and spinal cord; see Koob and Bloom, 1988). Nonetheless, many symptoms of opiate withdrawal are related to functions ascribed to the NA-LC system, including anxiety, hyperalgesia, enhanced startle, and altered sleep and waking (Gold et al., 1980; Redmond and Krystal, 1984). In addition, withdrawal symptoms in human addicts are substantially alleviated by administration of the α_2 adrenergic agonist clonidine (Gold et al., 1978, 1980), perhaps in part via inhibition of NA cell activity (Svensson et al., 1975; Foote et al., 1983). These findings underscore the potential importance of studies that elucidate the mechanism of activation of LC neurons during opiate withdrawal.

LC neurons in morphine-dependent rats were reported to be excited upon microiontophoretic application of the opiate antagonist naloxone (NLX) (Aghajanian, 1978). However, the excitation observed did not mimic in magnitude the effect of systemic opiate antagonists (Rasmussen and Aghajanian, 1989b; Rasmussen et al., 1990). Therefore, as pointed out by others (Christie et al., 1987), it was not clear whether this locally induced activation represented a simple reversal from the tonic inhibition produced by circulating morphine (if tolerance is incomplete) or a true, withdrawal-induced hyperactivity.

The possibility that withdrawal-induced hyperactivity of LC neurons is not induced by altered intracoerulear mechanisms was strongly supported by the lack of change observed during opiate withdrawal in LC neurons recorded in the *in vitro* slice (Andrade et al., 1983; Christie et al., 1987). This result suggested that afferents to LC may be the primary sites responsible for the robust activation of LC neurons precipitated by sudden opiate withdrawal. Two major afferents to LC (Aston-Jones et al., 1986, 1991c), and therefore prime candidates for mediating the withdrawal response in LC, are the nucleus paragigantocellularis (PGi) in the rostral ventrolateral medulla and the medial perifascicular area of the nucleus prepositus hypoglossi (PrH) in the dorsomedial rostral medulla. The PrH seems an unlikely candidate for mediating withdrawal-induced activation of LC,

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as it provides strong GABAergic inhibitory input to LC (Ennis and Aston-Jones, 1989a,b). The PGi, in contrast, provides potent excitatory amino acid (EAA) input to LC (Ennis and Aston-Jones, 1986, 1988; Aston-Jones and Ennis, 1988). Consistent with the possibility that PGi-EAA afferents may mediate opiate withdrawal hyperactivity in LC, recent studies have shown that such withdrawal-induced hyperactivity of LC neurons was attenuated by intracerebroventricular administration of the EAA antagonist kynurenic acid (KYN) (Rasmussen and Aghajanian, 1989b; Tung et al., 1990), or by lesion of the PGi, but not by PrH lesion (Rasmussen and Aghajanian, 1989b). However, it is not certain whether the reduction of LC hyperactivity observed in these studies was due to antagonism of the EAA pathway from PGi to LC. Indeed, coagulation lesions of PGi areas that attenuated the withdrawal response of LC were unable to block the excitation of LC neurons by sensory stimuli (Rasmussen and Aghajanian, 1989a,b), a response shown by our laboratory to be mediated by a direct EAA pathway to LC from PGi (Chiang and Aston-Jones, 1989; Aston-Jones et al., 1991b). This suggests that, in addition to the EAA pathway involved in sensory responses, there may be another pathway that mediates LC hyperactivity during opiate withdrawal. As intracerebroventricular injection of KYN is expected to block EAA neurotransmission in the entire brain, it was also unclear from the previous results whether the EAA synapse involved in opiate withdrawal–induced activation of LC is located within LC or in a distant afferent.

In the present study, we demonstrate that LC neurons in dependent animals are not provoked into opiate withdrawal hyperactivity by local microinjection of NLX into LC. In addition, we show that enhanced EAA neurotransmission within LC is responsible for most of the hyperactivity of NA-LC neurons induced by opiate withdrawal.

These data have been partially published in abstract form (Akaoka et al., 1990).

Materials and Methods

Chronic treatment

Male Sprague–Dawley rats of 300–400 gm (Taconic Farms) were used. Rats were implanted subcutaneously with osmotic minipumps (ALZA, model 2001) under light halothane anesthesia, using routine sterile conditions. On the day of implantation, morphine sulfate was dissolved in ultrafiltered water and loaded into the minipumps. Rats recovered from surgery quickly and were kept in normal housing conditions (12 hr:12 hr light/dark cycle, water and food ad libitum). Morphine sulfate was continuously infused from the implanted pump at a rate of approximately 2 mg/kg/hr. Electrophysiological experiments were carried out on the sixth day of chronic treatment.

Acute experiments

Animal surgery. On the sixth day of continuous exposure to morphine, rats were anesthetized with halothane (1.5–2% in oxygenated air) through a face mask. The right femoral vein and trachea were cannulated, and halothane was subsequently administered via spontaneous respiration through the tracheal cannula. The rectal temperature was maintained between 36°C and 38°C by a feedback-controlled heating pad for the entire experiment. Rats were mounted in a conventional stereotaxic frame, the skull surface was exposed, and the snout was lowered to place bregma 2 mm below the lambda suture point. The skull overlying LC was drilled and the dura carefully removed. In some experiments, a guide cannula (26 gauge) was inserted into the lateral ventricle. Intracerebroventricular injections were made through an inner cannula (30 gauge) extending 1 mm below the tip of the guide into the ventricle.

At the end of the surgery, the concentration of halothane was lowered to about 1%, where it remained for the duration of the experiment.

Single-cell recording. Glass micropipettes were made on a vertical puller (Narishige PE-2), and the tips were broken to an external diameter of 3–5 μm. They were filled with a sodium acetate solution (0.5 M, pH 7.4) containing 2% pontamine sky blue dye. For intracoerulear microinjection of substances, another glass micropipette was glued next to the recording micropipette as described below. The electrode was lowered into LC, and the extracellular electrical signal was fed to a high input-impedance preamplifier. Signals were filtered with a wide band pass (1 Hz to 10 kHz) to monitor field potentials in the cerebellum overlying LC and to identify the characteristic shape of the extracellular spike of LC neurons (Foote et al., 1983; Aston-Jones et al., 1991a), and with a high-pass filter (0.5–10 kHz) to discriminate neuronal spike activity.

LC neurons were tentatively identified following criteria described elsewhere (Cedarbaum and Aghajanian, 1976; Aghajanian, 1978; Foote et al., 1983; Aston-Jones et al., 1991a). Briefly, under wide band pass filtering they typically exhibited an entirely positive spike of relatively long duration (2–4 msec), often preceded by a positive notch. In addition, presumed NA-LC neurons responded to contralateral foot pinch or tail pinch with an initial activation followed by a prolonged postactivation inhibition.

The impulse activity of a single neuron was detected through a time–voltage discriminator, whose output was led to a chart recorder and fed to a computer via a CED 1401 interface (Cambridge Electronic Design, United Kingdom). Data were stored on floppy disks and analyzed off line by ad hoc programs (SPIKE 2, Cambridge Electronic Design). The effects of contralateral rear footpad stimulation (FS; see below) were also captured as peristimulus time histograms (PSTHs) with an Apple II computer (aquisition and analysis programs originally developed by K. Liebold, Scripps Research Clinic).

After recording sessions, dye was iontophoretically deposited from the recording micropipettes to verify histological location of recording sites (-7 μA, 4 sec pulse, 4 sec pause, for 10 min). All locations of presumed LC neurons marked by dye were found to originate from sites within the nucleus LC as revealed by neutral red counterstaining. As this nucleus is composed almost entirely of dopamine β-hydroxylase–immunoreactive (Foote et al.,1983) or tyrosine hydroxylase mRNA–positive cell bodies in the rat (Bérod et al., 1987), all recordings are considered to arise from NA-LC neurons.

Intravenous injection procedures. Opiate withdrawal was precipitated by intravenous injection of 0.1 mg/kg naloxone hydrochloride (Sigma), as commonly used in electrophysiological studies. Only one injection was made per animal. Morphine sulfate was acutely injected at doses of 3–5 mg/kg. The drugs (prepared fresh for each experiment) were dissolved in physiological saline and administered through the femoral vein cannula in a volume of 1 ml/kg.

Intracerebroventricular injection procedures. The vehicle for intracerebroventricular or intracoerulear injections, artificial cerebrospinal fluid (CSF), contained (in mM) NaCl, 122; KCl, 3.1; NaH_2PO_4, 0.4; $MgSO_4$, 1.2; $CaCl_2$, 1.3; $NaHCO_3$, 25; and glucose, 10. Five microliters of 0.1 M kynurenate (Sigma; pH 6.5–7.5) or artificial CSF alone were slowly infused intracerebroventricularly (typically over 1 min) with a Hamilton syringe. Only one injection was made, and one cell studied, per animal. The kynurenate solution for intracerebroventricular injection was freshly made for each experiment.

Intracoerulear pressure microinjection procedures. Local microinjections were made with a double pipette assembly, consisting of an injection micropipette of narrow inner diameter (Fisher) glued next to the recording pipette (Akaoka et al., 1987; Shiekhattar et al., 1991). Injection pipettes were pulled and broken to a tip diameter of 30–50 μm. These injection pipettes were heated and bent at an angle of 20–30° at about 10 mm from the tip. Using precision micromanipulators, the injection pipette was positioned parallel to the recording microelectrode (Curtis, 1968), so that the tip of the recording micropipette extended 100 μm beyond the injection pipette. The double pipette assembly was then secured using photopolymerizing resin (Silux, 3M). Generally, those compound pipettes were made in advance and could be stored for several weeks without noticeable change in recording or injection characteristics. Injection pipettes were filled by suction using a 10 ml syringe connected to the pipette via a three-way stopcock.

Intracoerulear microinjections were made as follows. Once the recorded neuron was tentatively characterized as a presumed LC neuron, the position of the electrode was carefully adjusted to obtain a stable recording with a signal at least four times the noise, which was typically 50 μV. Microinjections were performed using a solenoid-controlled pneumatic pressure device (Picospritzer, General Valve Inc.). The vol-

ume ejected was quantified on line under microscopic control (magnification, of 10–20×) by measuring the movement of the liquid column inside the pipette with a precision of 100 μm (corresponding to about 6 nl). Pressure (5–50 psi) was applied in pulses, 50–1000 msec in duration, given with a frequency of 1–10 Hz. The injection flow rate could be finely adjusted by varying pulse duration, pulse frequency, or both. Only one injection was made post-NLX, and one cell studied, per animal.

NLX, naloxone methiodide (MeNLX; Research Biochemicals, Inc.), KYN (Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, RBI), 2-amino-5-phosphonopentanoic acid (AP5; RBI) were prepared in artificial CSF. KYN, CNQX, and AP5 were stored at −20°C as stock solutions of 100, 2, and 1 mM, respectively, and diluted in artificial CSF on the day of use.

Microiontophoresis. Microiontophoresis of glutamate (Chouvet et al., 1988; Aston-Jones et al., 1991a; Charléty et al., 1991) was carried out with seven-barrel micropipettes (tip diameter, about 10 μm) to which a recording pipette was secured, as described above for pressure microinjection pipettes. The distances between the tips of recording and ejection pipettes were 15–20 μm.

Electrical stimulation of the rear footpad. A pair of 26 gauge needle electrodes were subcutaneously inserted into the medial aspect of the rear footpad of the rear footpad contralateral to LC recording for stimulation of the sciatic nerve. Monophasic, bipolar square pulses of 500 μsec (10 mA) were delivered every 2 sec, and PSTHs (8 msec bin width) were constructed from 50 consecutive stimuli.

Behavioral testing

Three groups were used in behavioral tests of withdrawal: (1) dummy pump implant followed by naltrexone (NTX) injection ($n = 2$), (2) chronic morphine followed by saline injection ($n = 2$), and (3) chronic morphine followed by NTX injection ($n = 11$). The following behavioral signs before (10 min) and following (45 min) NTX or saline injection were scored as described previously (Aston-Jones et al., 1991b). Here, NTX was used instead of NLX as its effect lasts much longer, ensuring the effectiveness of opiate withdrawal during the entire period.

Data analysis

Baseline spontaneous impulse activity was recorded for each cell for 3–5 min before drug injection. For the activation of LC neurons with opiate withdrawal, and its reversal by EAA antagonists, three values expressed as absolute discharge rate were collected for each cell: baseline activity, maximum activity following intravenous NLX, and minimum activity after administration of an EAA antagonist. The absolute increase in firing rate (FR) from baseline produced by intravenous NLX, and the absolute FR decrease produced by EAA antagonists from NLX-induced activation were calculated for each case. As local administration of EAA antagonists produced small decreases in the baseline discharge rate of LC neurons (see Results), we corrected each value of decrease from NLX-induced activation by subtracting the mean value of decrease in baseline observed for each EAA antagonist. We used values obtained from separate samples of cells as the effects of local EAA antagonists on the baseline activity could not be obtained from the cells tested for NLX-induced excitation. The degree of blockade of NLX-induced hyperactivity by subsequently administered agents was then computed as follows. We determined the mean increase of FR induced by NLX for all cells tested (a) and the mean of corrected (see above) decrease from NLX-induced excitation for each local EAA antagonist (b). The percentage of blockade was obtained by dividing b by a, for each EAA antagonist.

Excitation evoked by contralateral footpad stimulation (FS) was analyzed as described in an earlier publication (Aston-Jones and Bloom, 1981). The mean number and standard deviation (SD) of action potentials per bin were calculated for the prestimulus (baseline) period (504 msec). The baseline mean + 2 SD was used as the limit to detect the onset and offset of excitation. Response magnitudes were calculated as the total counts during the excitatory epoch minus the number of spikes that would be expected to occur during the same epoch if no stimulus was given, that is, response magnitude = (number of spikes in excitatory epoch) − (mean spikes per baseline bin × number of bins in the excitatory epoch). Excitation evoked by FS is expressed as the normalized number of action potentials elicited by one stimulus.

Statistical analyses were performed as follows. Differences in LC discharge between (1) baseline versus NLX-induced withdrawal, (2) NLX-induced withdrawal before versus after EAA antagonist administration (corrected for EAA antagonist-induced changes in baseline, described above), and (3) baseline versus NLX-induced withdrawal after EAA antagonist administration were tested by Student's paired t tests. The potencies of treatments on NLX-induced LC excitation between EAA antagonists were analyzed as follows: the mean absolute discharge rates obtained after EAA antagonists were compared by one-way analysis of variance (ANOVA) followed by the least-significant difference, multiple-range test. Student's t tests for two independent samples were used to compare the mean discharge rates in dependent versus naive rats. Paired Student's t tests were used to analyze (1) the effects of intracoerulear or intravenous injection of EAA or opiate antagonists on the spontaneous activity of LC neurons, and (2) the effects of intracoerulear microinjection of EAA antagonists on FS responses. The potencies of different EAA antagonist treatments on FS responses were compared using the ANOVA procedures described above. The excitatory responses to microiontophoretic applications of glutamate were analyzed as described in detail elsewhere (Aston-Jones et al., 1991a; Charléty et al., 1991).

Behavioral signs of opiate withdrawal were statistically analyzed using the Fisher–Pitman randomization test, which directly computes the probability of obtaining the observed values (Krauth, 1988). As animals in the control groups (I and II) exhibited no withdrawal symptoms (see Results), they were pooled for the statistical test.

Data are expressed as the mean ± standard error of the mean (SEM). All statistical tests were two tailed with a significance level of 0.05.

Results

Chronic treatment with morphine using osmotic minipumps

We verified that rats receiving morphine chronically from osmotic minipumps exhibited behavioral withdrawal when challenged on the sixth day by NTX (1 mg/kg, i.p.; Sigma). Strong behavioral symptoms of abstinence were consistently elicited only in experimental subjects belonging to group III (chronically exposed to morphine and challenged with NTX). The frequencies of jumping, wet shakes, chews, and teeth chatter were significantly greater than those in control (Fig. 1). Also, diarrhea (11/11), rhinorrhea (8/11), lacrimation (3/11), ptosis (9/11), and piloerection (6/11) were present only in the withdrawal group (3). None of the withdrawal signs assessed here were observed in rats belonging to the control groups (1 and 2).

We also verified that in rats receiving chronic morphine from osmotic minipumps, LC neurons exhibited the characteristic hyperactivity of discharge when opiate withdrawal was precipitated by intravenous NLX (0.1 mg/kg; $n = 19$, one cell tested per rat). The mean spontaneous activity in treated animals before NLX was 1.3 ± 0.2 Hz. This value was not statistically different from the mean obtained in naive animals (1.5 ± 0.2 Hz; $n = 16$, $p > 0.49$). NLX administration strongly activated the discharge rate of LC neurons in treated animals, yielding a mean rate (4.6 ± 0.4 Hz within 2 min after injection; $p < 0.001$ compared to pre-NLX) similar to that reported in another study (Rasmussen and Aghajanian, 1989b). In naive animals ($n = 8$), NLX had no significant effect on LC discharge rate (1.5 ± 0.2 Hz and 1.7 ± 0.1 Hz before and after intravenous NLX, respectively; $p > 0.4$).

Effects of intracoerulear microinjection of opiate antagonists

We studied the effects of local microinjection of NLX or MeNLX, a hydrophilic derivative of NLX, on the spontaneous activity of LC neurons in five dependent rats to evaluate whether local opiate withdrawal within LC may account for the hyperactivity of these cells induced by systemic NLX. Local microinjection of NLX (10 mM) failed to affect consistently the spontaneous discharge of LC neurons (mean change, 8 ± 9%; $p > 0.59$; $n = 8$; ejected volume, 42 ± 11 nl; Fig. 2*A*), with only one of eight cells robustly excited. Intracoerulear microinjection of MeNLX

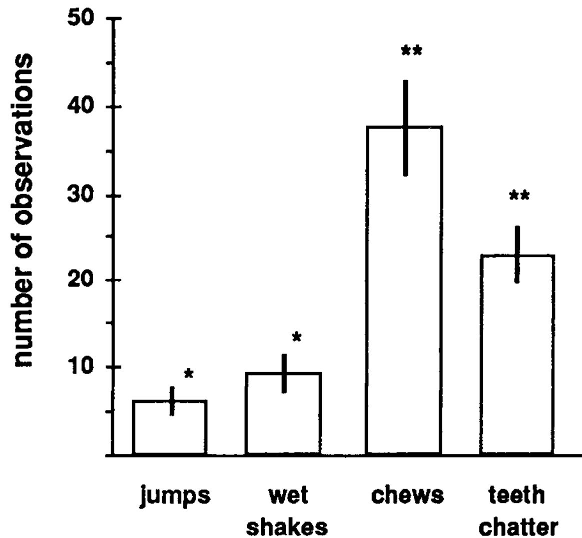

Figure 1. Behavioral measures of opiate withdrawal in rats treated chronically with morphine via osmotic minipumps. Behavioral signs of opiate withdrawal only occurred in the experimental group chronically exposed to morphine and challenged with NTX (see text; values for control animals were zero for all measures). The *ordinate* represents the number of epochs of each behavior observed during 45 min following NTX injection (1 mg/kg, i.p.). *, $p < 0.008$; **, $p < 0.002$.

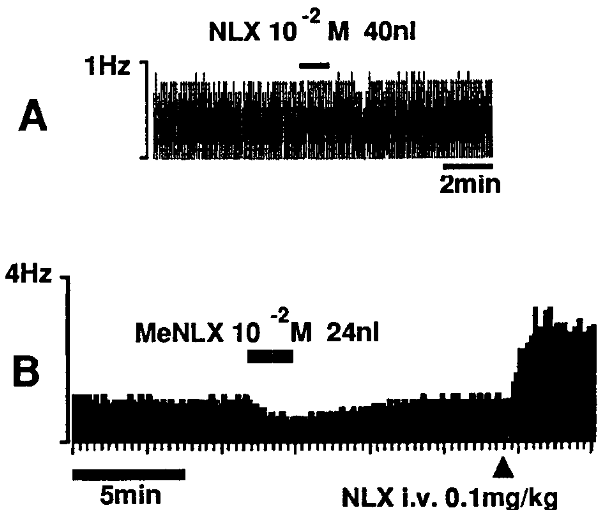

Figure 2. Rate meter records showing the effects of local microinjection of opiate antagonists into LC on the discharge of NA-LC neurons in morphine-dependent rats. *A,* Local opiate withdrawal by intracoerulear microinjection of NLX (40 nl, 10 mM; at *upper bar*) did not affect the firing rate of LC neurons. *B,* Intracoerulear microinjection of MeNLX (24 nl, 10 mM; at *upper bar*) slightly inhibited the discharge of LC neurons. However, this LC neuron was strongly activated by intravenous administration of NLX (0.1 mg/kg; at *arrowhead,* below). LC impulse activity was integrated over 5 sec (*A*) or 10 sec (*B*).

(10 mM) slightly, but consistently, inhibited LC neurons (mean change, $-25 \pm 15\%$; $p < 0.004$; $n = 7$; ejected volume, 20 ± 4 nl; Fig. 2*B*). Microinjection of vehicle alone (125 ± 16 nl; $n = 6$) had no effect on the discharge rate of LC neurons.

Similar microinjections of NLX (10 mM, less than 10 nl), however, completely and rapidly reversed the inhibition of LC neurons produced by systemic morphine in naive rats (3–5 mg/kg i.v.; $n = 3$; data not shown).

Effects of intracerebroventricular injection of KYN on NLX-induced activation of LC neurons

Intracerebroventricular administration of KYN (0.5 μmol) consistently and significantly antagonized the activation of LC neurons induced by intravenous NLX (Fig. 3*A*, Table 1). KYN attenuated the withdrawal-activated discharge of LC neurons from 5.3 to 2.1 Hz. Although substantial, this reversal of the NLX-induced activation was incomplete, as discharge post-KYN (2.1 ± 0.3 Hz) was significantly elevated compared to pre-NLX in the same cells (0.9 ± 0.2 Hz; $p < 0.04$). Injection of vehicle (artificial CSF, 5 μl) did not modify the hyperactivity of LC neurons induced by intravenous NLX ($n = 3$).

The effects of the same intracerebroventricular KYN injections were also examined on FS-induced activation for four of six cells tested above. While all four cells were strongly excited by FS prior to intracerebroventricular KYN, no significant excitation was observed during the 10 min period following KYN administration, as reported previously (Aston-Jones and Ennis, 1988; Ennis and Aston-Jones, 1988) (data not shown).

Effects of intracoerulear microinjection of EAA antagonists on withdrawal-evoked or sensory-evoked activation of LC neurons

Withdrawal-induced activation of LC neurons was significantly decreased by local infusion of each of the three EAA antagonists tested (Table 1, Fig. 3). However, the reversal produced by local EAA antagonists was never complete. As depicted in the rate meter records of Figure 3, irrespective of the drug infused, the onset of decrease was fast (typically within 10 sec); however, the new rate plateaued about 1 min later.

As shown in Table 1, local KYN (10 mM, 51 ± 18 nl; $n = 5$) significantly attenuated withdrawal-induced activation of LC neurons ($p < 0.04$; 63% blockade), returning the activity of tested cells to the level similar to that obtained by intracerebroventricular administration of the same antagonist (about 2 Hz). The mean FR obtained following local KYN (2.0 ± 0.2

Table 1. Effects of intracoerulear microinjection of EAA antagonists on LC hyperactivity induced by intravenous NLX-precipitated morphine withdrawal

	n	Ejected volume	Baseline	Intravenous NLX (0.1 mg/kg)	EAA antagonists
Intracerebroventricular KYN	6	—	0.9 ± 0.2 Hz	5.3 ± 1 Hz	2.1 ± 0.3 Hz[a]
KYN, 10 mM	5	51 ± 19 nl	0.9 ± 0.1 Hz	3.8 ± 0.6 Hz	2 ± 0.2 Hz[a]
CNQX, 100 μM	5	96 ± 35 nl	1.5 ± 0.4 Hz	4.4 ± 0.5 Hz	2.9 ± 0.4 Hz[a]
AP5, 100 μM	5	144 ± 20 nl	1.8 ± 0.2 Hz	4.6 ± 0.6 Hz	3.7 ± 0.6 Hz[a,b]

[a] Significantly different when compared to the corresponding discharge rates after intravenous NLX.

[b] Significantly different when compared to the discharge rates obtained after intracerebroventricular or local KYN.

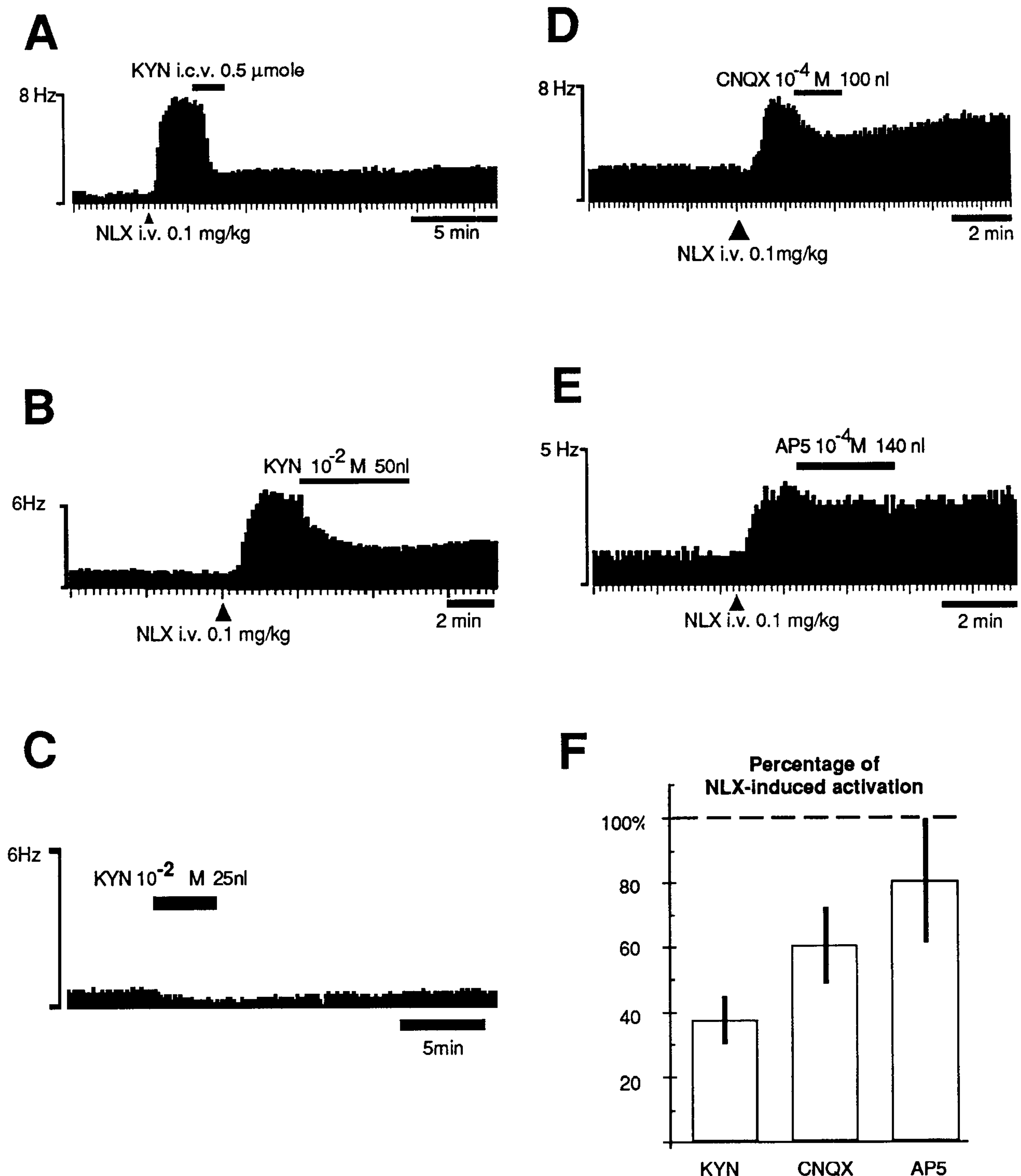

Figure 3. Effects of intracerebroventricular administration or intracoerulear microinjection of EAA antagonists on the activation of LC neurons induced by intravenous NLX-precipitated opiate withdrawal. *A,* Intracerebroventricular injection of KYN (0.5 µmol in 5 µl) strongly attenuated the withdrawal hyperactivity of LC neurons precipitated by intravenous NLX (0.1 mg/kg). *B, D,* and *E,* Local, intracoerulear microinjection of EAA antagonists attenuated in various degrees the withdrawal hyperactivity of LC neurons. Note the rapid onset of blockade, irrespective of the antagonist used (less than 10 sec in general). *C,* Effects of local, intracoerulear microinjection of KYN on the spontaneous activity of LC neurons in morphine-dependent rats. The decrease of baseline activity produced by local application of KYN was much smaller than that of NLX-induced activation (*B*), while the mean ejected volumes were similar (38 ± 9 vs 51 ± 19 nl). The two other EAA antagonists tested (CNQX and AP5) were less potent than KYN. Values plotted were corrected for decreased baseline activity elicited by EAA antagonists (see Materials and Methods). Once the discharge rate plateaued following local EAA antagonists, further infusion did not produce any additional decrease. *F,* Histogram illustrating the relative potencies of locally administered EAA antagonists in attenuating activation of LC neurons induced by intravenous NLX-precipitated morphine withdrawal. All local antagonists significantly, but incompletely, decreased the withdrawal hyperactivity of LC neurons (see Results). All tests were post- versus pre-EAA antagonist.

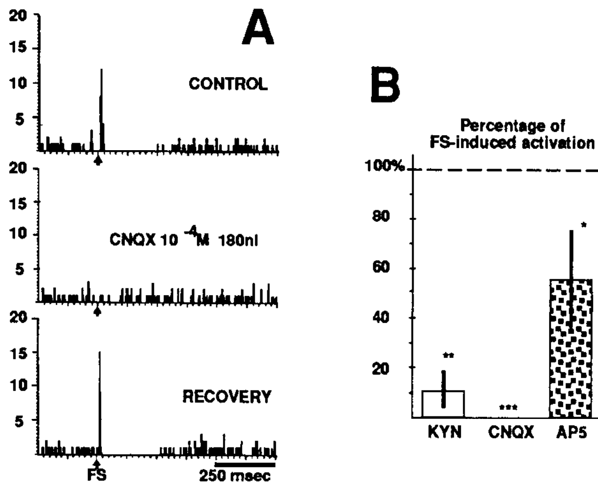

Figure 4. Effects of local, intracoerulear microinjection of EAA antagonists on the excitatory response of LC neurons in morphine-dependent rats to electrical stimulation of the contralateral rear footpad (*FS*). Withdrawal activation of these same cells was also decreased by local EAA antagonism (see Table 1). *A,* PSTHs showing the effect of CNQX microinjection on a typical LC neuron. CNQX completely suppressed the FS-evoked responses in all LC cells studied. Recovery PSTH was taken 25 min after CNQX injection. FS was presented at *arrow* in each PSTH. *B,* Histogram illustrating relative potencies of locally applied EAA antagonists in attenuating FS responses of LC neurons in morphine-dependent rats. Local KYN (10^{-2} M, 38 ± 9 nl) was nearly as potent as CNQX (10^{-4} M, 117 ± 30 nl), while AP5 (10^{-4} M, 108 ± 25 nl) was significantly less effective than the two other antagonists. *, $p < 0.02$; **, $p < 0.002$; ***, $p < 0.0005$; all tests were post- versus pre-EAA antagonist.

Hz) was significantly elevated compared to activity pre-NLX in the same cells ($p < 0.005$). Local microinjection of CNQX (100 μM, 96 ± 35 nl; $n = 5$) also substantially antagonized withdrawal-induced excitation of LC cells ($p < 0.02$; 39% blockade). The FR post-CNQX (2.9 ± 0.4 Hz; $n = 5$) was significantly higher than the spontaneous FRs observed pre-NLX ($p < 0.05$). Finally, local microinjection of AP5 (100 μM, 144 ± 20 nl; $n = 5$), while significantly attenuating withdrawal activation of LC neurons ($p < 0.05$), was the least potent among EAA antagonists tested ($p < 0.05$; 18% blockade; Table 1). The FR of LC neurons after AP5 (3.7 ± 0.7 Hz; $n = 5$) was also significantly higher than the spontaneous FR of the same neurons obtained pre-NLX ($p < 0.03$).

The spontaneous discharge of LC neurons in dependent rats was marginally decreased by intracoerulear microinjection of KYN (38 ± 9 nl; −0.5 ± 0.2 Hz; $p > 0.07$; Fig. 3C), CNQX (117 ± 30 nl; −0.4 ± 0.2 Hz; $p > 0.05$), or AP5 (108 nl ± 25 nl; −0.3 ± 0.2 Hz; $p > 0.08$). In contrast, excitatory responses of the same cells to FS were dramatically reduced by the same microinjection of KYN and CNQX, and less markedly, but still significantly, by AP5 (Fig. 4). AP5 was significantly less effective in decreasing FS response than CNQX or KYN ($p < 0.05$).

Neither withdrawal-induced hyperactivity nor FS-induced excitation of LC neurons was affected by local microinjection of vehicle (artificial CSF; 80 ± 19 nl, $n = 6$; and 125 ± 16 nl, $n = 6$, respectively).

Responsiveness of LC neurons to FS and iontophoretically applied glutamate in dependent versus naive animals

The results shown above indicate that the withdrawal hyperactivity of LC is substantially mediated by EAA neurotransmission within LC. To ascertain whether changes in the responsiveness of LC neurons to EAAs accompany withdrawal-induced hyperactivity, the effects of endogenous EAA release evoked by FS (Aston-Jones and Ennis, 1988; Ennis and Aston-Jones, 1988), or of iontophoretically applied Glu (Chouvet et al., 1988; Aston-Jones et al., 1991a; Charléty et al., 1991), were compared in dependent versus naive animals.

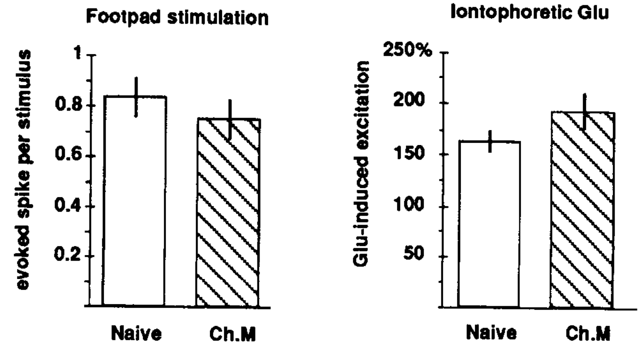

Figure 5. Histogram comparing the responsiveness of LC neurons to FS (*left*) or to iontophoretic application of Glu (*right*), in naive versus chronically morphine-treated rats, as indicated. The responsiveness of LC neurons to FS or to Glu was not significantly modified by chronic administration of morphine (*Ch.M*). See text for details.

The responsiveness of LC neurons ($n = 31$) to FS in morphine-dependent animals was very similar to that observed in naive animals (20 cells; $p > 0.42$), as shown in Figure 5 (left). In addition, chronic administration of morphine did not significantly modify the responsiveness of LC neurons ($p > 0.13$; Fig. 5, right) to Glu applied iontophoretically ($n = 9$ and 5, mean currents of 32 ± 2 nA and 38 ± 10 nA, respectively, in naive and dependent rats).

Discussion

Our results demonstrate that the hyperactivity of noradrenaline (NA)-containing neurons in the LC observed during opiate withdrawal precipitated by NLX is substantially mediated by direct EAA inputs to LC, as it is largely attenuated by intracoerulear microinjection of EAA antagonists. THe EAA inputs seem to act mainly on non-NMDA receptors, although the partial blockade obtained by a selective NMDA antagonist (AP5) suggests a significant contribution of NMDA receptor activation in the withdrawal-induced LC excitation.

As shown by both behavioral and electrophysiological experiments, the chronic administration of morphine using osmotic minipumps very reliably induced (100% of cases) opiate dependence, as also shown by others (Simson and Weiss, 1989; Adams and Holtzman, 1990). This procedure avoids repeated stressful stimuli accompanying morphine pellet implantation and, unlike administration in drinking water, allows an effective, regular, known intake of morphine by treated animals.

LC neurons in opiate-dependent rats were not activated by intracoerulear microinjection of NLX or MeNLX, although a relatively high concentration was used (10 mM). Such microinjections of NLX, however, effectively blocked opiate receptors in LC, as they reversed the inhibition of LC neurons produced by systemic morphine in naive animals. This is also consistent with the high specific affinity of NLX for opiate receptors (particularly the μ-subtype predominant within LC; see North et al., 1987; Valentino, 1987). Moreover, concentrations of NLX sim-

ilar to those used here have been found effective in blocking opiate receptors in other areas of the brain (Azami et al., 1982; Baumeister et al., 1988). In addition, the local doses of MeNLX used here (about 100 ng) have been shown to induce withdrawal behavior by intracerebral microinjection (Maldonado et al., 1990). The near lack of effect of local opiate antagonism in LC also suggests that LC neurons were almost completely tolerant to circulating morphine.

Our results differ slightly from another study *in vivo* (Aghajanian, 1978). There, microiontophoretic ejection of NLX activated LC neurons in dependent animals, although this excitation (about 100% increase) was lower than that induced by systemic administration of opiate antagonists reported in another article from the same laboratory (Rasmussen and Aghajanian, 1989b) or here (more than 200%). This discrepancy in results may be due to the different methods used to apply NLX in their study and in ours. In particular, the concentration of NLX applied is unknown with iontophoretic delivery, and it is possible that in their study concentrations were reached where NLX might have acted as a $GABA_A$ antagonist, as shown previously (Dingledine et al., 1978). The presently observed inability of pressure-applied, intracoerulear opiate antagonists to induce withdrawal activation of LC neurons is in complete agreement with previous *in vitro* studies. LC neurons in brain slices of morphine-dependent rats failed to exhibit any sign of withdrawal hyperactivity when perfused with morphine free- or NLX-containing bath solutions (Andrade et al., 1983; Christie et al., 1987).

One hypothesis proposed for the mechanism of LC hyperactivity during opiate withdrawal involves the upregulation of the G-protein/cAMP system observed within the LC region in dependent animals (Rasmussen et al., 1990). According to this hypothesis, upregulation of the cAMP system may "represent a homeostatic response to persistent opiate inhibition of the cells" (Rasmussen et al., 1990), which presumably results in LC discharge close to control levels. When the morphine is withdrawn or an opiate antagonist is administered, "the up-regulated cAMP system, unopposed by morphine, would increase the activity of LC neurons" (Rasmussen et al., 1990). The lack of effect of local, NLX-precipitated opiate withdrawal on LC discharge observed in dependent animals, both *in vitro* (Andrade et al., 1983; Christie et al., 1987) and in the present study *in vivo*, argues against that hypothesis.

The experiments using intracerebroventricular and intracoerulear administration of EAA antagonists were designed to evaluate the contribution of direct EAA inputs to LC in the withdrawal activation of LC neurons. Indeed, intracerebroventricular injection of a broad-spectrum EAA antagonist, KYN, prior to opiate antagonists, has been reported to prevent substantially the LC hyperactivity during withdrawal (Rasmussen and Aghajanian, 1989b; Tung et al., 1990). In our protocol, EAA antagonists were injected after LC activation by intravenous NLX. This procedure demonstrated that the tested neurons were indeed sensitive to opiate withdrawal, unlike in the previous studies (Rasmussen and Aghajanian, 1989b; Tung et al., 1990). In agreement with these studies, the effects of EAA antagonists we observed seem due mainly to antagonism of increased EAA neurotransmission subsequent to opiate withdrawal, as the basal activity of LC neurons is only slightly affected by intracerebroventricular (Ennis and Aston-Jones, 1988) or local application of EAA antagonists (see Results; see also Aston-Jones et al., 1991a). This conclusion is also supported by our preliminary experiments in which we locally infused KYN prior to NLX: the mean FR after withdrawal (2.2 Hz; $n = 3$) was considerably lower than the control FR post-NLX (4.6 Hz) and was very similar to the value obtained by KYN subsequent to NLX (2.0 Hz; see Table 1).

Results obtained by intracerebroventricular administration of KYN do not specify where in the brain EAAs are involved. Previous results (Rasmussen and Aghajanian, 1989b) indicate that the EAA pathway to LC from PGi that mediates sensory activation of LC (Aston-Jones and Ennis, 1988; Ennis and Aston-Jones, 1988) is not substantially involved in the LC hyperactivity induced by opiate withdrawal. This conclusion was based on the finding that tissue lesions in PGi, which attenuated the withdrawal-induced excitation of LC, did not suppress the FS-induced response. However, our results demonstrate that the withdrawal-induced hyperactivity of LC neurons is mediated by EAA synaptic interactions in the LC region. Taken together, these results (Aston-Jones and Ennis, 1988; Ennis and Aston-Jones, 1988; Rasmussen and Aghajanian, 1989a,b) may indicate that there are two EAA pathways from PGi to LC, one that mediates FS (and perhaps other sensory) responses and another that mediates opiate withdrawal–induced hyperactivity of LC neurons. Additional experiments are needed to test this hypothesis, but it would be consistent with recent anatomical (Guyenet and Young, 1987; Astier et al., 1990; Pieribone and Aston-Jones, 1991) and physiological (Aston-Jones et al., in press) evidence for functionally and anatomically distinct projections from PGi to LC.

Intracoerulear microinjection of all three EAA antagonists used significantly decreased the activation of LC neurons precipitated by intravenous NLX. This antagonism was of fast onset (less than 10 sec), which indicates that the EAA antagonists acted nearby the ejection site, that is, within LC. The delay of 1–2 min before a steady, lower discharge rate was obtained after antagonist administration may be explained by the necessity to block EAA receptors on distal LC dendrites, as has been shown for cerebellar or spinal cord cells (Chujo et al., 1975; Zieglgänsberger and Champagnat, 1979; Crepel et al., 1982; Hockberger et al., 1989a,b; Sugimori and Llinás, 1990).

Although all three EAA antagonists significantly attenuated the LC hyperactivity produced by opiate withdrawal, the magnitude of blockade obtained differed among the agents used. Local KYN was the most effective, followed by CNQX and AP5 (see Fig. 3*F*). We used concentrations of these EAA antagonists expected from other studies (Chiang et al., 1990; Shiekhattar and Aston-Jones, 1990) to achieve full or nearly full blockade of EAA-mediated responses in LC *in vivo*. Thus, microinfusion of 50 μM CNQX completely blocked FS responses to LC neurons, a response mediated primarily by non-NMDA receptors (Chiang et al., 1990; Shiekhattar and Aston-Jones, 1990). Similarly, microinfusion of 50 μM AP5 was found to block presumed NMDA-receptor–mediated responses in recent experiments (Chiang et al., 1990). In the present study, such tests were used to eliminate EAA responses as completely as possible, to ascertain if substantial non-EAA mechanisms may also contribute to LC hyperactivity during opiate withdrawal. Since in the present study only one concentration was tested for each EAA antagonist, the results obtained do not allow accurate comparisons of the involvement of different EAA receptors in the withdrawal-induced activation of LC. Nonetheless, some tentative conclusions may be drawn because previous findings indicate that the concentrations of EAA antagonists used may be relatively se-

lective for subtypes of EAA receptors. For example, in the brain slice preparation, substances ejected focally are typically used at a concentration 100 times (or more) greater than the pharmacologically selective bath concentration yielding similar, selective effects (Egan et al., 1983; Lacey et al., 1987), presumably because of substantial dilution. Concentrations used here for local microinjection were 3–20 times higher than those required for bath application in LC (Cherubini et al., 1988; Bobker and Williams, 1989) or hippocampus preparations *in vitro* (Nicoll et al., 1990). Also, as mentioned above, the concentrations used here are similar to those used in other *in vivo* studies in our laboratory that yielded effects selective for EAA receptor subtypes (Chiang et al., 1990; Shiekhattar and Aston-Jones, 1990). Assuming such selectivity for EAA antagonists locally ejected in the present experiments, our results suggest overall that the withdrawal-induced hyperactivity involves substantially non-NMDA receptors, with a smaller contribution of NMDA receptor mechanisms.

It has also been postulated that an increase in "intrinsic excitability" of LC neurons in dependent animals may be largely responsible for LC hyperactivity during opiate withdrawal (Rasmussen et al., 1990). If this were the case, as withdrawal-induced hyperactivity of LC is substantially mediated by EAA neurotransmission within LC, the activation of LC neurons by endogenous or exogenous EAAs (released by FS or iontophoretically ejected, respectively) should be significantly enhanced in dependent animals. The unchanged responsiveness of LC neurons to FS (an effect mediated by EAAs; see Aston-Jones and Ennis, 1988; Ennis and Aston-Jones, 1988) or to iontophoretic Glu suggests that LC hyperactivity during opiate withdrawal may be the result of heightened EAA release onto LC cells rather than increased excitability or responsiveness of LC neurons. Of course, such tests are not definitive; for example, comparisons of responses to iontophoretically applied agents are limited by variability in pipette characteristics, electrode position relative to receptive membrane, and other factors. Therefore, this conclusion must remain tentative until it receives further experimental support.

Our results suggest that enhanced EAA inputs substantially mediate the LC hyperactivity during opiate withdrawal. However, there also appear to be other inputs to LC that contribute to this excitation, as full blockade of LC hyperactivity was not achieved by any of the EAA antagonists tested. The possible involvement of corticotropin-releasing factor, an endogenous excitatory modulator of LC neurons, has been eliminated in a previous study (Valentino and Wehby, 1989). However, the possible contribution(s) of other excitatory neuropeptides or transmitters, such as substance P (Guyenet and Aghajanian, 1979), vasoactive intestinal peptide (Wang and Aghajanian, 1989), or ACh (Guyenet and Aghajanian, 1979; Egan and North, 1986; Chouvet et al., 1988; Aston-Jones et al., 1991a), remains unknown.

The present finding that enhanced EAA neurotransmission within LC is largely responsible for the hyperactivity of this NA system in withdrawal may indicate areas for future pharmacologic investigations. In recent studies (Aston-Jones et al., 1991b), however, we were unable to antagonize any of a variety of standard behavioral symptoms of opiate withdrawal (wet dog shakes, jumping, shivering, lacrimation, rhinorrhea) by injection of KYN intracerebroventricularly at doses sufficient to attenuate LC activation substantially, as demonstrated herein; similar negative results were obtained with intracerebroventricular AP5 or CNQX. In other recent studies, acute peripheral administration of MK-801 (a noncompetitive NMDA antagonist) yielded contradictory findings, differing between species studied. Such MK-801 administration was reported effective in blocking the jumping during opiate withdrawal in mice and guinea pigs (Tangenelli et al., 1991), but not in rats (Trujillo and Akil, 1991).

These behavioral results using EAA antagonists do not rule out the possible involvement of EAA neurotransmission and LC hyperactivity in opiate withdrawal symptomatology. LC hyperactivity may be involved in aspects of opiate withdrawal other than the gross behavioral signs readily observed in typical withdrawal assays. Indeed, previous studies linked LC activation to withdrawal symptoms such as hyperarousal, anxiety, emotional disturbances, or sensory response deficits (Gold et al., 1980; Redmond and Krystal, 1984). Thus, further investigation appears necessary to discern the behavioral and cognitive contribution of LC hyperactivity and EAA neurotransmission to the overall opiate withdrawal syndrome.

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