

Short communication

Local opiate withdrawal in locus coeruleus in vivo

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Abstract

Hyperactivity of noradrenergic locus coeruleus (LC) neurons following withdrawal from chronic opiates has been implicated in the opiate withdrawal syndrome. Here, we report that local withdrawal induced in vivo by microinfusion of an opiate antagonist into the LC of morphine-dependent rats marginally, but significantly, activated LC neurons above the level obtained with local naloxone microinfusion in naive rats. This local withdrawal response contributes a significant fraction (~19%) of the total LC hyperactivity induced by systemic naloxone. © 1997 Elsevier Science B.V.

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Noradrenergic locus coeruleus (LC) neurons in rats chronically treated with morphine exhibit pronounced hyperactivity in response to intravenous (i.v.) injection of opiate antagonists such as naloxone [1,6]. This hyperactivity in LC neurons has been proposed to play an important role in opiate withdrawal because drug treatments such as opiates or clonidine which reduce this hyperactivity also reduce behavioral and subjective opiate withdrawal symptoms [1,9,17]. However, these drugs also have undesirable side effects (e.g., clonidine produces hypotension, and opiates such as methadone as well as clonidine produce dependence). Thus, it would be useful to determine the mechanisms that elevate LC activity during opiate withdrawal to develop better pharmacologic treatments for opiate abuse.

Previous studies demonstrated that excitatory amino acid (EAA) inputs to the LC are an important element in the LC withdrawal response, as the bulk of LC hyperactivity during morphine withdrawal was eliminated by local or intraventricular EAA antagonist administration [2,14]. However, LC hyperactivity was not completely abolished

by these treatments, indicating that the EAA inputs may not entirely account for the LC withdrawal response [2,14]. A previous study in vivo reported that naloxone iontophoresed onto LC neurons of morphine-dependent rats substantially increased discharge rates [1]. However, in this study it was not determined whether this increase was due to antagonism of the inhibition of LC neurons by circulating morphine. Previous in vitro studies yielded contrasting results as to whether intrinsic changes in LC neurons could account for a significant fraction of LC hyperactivity during opiate withdrawal. Kogan et al. [12] reported that the discharge rate of LC neurons in vitro was higher in slices taken from morphine-dependent rats than in slices taken from morphine-naive animals. However, this report conflicted with previous findings from the same laboratory [3] and other investigators [8] who found no effect of opiate withdrawal on LC neurons recorded in brain slices. These conflicting results left it unclear as to what extent hyperactivity in the LC nucleus during opiate withdrawal may be due to a withdrawal-induced changes within the LC. This question is particularly important in view of recent findings that local withdrawal in the LC area of intact rats results in substantial withdrawal behaviors [13]. Therefore, in the present study we tested the sensitivity of LC neurons to local morphine withdrawal in vivo, precipitated by microinfusion of naloxone methiodide into the LC. Results of this study have been previously presented in abstract form [10].

Male Sprague–Dawley rats (300–400 g, Taconic Farms, Germantown, NY) were subcutaneously implanted with

Abbreviations: aCSF, artificial cerebrospinal fluid; EAA, excitatory amino acid; i.v., intravenous; KYN, kynurenate; icv, intracerebroventricular; LC, locus coeruleus; MeNLX, naloxone methiodide; NaI, sodium iodide; NLX, naloxone

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osmotic minipumps (ALZA, model 2ML1, containing 100 mg morphine sulfate in 2 ml of sterile saline) under halothane anaesthesia using sterile procedures. The rats recovered from surgery quickly and were placed in a normal housing condition (12 h:12 h light/dark cycle, water and food ad libitum). Morphine sulfate was continuously infused from the pumps at a rate of ~ 137 mg/kg/day for 6 days. Electrophysiological experiments were performed on the 6th day of morphine treatment. Our previous studies found that this treatment produced strong morphine dependence [2]. Naive rats implanted with dummy (inactive) pumps were used as control subjects.

Experimental procedures were as previously described [11]. During initial surgical procedures, animals were anaesthetized with 2% halothane anaesthesia administered in air via spontaneous respiration through a trachea cannula. The right jugular vein was catheterized for drugs and electrolyte injection. In some animals, the right common carotid artery was also catheterized for blood pressure measurement. Body temperature was maintained between 36.5 and 37.5°C by a feedback controlled heating pad.

The animal was placed in a stereotaxic instrument with the incisor bar lowered to position the bregma skull suture point 2 mm below lambda. A small craniotomy was performed over the coordinates for the LC (~ 3.8 – 4.5 mm caudal and 1.0–1.5 mm lateral to the lambda suture crossing) and the dura was removed. A double-barrel glass micropipette was used to record impulses from single LC neurons extracellularly, and to simultaneously microinfuse solutions locally into the recording site area, as previously described [2,16]. This assembly consisted of a recording micropipette (tip broken to an outer diameter of 2.5–5.0 mm, 10–20 M Ω), and an infusion micropipette of narrow inner lumen (Fisher 5 μ l measuring pipette; 1 μ l/15 mm shaft length) which was broken to an outside tip diameter of 30–50 μ m. The infusion pipette tip was recessed ~ 125 – 150 μ m from the tip of the recording micropipette and the pipettes were glued together with photopolymerizing resin (Silux, 3M). LC neurons were recorded under 1.0–1.5% halothane anaesthesia using conventional electrophysiological recording methods and physiologically identified as previously described [4,11]. Once an LC neuron was isolated, its spontaneous discharge rate was recorded for 5–10 min (pre-drug control) and then naloxone methiodide (MeNLX; Research Biochemicals Int.; 1 μ M in artificial cerebrospinal fluid, aCSF), sodium iodide (NaI; Sigma; 10 mM in aCSF) or aCSF alone was slowly ejected through the infusion pipette with a pneumatic pressure device (Picospritzer, General Valve, Inc.) over 2–4 min. The volume of infusion was measured by observing the movement of the meniscus inside the pipette (~ 6 nl per 0.1 mm) and totaled 60–140 nl. Previous studies using this method indicated that this should effectively bathe the LC neuron being recorded with the infused solution [16]. The composition of aCSF was the following (in mM): NaCl, 122; KCl, 3.1; MgSO₄, 1.2; CaCl₂, 1.3;

NaH₂PO₄, 0.4; NaHCO₃, 25 and glucose, 10. MeNLX was most often used because such a polar form of NLX exhibits less diffusion through lipid membranes and is reported to be more effective with local intraparenchymal injections [13,15]. However, there was no statistical difference between the values for local microinfusions of MeNLX vs. NLX in dependent rats, and therefore these data were pooled (values post-infusion = 1.8 ± 0.2 spikes/s, $n = 10$, vs. 1.7 ± 0.1 spikes/s, $n = 3$, respectively; $p > 0.1$). Intracereulear microinfusions of NaI were used to control for the methiodide salt of the MeNLX solution.

In some animals, kynurenate (5 μ l; 0.1 M; Sigma, pH 6.5–7.5) was administered intracerebroventricularly (i.c.v.) through a 30 gauge cannula inserted into a guide cannula (26 gauge) placed in the lateral ventricle. Intravenous (i.v.) injections of naloxone HCl (NLX; 0.1 mg/kg; Sigma) were made via a cannula inserted in the jugular vein and injected in a volume of 1 ml/kg in physiological saline. At the termination of the experiment, pontamine sky blue dye was iontophoretically deposited from the recording electrode (-7 μ A for 10 min) to mark the recording site. All loci for the neurons analyzed in this report were histologically verified in neutral red-stained tissue to be within the LC nucleus.

In all experiments, pre-drug discharge rates were based on the spontaneous activity during a 3 min period, and the post-drug rates were taken for a 20 s period during maximum mean frequency (visually determined from the ratemeter plot). Discriminated neuronal impulses were collected and analyzed using Spike 2 software and its associated hardware (CED; UK). The values for discharge rates were expressed as mean \pm standard error of the mean (S.E.M.). Two-tailed *t*-tests were used to determine statistical significance for pre- vs. post-drug and between-group comparisons. Some animals tested with local 1 μ M NLX or MeNLX were also pretreated with kynurenate; there was no statistically significant difference between the responses of these cells to MeNLX as a function of kynurenate pretreatment ($p > 0.1$), and thus these two groups were pooled.

Experimental procedures were conducted so as to minimize animal suffering and the number of animals used, and to employ alternatives to in vivo techniques, when available. These procedures were approved by the animal welfare committee of Allegheny University and were in accordance with the guidelines established by the NIH.

A total of 133 single LC neurons were analyzed for this study (from 91 morphine-treated and 39 control rats). Only one cell was recorded from each animal except for 1 μ M MeNLX local microinfusions in naive animals (12 cells/10 rats) and aCSF local microinfusions in dependent animals (4 cells/3 rats). When more than one cell was obtained from a rat, at least 40 min lapsed between microinfusions. Fig. 1 displays ratemeter records from two typical LC neurons recorded in chronically morphine-treated rats,

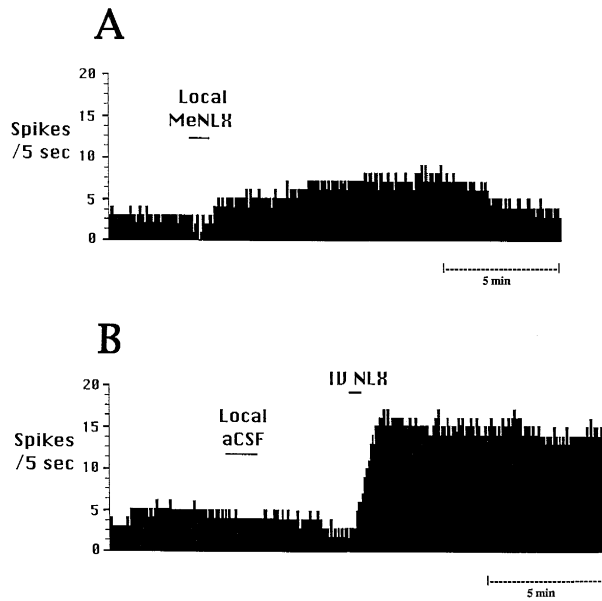


Fig. 1. Effect of local vs. systemic opiate withdrawal on LC impulse activity in rats chronically treated with morphine. A: ratemeter record showing modest increase in activity for a typical LC neuron following MeNLX ($1 \mu\text{M}$) locally microinfused into the LC (60 nl, indicated at bar). Activity increased from 0.6 to 1.6 spikes/s. Note that a substantial part of this increase was produced by antagonism of the inhibitory effect of morphine still present in the LC – additional comparisons were done to determine the contribution of local withdrawal processes specifically (see Table 1). B: ratemeter record showing increased activity in a different LC neuron following i.v. naloxone (0.1 mg/kg). Activity increased from 0.8 to 3.2 spikes/s. Note lack of effect of locally infused artificial cerebrospinal fluid (aCSF; 60 nl, indicated at bar).

showing the magnitudes of activation precipitated by local MeNLX microinfusion or by systemic NLX injection. Note also in Fig. 1B the lack of effect by local infusion of aCSF.

As shown in Table 1, systemic NLX injection into morphine-dependent rats markedly activated LC neurons as previously reported [1,2]. Local MeNLX infusion into

the LC of dependent rats also activated LC neurons but substantially less than systemic NLX (Table 1). Note that part of the increase observed for both systemic NLX and local MeNLX was due to antagonism of the inhibitory effect of circulating morphine, as revealed by the lower pre-NLX discharge rate of LC cells in morphine-dependent rats (compared to naive rats (Table 1; $p < 0.01$). Note also that local NLX application in naive rats significantly suppressed LC activity ($p < 0.01$), as did i.v. NLX in naive animals (Table 1; $p < 0.05$). Therefore, changes in LC activity due specifically to dependence are best determined with between-group comparisons (morphine-treated vs. naive rats) of post-infusion data.

As shown in Table 1, a between-group comparison of LC discharge rates for naive vs. morphine-dependent rats following systemic or local MeNLX administration revealed that there was a rate increase of 2.7 spikes/s in the systemic NLX group, and 0.5 spikes/s in the local MeNLX group. Both of these increases were statistically significant ($p < 0.01$ and 0.05 , respectively). Moreover, the increase with systemic NLX was significantly greater than that with local MeNLX ($p < 0.001$). This between-group comparison of post-NLX results confirms that local withdrawal within the LC significantly activates LC neurons, but much less than does systemic withdrawal. This comparison indicates that, of the activation seen with local naloxone in dependent animals (from 0.8 to 1.8 spikes/s), about half is due to antagonism of inhibition by circulating morphine (compare to 1.3 vs. 1.8 spikes/s in naive vs. dependent rats post-NLX). Comparing the increases in rates for the two modes of NLX administration reveals that local opiate withdrawal in the LC may account for about 19% of the increased LC rate seen with systematically precipitated withdrawal (0.5 spikes/s compared to 2.7 spikes/s).

As described above, in naive rats local MeNLX reduced tonic LC activity whereas local aCSF microinfusion did not alter LC impulse rate (Table 1). Decreased activity was also induced by local microinfusion of NaI compared to

Table 1

Average (\pm S.E.M.) discharge rates of LC neurons for NLX (0.1 mg/kg, i.v.) or MeNLX (local infusion) treatments in either naive or morphine-dependent rats, as indicated

	i.v. NLX		1 μM MeNLX		aCSF		NaI	
	Pre-Inf	Post-Inf	Pre-Inf	Post-Inf	Pre-Inf	Post-Inf	Pre-Inf	Post-Inf
Naive	1.6 \pm 0.1	1.4 \pm 0.1	1.8 \pm 0.2	1.3 \pm 0.2	1.8 \pm 0.3	1.9 \pm 0.3	1.6 \pm 0.3	1.1 \pm 0.3
Dependent	1.1 \pm 0.1	4.1 \pm 0.2	0.8 \pm 0.1	1.8 \pm 0.1	1.1 \pm 0.2	1.0 \pm 0.2		

Pre-Inf, pre-infusion; Post-Inf, post-infusion. Note the following: *i.v. NLX groups*: systemic withdrawal induced marked activation of LC neurons ($p < 0.01$), substantially more than local withdrawal. Also, i.v. NLX in naive rats significantly decreased LC firing rate ($p < 0.05$). Finally, note that the pre-NLX activity of LC cells in dependent rats was less than that in naive rats indicating that tolerance was incomplete (see also aCSF groups in this table). $n = 7$ and 59 cells in naive and dependent rats, respectively. *1 μM MeNLX groups*: a between-group comparison of LC impulse rates post-local MeNLX ($1 \mu\text{M}$) in dependent vs. naive rats revealed a small but significant activation of LC neurons ($p < 0.05$). Note that local infusion of MeNLX suppressed LC activity in naive animals (perhaps due to the iodide salt, as indicated by results with NaI infusion; see below). $n = 12$ and 13 cells in naive and dependent rats, respectively. *aCSF groups*: tolerance to morphine was incomplete after this chronic treatment ($p < 0.01$ for naive vs. morphine-treated animals). $n = 11$ and 10 cells in naive and dependent rats, respectively. *NaI groups*: Local microinfusion of an iodide salt in aCSF significantly depressed LC tonic activity ($p < 0.01$). This may explain why local microinfusion of MeNLX (an iodide salt of NLX) into the LC of naive rats suppressed tonic discharge. $n = 4$ cells in naive animals.

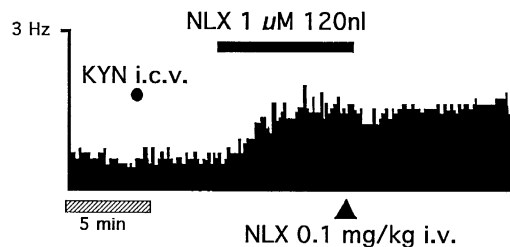


Fig. 2. Ratemeter display showing activation of a typical LC neuron by MeNLX locally microinfused into the LC ($1 \mu\text{M}$, at upper bar). In this animal, kynurenic acid (KYN, $5 \mu\text{mol}$) was injected into the lateral ventricle (at upper circle) before the local MeNLX infusion to prevent activation of LC neurons by EAA inputs [2]. Note that local NLX was effective in activating the cell when EAA receptors are blocked. Note also that subsequent i.v. NLX (at triangle below) did not elicit an additional increase in activity of this cell. This indicates that EAA inputs and local withdrawal mechanisms may fully account for the withdrawal-induced activation of this neuron.

aCSF in naive animals (Table 1; $p < 0.01$, two-tailed t -test), indicating that the decrease produced by local MeNLX may have been produced by the action of the methiodide in MeNLX on LC cells.

We also tested whether non-EAA inputs participate in the activation of LC neurons during morphine withdrawal. For this, 6 LC neurons in morphine-dependent rats were recorded following i.c.v. kynurenic acid and local microinfusion of MeNLX ($1 \mu\text{M}$, about 5 min after the kynurenic acid) to block EAA transmission and remove morphine from opiate receptors in the LC. Less than 1 min after termination of the local MeNLX microinfusion, NLX was administered i.v. while recording these same cells to determine if subsequent systemic opiate withdrawal would further elevate LC activity. Fig. 2 and Table 2 show that in these cells i.v. NLX did not significantly activate LC neurons above the level produced by the preceding local microinfusion of MeNLX. Kynurenic acid appeared to reduce LC activity, but this difference was not statistically significant ($p > 0.1$). Furthermore, Table 2 shows that activity in these 6 cells after i.c.v. kynurenic acid plus $1 \mu\text{M}$ local MeNLX was the same as the value for $1 \mu\text{M}$ local MeNLX alone, and demonstrates also that the $1 \mu\text{M}$ concentration was sufficient to fully antagonize local opiate receptors.

The present study found that local microinfusion of naloxone produces a modest but significant increase in LC neuronal activity in animals made dependent with chronic morphine treatment. The increase by local naloxone was

much less than that induced by systemic naloxone injection. The increased LC activity with local withdrawal was beyond that expected from antagonism of circulating morphine, indicating that this effect may derive from local changes that occur within the LC during chronic morphine treatment. Local microinfusion of NLX also produced modest activation of LC neurons after KYN pretreatment, and systemic NLX failed to increase activity in these cells beyond this level, indicating that activation of LC neurons with systemically induced withdrawal is accounted for by EAA inputs (primarily) and other local changes at the level of the LC.

In contrast to these results with $1 \mu\text{M}$ MeNLX, similar previous experiments with 10 mM MeNLX in the microinfusion pipette revealed no increase in LC activity after local MeNLX microinfusion [2]. However, in those experiments the pre-infusion discharge rate for LC neurons in morphine-dependent rats (2.1 ± 0.3 spikes/s) was somewhat higher than that in naive rats (1.6 ± 0.1 spikes/s, $p > 0.1$), in contrast to the present results where pre-infusion rates for dependent rats were significantly less than in naive rats (described above). We tentatively conclude that this difference resulted from substantial leakage of MeNLX with the high concentration in the micropipette in the previous experiments, which antagonized opiate receptors in the LC before application of pressure to microinfuse the solution. Such leakage may explain why a local withdrawal response was not apparent in that previous study.

The present finding that systemic NLX did not produce activation beyond that induced by local MeNLX after i.c.v. treatment with the EAA antagonist KYN indicates that blockade of EAA receptors eliminated activation of LC neurons by extrinsic inputs during opiate withdrawal. Thus, a non-EAA-mediated activation of LC cells during withdrawal appears to be unlikely. These results indicate that the activation of LC neurons following systemic NLX that is specifically mediated by opiate withdrawal is accounted for largely, or entirely, by two factors: (i) EAA inputs (accounting for more than 80% of the activation), and (ii) a local withdrawal reaction within the LC (accounting for less than 20% of the activation). Note that these results are from comparing data for dependent vs. naive rats post-NLX, and therefore the activation of LC cells by disinhibition from circulating morphine (about 0.5 spikes/s) is excluded in these figures.

Previous studies with intracellular recording in vitro found no change in membrane properties of LC neurons in

Table 2

Average (\pm S.E.M.) discharge rates of 6 LC neurons in morphine-dependent rats after different drug treatments, administered sequentially as indicated

	Pre-drug	KYN	KYN + MeNLX	KYN + MeNLX + i.v. NLX
LC rate	1.2 ± 0.4	0.8 ± 0.1	1.8 ± 0.3^a	2.1 ± 0.5

Note that the i.c.v. injection of KYN did not prevent the LC discharge increase seen with local MeNLX infusion, but abolished the marked increase in LC firing normally seen after intravenous NLX injection in these animals. Although KYN tended to decrease activity compared to pre-drug rates, this was not significant. ^a $p < 0.01$, KYN compared to KYN + MeNLX, paired two-tailed t -tests.

morphine-dependent rats [3,8]. Although our *in vivo* approach is important because it examines this issue in the intact animal, we cannot determine from this study whether the local withdrawal-induced activation we found is due to intrinsic changes within LC neurons with dependence, or to an unknown extrinsic influence (perhaps mediated by altered transmitter release from terminals in the LC). Nonetheless, our findings are important as they indicate that the activation of LC neurons that occurs with local withdrawal is beyond that expected from antagonism of circulating morphine, and this response may underlie findings that local naloxone injection into the LC of intact dependent rats elicits substantial withdrawal behaviors [13]. It is noteworthy that the activation of LC neurons via local antagonism of circulating morphine may also play a significant role in the behavioral response observed in the above referenced study, in view of the changes that may occur throughout LC postsynaptic areas with continuous morphine administration over days.

In our study, pre-drug discharge rates for morphine-dependent rats were significantly lower than those for naive rats in all groups. This indicates that tolerance to morphine was incomplete after our chronic treatment regimen. Aghajanian [1] reported that although chronic treatment with morphine pellets produced baseline LC discharge rates that were lower than those in naive rats, this difference was not significant, indicating more complete tolerance had developed than we observed. This difference may be due to differences in anaesthetic (chloral hydrate vs. halothane) or chronic morphine treatment procedures in the two studies (pellets vs. osmotic minipumps).

An important aspect of our paper is the between-groups comparison (naive vs. morphine-dependent rats) of LC discharge rates after local naloxone microinfusion. Because morphine is present and continues to inhibit LC activity during recordings, the degree of withdrawal activation cannot be ascertained by comparing activity within cells in dependent animals before and after naloxone administration; a component of the resulting activation would be due to antagonism of morphine that is still present. There has been one previous report of activation of LC neurons by locally administered naloxone in dependent rats [1]. That study did not perform a between-groups analysis to permit assessment of the degree of activation due to antagonism of circulating morphine. In our study, it appears that about half of the activation of LC neurons with local naloxone in dependent rats is due to antagonism of extant morphine acting at opiate receptors in the LC.

Our findings of locally precipitated withdrawal in LC neurons *in vivo* indicate that changes within the LC during chronic opiate treatment may contribute to activation of LC neurons during systemic withdrawal. However, it is important to note that this locally induced response is modest (about 0.5 spikes/s). This value agrees closely with the increase reported for LC neurons in slices taken from morphine-dependent rats compared to naive rats [12].

As LC neurons increase activity by about 3 spikes/s after withdrawal evoked by systemic NLX, this indicates that local withdrawal processes account for less than 20% of the total activation of these cells during opiate withdrawal. Although modest, this tonic activation could have functional significance for behavioral or other responses to opiate withdrawal (see, e.g., [5,7,13]).

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