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ORIGINAL INVESTIGATION

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A comparison of the effects of clonidine and CNQX infusion into the locus coeruleus and the amygdala on naloxone-precipitated opiate withdrawal in the rat

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Abstract Both the locus coeruleus (LC) and the amygdala have been implicated in aspects of opiate dependence and withdrawal. The LC is known to be one of the most sensitive sites for precipitating withdrawal behaviors after local opiate antagonist infusions in morphine-dependent subjects. The amygdala is also known to mediate antagonist-induced withdrawal behaviors and aversive motivational states. The goal of the present study was to evaluate directly the ability of noradrenergic agonists and glutamatergic antagonists to attenuate naloxone-precipitated withdrawal behaviors when infused into the LC or the central nucleus of the amygdala (CeA). The alpha-2-noradrenergic agonists clonidine or ST-91 were infused into the CeA to compare the effects of noradrenergic activation in the CeA to the attenuation of withdrawal previously observed in rats infused with clonidine into the LC, since the LC and CeA are known to contain co-localized opiate and noradrenergic receptors. The effects of microinfusions of the non-NMDA excitatory amino acid antagonist 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) were also infused into the LC and CeA since opiate withdrawal is associated with increased glutamatergic transmission. Intra-CeA clonidine or ST-91 (2.4 µg/0.5 µl or 1.0 µl) produced significant reductions primarily in the occurrence of irritability. Conversely, intra-CeA or intra-LC infusions of CNQX (2.5 µg/0.5 µl) significantly attenuated naloxone-precipitated withdrawal, an effect similar to the attenuation previously observed after intra-LC clonidine infusions. These data demonstrate the specific behavioral effects of altering glutamatergic and noradrenergic neurotransmission in the LC or CeA during naloxone-precipitated opiate withdrawal. Elucidation of the neuroanatomical circuitry involved in opiate withdrawal should increase our understanding of the neuroadaptations associated with drug dependence and subsequent withdrawal behavior.

Key words Clonidine · ST-91 · CNQX · LC · Amygdala · Intracerebral infusion · Withdrawal · Naloxone · Morphine · Opioid · Rat

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Introduction

Withdrawal from chronic opiate administration induces neuroadaptations that result in increased noradrenergic neuronal firing (Aghajanian 1978; Crawley et al. 1979; Laverty and Roth 1980; Zigun et al. 1981; Roth et al. 1982), increased c-fos expression (Rasmussen et al. 1995), an upregulated cAMP pathway (Duman et al. 1988; Nestler and Tallman 1988; Rasmussen and Aghajanian 1989; Rasmussen et al. 1990; Kogan et al. 1992; Nestler 1992, 1996; Matsuoka et al. 1994), and increased glutamatergic activity (Akoaka and Aston-Jones 1991; Zhang et al. 1994). Understanding the specific contributions of these adaptive changes and the neural circuits associated with opiate dependence and withdrawal is important for the development of effective pharmacological strategies that may attenuate or prevent dependence and withdrawal in man. The goal of our study was to examine the ability of altered noradrenergic and glutamatergic neurotransmission in the locus coeruleus (LC) and central nucleus of the amygdala (CeA) to attenuate behavioral signs of naloxone-precipitated morphine withdrawal since adaptations have been found in these brain regions.

The LC, the main source of noradrenergic (NE) innervation in brain, sends projections to several brain regions including the amygdala. The LC and the amygdala contain co-localized opioid and noradrenergic receptors (Freedman and Aghajanian 1985). Clonidine, an alpha-2-noradrenergic agonist, is known to inhibit the firing of LC neurons (Aghajanian 1978) and the activation of amygdala and LC neurons elicited upon opiate with-

drawal (Freedman and Aghajanian 1985). The efficacy of clonidine to treat opioid dependency by reducing the occurrence of withdrawal signs and symptoms (Gold et al. 1978, 1980; Uhde et al. 1980; Washton et al. 1980; Charney et al. 1981, 1982; Redmond 1981) may be due to the fact that alpha-2 adrenoceptor and opiate receptor agonists operate through common intracellular effector mechanisms in regions such as the LC and amygdala. Stimulation of either alpha-2 or opiate receptors is known to mediate alterations in the cAMP pathway, as clonidine- or morphine-induced hyperpolarization of LC neurons can be reversed by administration of cAMP analogs which activate protein kinase A (PKA; Aghajanian and Wang 1987). In addition, chronic morphine administration increases levels of adenylyl cyclase and PKA in the LC (see Nestler 1996) and PKA activity in the amygdala (Terwilliger et al. 1991), suggesting morphine-induced adaptations in these regions.

Clonidine is also known to suppress naloxone-precipitated morphine-withdrawal induced c-fos expression in the amygdala (Rasmussen et al. 1995). Behavioral studies using microinfusion techniques have also implicated the amygdala in physical or somatic signs of withdrawal (Lagowska et al. 1978; Calvino et al. 1979; Maldonado et al. 1992) as well as in aversive motivational states (Stinus et al. 1990; Koob et al. 1992). Infusions of the non-lipophilic opiate antagonist methyl-naloxonium into the amygdala of morphine-dependent rats produces place aversion (Stinus et al. 1990). Place aversion can be attenuated by clonidine injections (Kosten 1994) and antagonist infusions into the amygdala can also induce some physical signs of withdrawal – though less than infusions into the LC (Maldonado et al. 1992). The ability of intra-LC infusions of clonidine to attenuate the behavioral signs and neurochemical correlates associated with opiate-antagonist precipitated withdrawal has been demonstrated (Taylor et al. 1988); however, the effects of intra-amygdala clonidine on the physical signs of opiate withdrawal have not been examined.

In addition to noradrenergic hyperactivity, increased glutamatergic activation may contribute to the role of the LC and CeA in withdrawal behaviors. Previous studies have found increased levels of glutamate in the LC of rats treated with naloxone after chronic morphine (Aghajanian et al. 1994; Zhang et al. 1994). In vitro studies also have shown no changes in sensitivity to glutamate in LC of morphine dependent rats, an indication that these effects are due to increased input from efferent terminals (Kogan et al. 1991). Furthermore, lesions of the nucleus paragigantocellularis (PGi), the major afferent to the LC (Aston-Jones et al. 1986), have been shown to attenuate withdrawal-induced activation of LC neurons (Rasmussen and Aghajanian 1989). Intracerebroventricular (ICV) infusions of excitatory amino acid (EAA) antagonists have been demonstrated to have powerful anti-withdrawal actions (Rasmussen et al. 1991a,b; Rasmussen 1995), and non-NMDA receptor antagonists have been shown to be the most effective EAA antagonists to attenuate withdrawal-induced firing of the LC (Akaoka and Aston-Jones 1991). If the glutamatergic activation of the LC during withdrawal is important for eliciting symptoms of withdrawal, then intra-LC infusions of non-NMDA antagonists, such as 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), would be predicted to attenuate behavioral signs of opiate withdrawal.

It is also known that glutamatergic projections from the cortex and other amygdaloid nuclei mediate synaptic transmission in the CeA (see Davis et al. 1994 for review). Morphine withdrawal results in elevated c-fos expression in the amygdala, an effect that can be blocked by pretreatment with the NMDA antagonist MK801 (Rasmussen, 1995). It is also of note that non-NMDA receptors in the amygdala are known to be involved in the conditioned and unconditioned expression of emotional behavior associated with fear and anxiety (see for review Davis 1992; LeDoux 1992). Thus it is possible that glutamate transmission in the amygdala may be linked to some of the psychological aspects of withdrawal. We sought to determine whether non-NMDA receptor activity may contribute to the role of the CeA and the LC in the expression of somatic and emotional behaviors associated with opiate withdrawal.

Materials and methods

Procedures, materials and methods were based on those used in Taylor et al. (1988).

Subjects and apparatus

Male Sprague-Dawley rats (Camm, N.J., USA), which weighed approximately 275–300 g at the start of the experiment, were used. They were housed in pairs in plastic cages containing "Beta" chips. Food and water were continuously available. Subjects were maintained on a 12/12-h light/dark cycle. Plastic boxes (37×28×29 cm) were used as test chambers. They were separated into quadrants with markings so that activity could be measured by counting crossings of a section. A layer of Beta chips served as bedding.

Intracranial surgical procedures

Stereotaxic surgery was conducted under Equithesin (4.32 mg/kg IP) anesthesia (sodium pentobarbital and chloral hydrate). Subjects were implanted bilaterally with stainless-steel guide cannulae (25 gauge) aimed to give access to the LC [anterior-posterior (AP) –0.6 from lambda, lateral from midline (Lat)±1.2, dorsal-ventral (Vent) 5.0 from dura] or the central nucleus of the amygdala, CeA (AP –2.3 from bregma, Lat±4.0, Vent 6.0 from dura). Stereotaxic coordinates were determined from Paxinos and Watson (1982). Following surgery, stylettes were inserted into the guide cannulae to keep the tubing patent. After surgical implantation of the guide cannulae, all subjects were allowed at least 1 week for recovery.

Drug treatments and infusions

To produce dependence on morphine, pellets containing 75 mg morphine base (NIDA) were implanted SC into the subjects' back. Pellet implantation was conducted under halothane anesthesia. The wound was cleaned with antiseptic solution and closed with a stainless-steel skin clip. All subjects received three pellets before

the test (day 1, one pellet; day 4, one pellet; day 5, one pellet; day 6, test). Plasma morphine levels after this number of pellets is estimated to be greater than 150 ng/ml (see Gold et al. 1994).

Clonidine and ST-91 (Boehringer Ingelheim) doses were determined from our previous study of these drugs (Taylor et al. 1988). Drug weights were calculated as the salt and all were dissolved in 0.9% sterile saline, which was used as a vehicle (control) solution. The dose of CNQX (Research Biochemicals Inc., Natick, Mass., USA) infused was determined from pilot studies and calculated in terms of its base, dissolved in dimethyl sulfoxide (50%) and saline at physiological pH, which was used as a vehicle. Withdrawal was precipitated by an IP injection of 1.0 mg/kg naloxone hydrochloride (Endo Labs).

Intracerebral infusions were made bilaterally. Rats were handheld while 31-gauge injection needles were placed into the surgically implanted guide cannulae. The injection needles protruded 2.0 mm beneath the guide cannulae, terminating in the CeA (8.0 mm from dura) or the dorsal LC (7.0 mm from dura). The injection needles were attached to syringes (Hamilton 10 µl) by tubing (PE 20) filled with the drug or vehicle solution and infusions of 0.5 or 1.0 µl were delivered by Harvard pump over a 2- or 4-min period, respectively. After the infusion, 2 min was allowed to elapse prior to the removal of the injection needles. CNQX or vehicle was infused into the LC or CeA (2.5 µg/0.5 µl). Clonidine hydrochloride (2.4 µg/0.5 or 1.0 µl) or the non-lipophilic alpha-2-agonist ST-91 (2.4 µg/0.5 or 1.0 µl) were infused into the CeA. In order to make direct comparisons with our previous study, we infused these drugs in a larger volume of 1 µl, as well as a smaller volume of 0.5 µl to limit the spread of the drug to nearby structures (see Taylor et al. 1988).

Behavioral ratings and analyses

All subjects received a 15-min habituation session to the test environment (containing fresh bedding) prior to surgery. On the test day, each animal was weighed and the intracerebral infusion was followed immediately by the IP injection. The subject was then returned to the holding cage for 5 min before being placed into the test box for the 15-min behavioral rating. After the test session, the subject was re-weighed to determine weight loss. The morphine-treated subjects were randomly assigned to the groups before the test and the order of testing was randomized for subjects in the groups during the test day. All injections and ratings were performed by an observer who did not know what experimental treatment had been administered over a 15-min period in a quiet, temperature-maintained (20°C) room. The scored signs, defined in Table 1, were modified from those described by Bläsig et al. (1973). The presence of each checked sign and the frequency (number per time bin) of each counted sign were noted on the score sheet in 5-min time bins. Checked signs were examined qualitatively as a proportion of subjects showing the sign during the test and quantitatively as all the checked signs combined (total of seven). Counted signs were examined as the frequency the sign was observed in each 5-min time bins. The incidence of checked and counted signs (total of 18) were examined as total signs. Behavioral signs generally measured somatic aspects of withdrawal, with the exception of irritability which may reflect emotional behavior.

The mean frequency of each of the counted, all checked or counted signs, total signs, and average weight lost were evaluated using analysis of variance (ANOVA) and post hoc comparisons when multiple groups were analyzed (Bonferroni tests, a method which adjusts multiple comparisons for type 1 error) between treatment groups (drugs). When there were two sites examined, these data were initially analyzed using a site (LC versus CeA)×drug (saline versus CNQX) two-way ANOVA. The presence of each of the checked signs was analyzed using non-parametric statistics (Chi-square, χ^2, as recommended by Siegel 1956) individually and when multiple group comparisons were made a more conservative significance value was used ($P<0.01$).

Histological analyses

At the completion of the behavioral testing, histological analyses were performed. Subjects were killed by decapitation after being fully anesthetized with an overdose injection of sodium pentobarbital. Brains were removed, soaked in 10% formalin and then stored in the formalin-sucrose solution for the histological preparation. Sections of fixed brain were cut (60 µm) on a freezing microtome and mounted using procedures detailed in Wolf (1971). Sections were mounted on treated slides to aid adhesion to the glass and then stained with cresyl violet. When the sections were examined, using a light microscope set at ×10–40 to determine the location of the injection site, the observer was blind to the experimental treatment.

Results

Histological analyses

Estimated sites for clonidine and ST-91 infusions into the CeA are depicted in Fig. 1. Some infusions into the

Table 1 Morphine withdrawal signs used for behavioral ratings are defined for "counted signs" which indicate the frequency (number) and "checked signs" which indicate presence are shown

Counted sign	
Activity	crossing of a quadrant mark
Rear	lifting the forepaws off the ground
Chat	teeth grinding or rapidly opening/closing the jaws
Wet-dog shake	shaking of head and body resulting in loss of balance
Groom	using limbs to manipulate the head or body
Jump	raising all limbs off the ground rapidly
Dig	using the forepaws to displace the bedding
Freeze	immobility for more than 10 s
Chew	masticating the bedding or fecal material
Backwards walk	backwards locomotion
Rub jaw/flat posture	moving the jaw or the torso on the ground
Checked sign	
Diarrhea	
Ptosis	squinting of the eyes
Irritability	vocalizing when placed into or out of the test box
Lacrimation	appearance of a brown secretion from the eyes
Rhinorrhea	appearance of a brown secretion from the nose
Abnormal posture	lying of the side writhing or hunching of body
Penile erection	evidence of protrusion of the penis before or after test

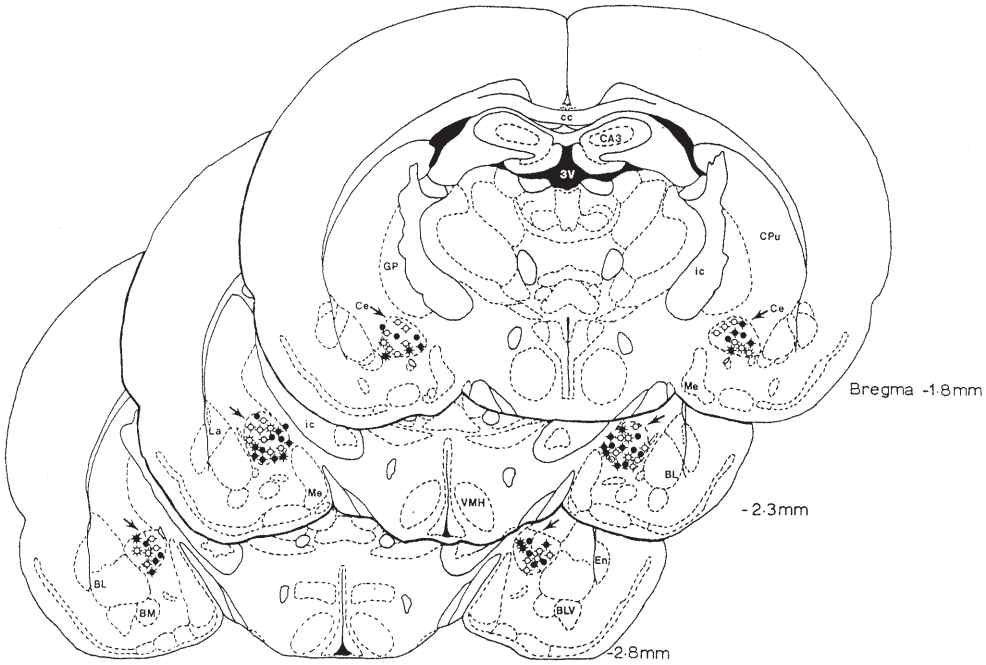

Fig. 1 Brain sections modified from Paxinos and Watson (1982) indicating individual rat bilateral injection tips estimated from the histological analysis. Infusion sites for the six groups are illustrated as follows: saline+intra-amygdala saline (-○-), *n*=9; naloxone+ intra-amygdala saline (●), *n*=10; naloxone+2.4 µg/0.5 µl clonidine (-○-), *n*=6; naloxone+ 2.4 µg/1.0 µl clonidine (-●-), *n*=8; naloxone+2.4 µg/0.5 µl ST-91 (-✸-), *n*=5; naloxone+ 2.4 µg/1.0. µl ST-91 (-☼-), *n*=5. The distance (mm) of each section, anterior-posterior, from the interaural line is shown. The intended site of the central nucleus of the amygdala for all groups was between –1.8 mm and –2.8 mm. *Abbreviations: Ce*, Central amygdaloid nucleus; *BL*, basolateral amygdaloid nucleus; *Me*, medial amygdaloid nucleus; *BM*, basomedial amygdaloid nucleus; *Cpu*, caudate putamen

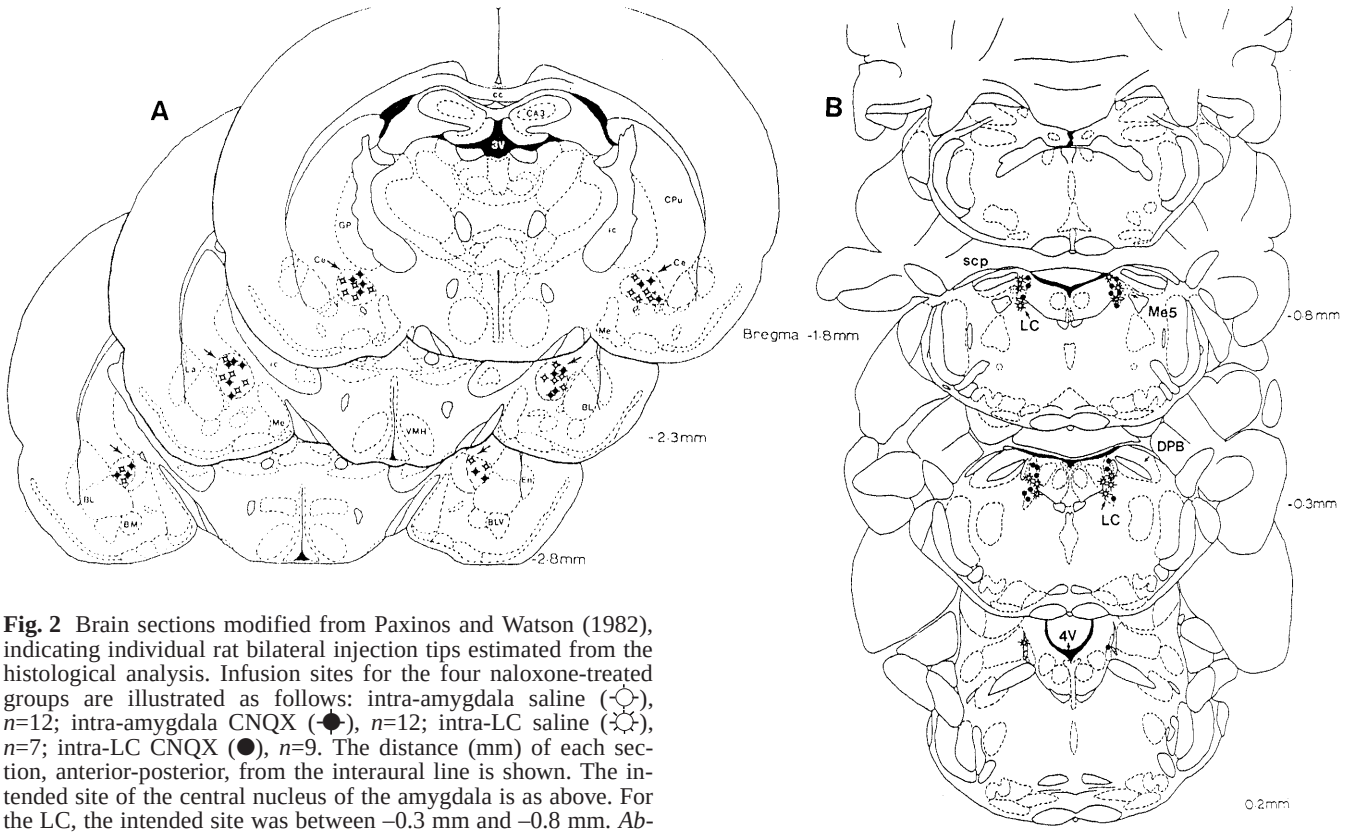

Fig. 2 Brain sections modified from Paxinos and Watson (1982), indicating individual rat bilateral injection tips estimated from the histological analysis. Infusion sites for the four naloxone-treated groups are illustrated as follows: intra-amygdala saline (-○-), *n*=12; intra-amygdala CNQX (-●-), *n*=12; intra-LC saline (-☼-), *n*=7; intra-LC CNQX (●), *n*=9. The distance (mm) of each section, anterior-posterior, from the interaural line is shown. The intended site of the central nucleus of the amygdala is as above. For the LC, the intended site was between –0.3 mm and –0.8 mm. *Abbreviations: LC*, Locus coeruleus; *4V*, fourth ventricle; *Me5*, mesencephalic trigeminal nerve; *scp*, sup cerebellar peduncle; *DPB*, dorsal parabrachial nucleus

Table 2 Differences in the percent of subjects in the groups displaying each of the checked signs are shown: diarrhea, χ^2=24.63, P<0.01; ptosis, χ^2=25.35, P<0.01; rhinorrhea, χ^2=22.58, P<0.01; lacrimation, χ^2=10.89, P<0.05; abnormal posture, χ^2=21.13, P<0.01; penile erection, χ^2=15.53, P<0.01; df=5, respectively). The six groups are shown (n=9, 10, 6, 8, 5, 5, respectively). All of the signs were increased in the naloxone group compared with the saline control group, except lacrimation, and the incidence of the other behaviors after clonidine or ST-91 was increased. [Significant differences *P<0.01 compared with control (*S/S*) and significant differences +P<0.01 compared with naloxone-treated subjects (*N/S*), χ^2>6.54, P<0.01] for the six groups – saline + intra-amygdala saline (*S/S*), n=9; naloxone+intra-amygdala saline (*N/S*), n=10; naloxone+2.4 µg/0.5 µl clonidine (*N/C0.5*), n=6; naloxone+2.4 µg/1.0 µl clonidine (*N/C1.0*), n=8; naloxone+2.4 µg/0.5 µl ST-91 (*N/ST0.5*), n=5; naloxone+2.4 µg/1.0 µl ST-91 (*N/ST1.0*), n=5. The mean incidence of checked signs is also shown

% Subjects behavior	S/S	N/S	N/C 0.5 µl	N/C 1.0 µl	N/ST 0.5 µl	N/ST 1.0 µl
Diarrhea	0	90*	83*	75*	100*	80*
Ptosis	0	100*	67	63	80*	100*
Rhinorrhea	0	70*	50	75*	100*	60
Lacrimation	0	50	50	25	80*	40
Abnormal posture	0	80*	83*	63	100*	80*
Penile erection	0	80*	67	75*	40	60
Mean checked signs	0	5.8(0.5)*	4.3(0.7)*	4.0(.5)*+	5.5(0.4)*	3.9(0.4)*+

Table 3 Mean differences between the experimental groups after intra-CeA infusions for each of the counted signs and weight loss. *Significant differences (P<0.01) compared with control (*S/S*). Mean behaviors during the 15-min test session in morphine-dependent rats (±SEM) (see Table 2 for group definitions)

Mean behavior	S/S	N/S	N/C 0.5 µl	N/C 1.0 µl	N/ST 0.5 µl	N/ST 1.0 µl
Wet dog shakes	0	13.0(2.4)*	10.5(0.8)*	12.0(2.3)*	15.3(3.3)*	16.1(1.2)*
Teeth chattering	0.3(0.2)	11.7(5.2)*	2.0(1.8)	8.0(2.4)	2.5(1.4)	8.9(2.5)
Rearing	18.6(5.4)	5.0(1.7)*	3.9(2.5)*	6.1(2.2)	2.2(1.4)*	2.1(0.8)*
Grooming bouts	3.0(0.6)	5.7(1.2)	4.6(0.6)	3.8(1.2)	3.9(0.9)	4.2(1.1)
Weight loss	0.3(0.1)	8.6(2.0)*	7.0(1.8)*	7.9(1.3)*	6.9(1.8)*	6.5(1.2)*

amygdala appeared not to be confined to the central nucleus, bordering on either the medial or basolateral nucleus. Two subjects whose cannulae tips were outside the CeA and one subject whose cannula was blocked and therefore received a unilateral infusions were excluded from the analysis and are not shown. Estimated infusions sites for CNQX into the CeA and LC are depicted in Fig. 2a, b. Two subjects with LC infusions had evidence gross histological damage (evidence of severe cell loss or gliosis) and were excluded from the analysis.

Behavioral analyses

Infusions of clonidine or ST-91 into the CeA

There were overall differences between the groups when the average of all the checked signs (total of seven) observed during the test session were examined (F=29.95, df 5,37, P<0.01, Table 2). Checked signs were never observed in subjects in the control group (i.e., morphine-dependent but not naloxone injected). Naloxone produced a significant increase in checked signs when compared with control subjects (P<0.01, df 17, 13, 15, 12, 12), indicating the precipitation of withdrawal. Furthermore, only subjects that received 1.0 µl infusions of either clonidine or ST-91 prior to naloxone injections had significantly fewer checked signs than subjects injected with naloxone alone (Table 3, P<0.01, df 15,12).

Chi-square analyses were also performed on each of the checked signs to determine significance and comparisons between groups (Table 2). Only irritability was significantly reduced by intra-CeA infusions of clonidine or ST-91 compared with subjects with naloxone alone (Fig. 3). Irritability was present in 100% of subjects receiving naloxone compared with its absence in controls (χ^2=15.21, P<0.01). Intra-CeA administration of 2.4 µg clonidine (0.5 or 1.0 µl) or ST-91 (0.5 or 1.0 µl) reduced irritability compared with subjects given naloxone alone, being observed in 17, 12, 40, or 20% of subjects, (χ^2=8.55, P<0.01; χ^2=10.87, P<0.01; χ^2=4.22, P<0.05; χ^2=7.20, P<0.01). Irritability was reduced in all these four groups to levels not different from saline-treated control subjects. No other naloxone-induced checked sign was reduced (Table 2).

Intra-CeA infusions of clonidine or ST-91 failed significantly to attenuate any of the counted signs induced by naloxone during withdrawal. Significant main effects of group, nonetheless, were found for wet-dog shakes (F=10.35, df 5,37, P<0.01), teeth chattering (F=2.49, df 5,37, P<0.05), rearing (F=3.92, df 5,37, P<0.01), and weight loss (F=4.29, df 5,37, P<0.01). These differences were due to naloxone-induced significant increases in wet-dog shakes (df 17, P<0.01), teeth chattering (df 17,

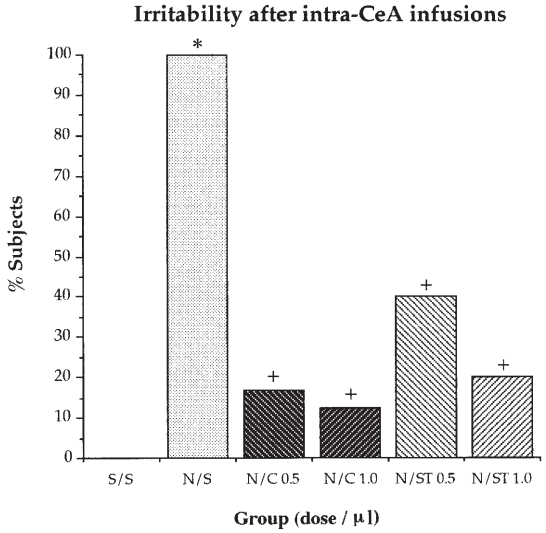

Fig. 3 Percentage of morphine-treated subjects in the groups displaying irritability during the test session (irritability, χ^2=26.67, *df*=5, *P*<0.01): *Ordinate*: percentage (%) of subjects with the sign present. *Abscissa*: the six groups – saline+intra-amygdala saline (*S/S*), *n*=9; naloxone+intra-amygdala saline (*N/S*), *n*=10; naloxone+2.4 µg/0.5 µl clonidine (*N/C0.5*), *n*=6; naloxone+2.4 µg/1.0 µl clonidine (*N/C1.0*), *n*=8; naloxone+2.4 µg/0.5 µl ST-91 (*N/ST0.5*), *n*=5; naloxone+2.4 µg/1.0 µl ST-91 (*N/ST1.0*), *n*=5. *Significant increase from saline controls (*S/S*). +Significant decrease from naloxone only treated subjects (*N/S*)

P<0.01) and weight losses (*df* 17, *P*<0.01) compared with saline-treated control subjects (Table 3). Clonidine and ST-91, given in infusion volumes of 0.5 µl, did not decrease teeth chattering; however, the mean was not increased when compared with saline control subjects. Neither grooming or weight loss were significantly reduced by intra-CeA infusions of clonidine or ST-91. Although general activity levels or jumping were not different in the groups, injection of naloxone reduced rearing compared with controls (*df* 17, *P*<0.05) in all animals except the groups receiving intra-CeA infusions of 1.0 µl clonidine (Table 3). Although the counted signs observed in the 5-min time periods (*F*=5.99, *df* 5,37, *P*<0.01) and the incidence of total signs (counted and checked, *F*=8.29, *df* 5,37, *P*<0.01) were increased by naloxone, there were no reductions in signs after intra-CeA clonidine or ST-91 (data not shown).

Infusions of CNQX into the LC or CeA

There was a significant interaction between site (LC versus CeA) and drug (vehicle versus CNQX) for checked signs (*F*=5.01, *df* 1,36, *P*<0.03) after naloxone-precipitated withdrawal. Checked signs were reduced after infusions of CNQX into the LC (*F*=6.90, *df* 1,22, *P*<0.02) or CeA (*F*=46.01, *df* 1,14, *P*<0.001) compared with naloxone-treated subjects given intra-LC or intra-CeA vehicle infusions, as shown in Table 4. The reduction was greater after infusions of CNQX into the CeA than after infusions into the LC (*F*=13.39, *df* 1,19, *P*<0.001).

Table 4 Percentage of subjects in the groups displaying the checked signs diarrhea, irritability, ptosis, rhinorrhea, lacrimation, penile erection (χ^2=14.59; χ^2=11.48; χ^2=7.49; χ^2=15.14; χ^2=17.72; χ^2=9.51, *df* 3) and abnormal posture. The four groups are shown (*n*=12, 12, 7, 9, respectively) and significant reductions are shown compared with their respective control groups, i.e., CeA-SAL or LC-SAL compared with CeA-CNQX or LC-CNQX, respectively (**P*<0.01, χ^2>9.44). There were four naloxone-treated groups – intra-CeA saline, *n*=12; intra-CeA CNQX, *n*=12; intra-LC saline, *n*=7; intra-LC CNQX, *n*=9. The mean incidence of checked signs are also shown. +*P*<0.01 difference between CeA-CNQX and LC-CNQX

% Subjects behavior	CeA-SAL	CeA-CNQX	LC-SAL	LC-CNQX
Diarrhea	88	44	91	9*
Irritability	71	11*	83	50
Ptosis	85	11*	50	66
Rhinorrhea	100	56	100	41*
Lacrimation	86	0*	83	41
Abnormal posture	100	56	60	75
Penile erection	71	11*	75	60
Mean checked signs	6.1(0.5)	1.7(0.4)*+	6.2(0.5	4.3(0.5)*

Table 5 Mean differences (±SEM) between the experimental groups after intra-CeA or -LC infusions for each of the counted signs and weight loss. *Significant differences (*P*<0.01) compared with control-infused naloxone-treated subjects (CeA-SAL or LC-SAL). Mean number of behaviors during the 15-min test session in morphine-dependent rats (±SEM) See Table 2 for group definitions

% Subjects behavior	CeA-SAL	CeA-CNQX	LC-SAL	LC-CNQX
Wet dog shakes	20.1(4.4)	6.2(3.1)*	19.5(2.1)	11.5(1.3)*
Teeth chattering	8.4(2.3)	3.6(1.2)*	12.7(1.9)	8.9(1.6)*
Rearing	7.4(2.1)	6.7(1.2)	5.2(0.8)	4.5(0.9)
Grooming bouts	5.6(1.6)	3.6(1.1)	2.1(1.0)	0.8(0.3)
Weight loss	9.9(0.8)	5.9(1.3)*	5.9(0.9)	3.8(0.6)*

CNQX infusions into the CeA significantly reduced ptosis, irritability, lacrimation, and penile erections during withdrawal, as shown in Table 4. Conversely, CNQX infusions into the LC reduced diarrhea and rhinorrhea (Table 4). Significant reductions in checked signs in both groups were thereby represented by different behaviors. Abnormal posture was the only behavior not affected by these treatments.

There were significant differences between the LC, CeA and vehicle-control groups for wet-dog shakes, teeth chattering, grooming, weight lost, counted signs, and total signs, but not for activity, rearing, jumping, or any of the other counted signs. Comparisons shown in Table 5 are detailed below. Infusions of CNQX into the LC or the CeA significantly reduced the number of wet-dog shakes that were observed during naloxone-precipitated withdrawal, as suggested by a main effect of drug (*F*=17.30, *df* 1,36, *P*<0.001) but no effect of site or drug×site interaction. Teeth chattering was lower in CeA-infused subjects compared with LC-infused subjects (*F*=6.66, *df* 1,36, *P*<0.02), regardless of whether

CNQX or saline was infused. There was also a main effect of drug, such that CNQX infusions into either the LC or CeA reduced teeth chattering (F=5.40, df 1,36, P<0.01). In contrast, grooming bouts were lower in the LC-infused subjects compared with CeA-infused subjects (F=9,33, df 1,36, P<0.01), averaging less than one bout compared with approximately four bouts; however, they were not reduced by CNQX infusions into either site. The effects of site of infusion for teeth chattering (<CeA subjects) and grooming (<LC subjects) could be due to some damage caused to the overlying tissues; however, most other withdrawal behaviors were not differently affected. In addition, these behaviors are exceedingly difficult to score since they are measured as "bouts" and the end and subsequent beginning of a bout is not always distinct. Thus, these differences may reflect problems in behavioral ratings. Infusions of CNQX into either the LC or CeA reduced the average weight loss (F=10.75, df 1,36, P<0.01), perhaps due to reductions in diarrhea. There was also a main effect of site indicating that subjects given LC infusions lost less weight than subjects given intra-amygdaloid infusions (F=10.55, df 1,36, P<0.01). This was probably due to the fact that after LC surgery, subjects did not gain weight as rapidly as subjects after CeA surgery, and thus LC subjects were lighter and thus these differences may be related to a "floor effect". Both the frequency of counted signs during the 5-min time periods (F=5.58, df 1,36, P<0.03) and incidence of total signs (F=39.26, df 1,36, P<0.0001) observed during the session were also attenuated by either CNQX infusions into the CeA or LC (data not shown).

Discussion

This study provides direct support for the hypothesis that altered noradrenergic and glutamatergic transmission in the LC and CeA contributes to the role played by these brain regions in the behavioral signs of opiate withdrawal. Attenuation of behavioral signs of opiate withdrawal were observed after intra-LC administration of alpha-2 agonists or CNQX. These data are consistent with several previous studies suggesting that the LC is an important part of the brain circuitry which subserves withdrawal behavior and that LC neuronal hyperactivity during withdrawal is mediated in part by augmented EAA input to the LC during withdrawal (Rasmussen and Aghajanian 1989; Akaoka and Aston Jones 1991; Rasmussen 1995). For example, intra-LC infusions of AP5 (an NMDA receptor antagonist), kynurenate (a non-selective glutamate receptor antagonist), and most effectively CNQX attenuated naloxone-induced activation of LC neurons in morphine-dependent rats (Akaoka and Aston Jones 1991). This is the first demonstration that non-NMDA receptor antagonists infused directly into the LC could attenuate behavioral signs of naloxone-precipitated withdrawal.

Infusions of CNQX into the CeA also markedly attenuated physical signs of withdrawal, though affecting somewhat different withdrawal behaviors compared with intra-LC infusions. This effect may be due to two factors. The amygdala is known to have connections with several brain regions importantly involved in opiate withdrawal, anxiety and fear, including the LC, periaqueductal gray (PAG), and hypothalamus (see Davis 1992; Maldonado et al. 1992; Maldonado 1997), and the amygdala is implicated in opiate withdrawal-induced place aversion (Stinus et al. 1990), a measure of negative affect. The amygdala is a site where microinfusions of opiate antagonists elicit aversive motivational states (Stinus et al 1990) and lesions of the amygdala can block these place aversions (Kelsey and Arnold 1994). These anatomical and behavioral studies suggest that the amygdala and LC may be part of a neural circuit (Aston-Jones et al. 1986; Davis 1992) subserving the physical as well as emotional components of opiate withdrawal.

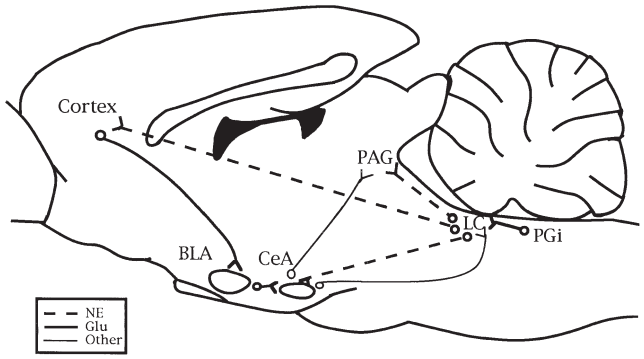

Fig. 4 A sagittal section of the rat brain illustrating neuroanatomical connections between the LC and amygdala and other sites possibly important in withdrawal. Noradrenergic and glutamatergic containing neurons are shown by *dashed* and *solid lines*. Increased LC-NE transmission during withdrawal activate the cortex, PAG and CeA. Glutamatergic projections from the cortex to the BLA, BLA to CeA, and PGi to LC are shown in *bold lines*. Shown also are connections to the LC and PAG from the amygdala, perhaps important in the role of the amygdala in somatic signs of withdrawal. *NE*, Noradrenergic; *Glu*, glutamate

Figure 4 illustrates some of the glutamatergic and noradrenergic connections between the LC and CeA and other brain regions such as the PAG thought to be involved in opiate withdrawal. EAA mediated synaptic transmission in the amygdala originating from both cortical and subcortical inputs, and intrinsic amygdaloid connections seem to be involved in the expression of fear-related (Davis et al. 1992) and withdrawal-related behaviors. Within the amygdaloid complex, afferents from the lateral and basolateral nucleus of the amygdala to the CeA are also glutamatergic and NMDA and non-NMDA receptors in the amygdala are known to be involved in anxiety- and fear-related behaviors (see Davis 1992). For example, the expression of aversive cue conditioning can be blocked by non-NMDA antagonists such as CNQX (Campeau et al. 1992). Non-NMDA EAA receptors in the CeA may be involved in aversive states such as opiate withdrawal as well as anxiety and fear. Furthermore, cortico-amygdaloid glutamatergic afferents may be modulated by NE transmission in cere-

bral cortex (Davis et al. 1994). These are two potential circuits by which NE and glutamate may interact in the expression of opiate withdrawal. Moreover, recent studies have suggested that cortical glutamatergic afferents regulate LC neuron activity at the somatodendritic level.

Interestingly, clonidine infusions into the CeA only attenuated irritability, a sign indicative presumably of the aversive emotional effects of withdrawal. Intra-CeA clonidine did not attenuate somatic signs of withdrawal, despite the fact that clonidine given either systemically or directly into the amygdala has been shown partially to attenuate the activation of amygdaloid neurons upon withdrawal (Freedman and Aghajanian 1985). The failure of intra-CeA clonidine, or ST-91, to attenuate withdrawal may be due to a number of factors. First, although opiate antagonist infusions into the amygdala do elicit some physical signs of withdrawal, these effects are less dramatic than after infusions into other brain sites (Maldonado et al. 1992). Second, there may be different or weaker intrinsic adaptations in amygdaloid neurons after opioid administration compared with those known to occur in LC neurons. Although chronic morphine increases PKA activity in the amygdala, as well as the LC, this effect is much smaller than that observed in the LC and it is not accompanied by an increase in adenylyl cyclase (Terwilliger et al. 1991). Moreover, infusions of PKA inhibitors into the LC or PAG do attenuate, but similar infusions into the CeA do not significantly attenuate, opiate withdrawal behaviors (Punch et al. 1997). Finally, the reduction of withdrawal behaviors after intra-LC clonidine infusions (Taylor et al. 1988) may be the result of a potent inhibition of LC neurons produced by alpha-2-agonist administration at the somatodendritic region via stimulation of impulse-regulating autoreceptors. In contrast, alpha-2 receptors (presumably release-regulating autoreceptors) located on terminals within target fields such as the amygdala may not have the same potential for reducing NE transmission. Therefore, the failure of intra-CeA clonidine infusions to attenuate withdrawal may not indicate that the CeA is not part of brain withdrawal circuitry or that NE activity is not involved but may suggest that the CeA is not a primary site for the induction of physical signs of withdrawal.

Other brain regions, such as the PAG and peripheral sites, are known to be altered by opiate withdrawal, and some evidence for the involvement of the LC has been suggested to be attributable to diffusion of drugs, such as clonidine, from the LC to other sites such as the PAG (Christie et al. 1997). We therefore attempted to limit drug diffusion in order to identify critical brain sites associated with opiate withdrawal. The non-lipophilic alpha-2-agonist ST-91, in addition to clonidine, was used and these agonists were given in two volumes (1.0 and 0.5 µl). No behavioral differences between the two agonists or the two volumes were found, except for attenuation of checked signs, where only the larger volumes had significant effects. Notably, irritability was reduced by the drugs at both volumes. Nevertheless clonidine had weak effects in the CeA, and these effects appeared to be confined to "emotional" behaviors. Thus, NE in the CeA may not be a substrate for the initiation of somatic signs but may play a role in mediating emotional or reward circuitry that drives somatic and aversive consequences of opiate withdrawal (see Harris and Aston-Jones 1994).

The ability of CNQX, but not clonidine, to attenuate physical withdrawal signs when given into the CeA suggests an important contribution for glutamatergic activation of the CeA from regions like the cortex and basolateral amygdaloid nucleus in opiate physical withdrawal behaviors. The ability of CNQX to attenuate withdrawal signs when given into the LC is consistent with the increased EAA transmission in LC during withdrawal and with a role for NE neurons of the LC in opiate withdrawal. These data provide behavioral evidence to support the hypothesis that the contribution of the LC and CeA to opiate withdrawal signs is mediated by altered glutamatergic projections to the LC and CeA and provide further support for the role of altered noradrenergic activity. The mechanism by which long-term exposure to morphine produces adaptations in subpopulations of opiate-response neurons in brain is not known, but identifying the circuitry associated with reducing the behavioral expression of withdrawal should help identify and develop treatment strategies. Withdrawal signs may be differentiated by whether they are caused by "within-system adaptations" that may involve intrinsic changes in regions in brain and periphery that mediate the "primary pharmacological actions of the drug", and "between-system adaptations" where primary effects in turn affect other anatomically connected brain sites (Shulteis and Koob 1996). Because these changes in noradrenergic and glutamatergic systems may result from independent adaptations, we are currently investigating if there would be additive effects in combining noradrenergic and glutamatergic treatments in the development of novel anti-withdrawal strategies.

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