Nor-binaltorphimine Precipitates Withdrawal and Excitatory Amino Acid Release in the Locus Ceruleus of Butorphanol—but Not Morphone-Dependent Rats

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ABSTRACT
The relative involvement of kappa opioid receptors in the mediation of behavioral and neurochemical responses to withdrawal from chronic drug treatment with the opioid analgesic butorphanol was studied using in vivo microdialysis to detail extracellular fluid concentrations of glutamate and aspartate within the locus ceruleus. Sprague-Dawley rats were rendered opioid dependent after 3 days of intracerebroventricular (i.c.v.) infusion of butorphanol (26 nmol/μl/hr) or morphine (26 nmol/μl/hr) and after i.c.v. infusion of saline vehicle (1 μl/hr). Acute withdrawal was precipitated by i.c.v. injection of the selective kappa opioid receptor antagonist nor-binaltorphimine (48 nmol/5 μl) after the 3-day period of infusion. Behavioral signs of withdrawal were detected after nor-binaltorphimine only in butorphanol-dependent rats. Basal levels of glutamate and aspartate were not different between treatment groups. Nor-binaltorphimine in the butorphanol-dependent rats increased glutamate to 227% and aspartate to 158% in the initial 15-min sample (P < 0.01). Nor-binaltorphimine did not increase glutamate or aspartate concentrations in the morphine-dependent or saline-treated groups. These results indicate a significantly greater participation of kappa opioid receptors in the development of butorphanol, rather than morphine, dependence and identify a differential neurochemical response to butorphanol withdrawal within a defined brain region, the locus ceruleus.

Butorphanol (17-cyclobutylmethyl-3,14-dihydroxymorphinan) tartrate is available for clinical use as an unscheduled opioid analgesic agent under the trade name of Stadol. A member of the phenanthrene class of opioid analogues, butorphanol is characterized as a mixed agonist-antagonist that exerts an analgesic action with a potency seven times greater than that of morphine (Dobkin et al., 1976). Indicated for the relief of moderate to severe pain (Wilkinson, 1987), butorphanol is considered to have a low abuse potential when used according to therapeutic recommendations. Nevertheless, several clinical reports document the development of dependence on butorphanol (Brown, 1985; Evans et al., 1985; Jasinski et al., 1976), significant illegal diversion of the drug from hospitals within the United States (Hoover and Williams, 1985) and frank abuse by teenagers (Smith and Davis, 1984). The recent release of a formulation for intranasal application of butorphanol may increase the likelihood of diversion and abuse of the agent, particularly among medical personnel (Jasinski et al., 1988). The opioid receptor interaction profile of butorphanol differs significantly from that of the classic opioid analgesic morphine. Butorphanol has been shown to interact with the kappa as well as the mu and delta opioid receptors (Horan and Ho, 1989b), whereas morphine binds as an agonist to primarily the mu and delta opioid receptors (Abdelhamid et al., 1991; Gulya et al., 1988; Miyamoto et al., 1993). Although it is clear that the actions of butorphanol at the mu and delta opioid receptors are important to the development of dependence (Jaw et al., 1993; Oh et al., 1992), evidence indicates that interaction with the kappa opioid receptor contributes significantly to the response (Feng et al., 1994a, 1994b; Jaw et al., 1993, 1994). Additional data are needed to establish the relative degree of the involvement of kappa opioid receptors in mediation of specific aspects of butorphanol dependence, particularly with respect to assessment of withdrawal from dependence.

Excitatory amino acid neurotransmitters, including glutamate and aspartate, participate in the opioid withdrawal syndrome, an involvement that is evident particularly within the pontine locus ceruleus (Rasmussen et al., 1990; Tanganelli et al., 1991). Recently, it has been recognized that naloxone-precipitated withdrawal from dependence on both morphine and butorphanol is associated with increased extracellular fluid levels of glutamate and aspartate within the locus ceruleus (Aghajanian et al., 1994; Feng et al., 1995).

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ABBREVIATIONS: i.c.v., intracerebroventricular; i.p., intraperitoneal; CTOP, d-Pen-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2; NMDA, N-methyl-D-aspartate; s.c., subcutaneous.
Both behavioral signs of withdrawal and increased locus ceruleus concentrations of glutamate and aspartate can be elicited in butorphanol- and morphine-dependent rats after i.c.v. challenge injections of selective antagonists at mu or delta opioid receptors; no differences in the magnitude of the responses can be distinguished between rats dependent on either opioid analgesic (Feng et al., 1996). These similarities, and the documented differences between butorphanol and morphine with respect to kappa opioid receptor stimulation, suggest that it is of interest to examine the relative role of the kappa opioid receptor in mediation of the behavioral and neurochemical responses to withdrawal from dependence on butorphanol in comparison with withdrawal from morphine dependence. To accomplish this objective, i.c.v. injections of the selective kappa opioid receptor antagonist nor-binaltorphimine were used to precipitate withdrawal in butorphanol- and morphine-dependent rats. Measurements of glutamate and aspartate concentrations from within the locus ceruleus were performed using in vivo microdialysis in conscious rats and correlated with the behavioral signs of withdrawal elicited after nor-binaltorphimine challenge.

**Methods**

**Surgical procedures.** Male Sprague-Dawley rats (250–300 g; Charles River, Wilmington, MA) were purchased and maintained under conditions of 21±2°C with a 12 hr/12 hr light/dark cycle for 1 week before surgery. Each rat was anesthetized with Equithesin (4.25 g of chloral hydrate, 2.23 g of MgSO₄ · 7H₂O, 0.972 g of pentobarbital Na, 44.4 ml of propylene glycol, 10 ml of 95% ethanol and distilled water to make a final volume of 100 ml; 0.3 ml/100 g of body weight i.p.), and then placed in a stereotaxic apparatus. A dorsal midline skin incision was made on the cranium, and soft tissue was cleared from the skull by blunt dissection. The position of the incisor bar was adjusted so that the skull sutures, bregma and lambda, were at the same vertical position, leveling the dorsal skull surface. A burr hole was made in the skull, the dura was incised and a stainless steel cannula (26 gauge, 10 mm long) was implanted into the right lateral cerebral ventricle to permit i.c.v. injection or infusion. A stylet (32-gauge sealed stainless steel tubing) was inserted into the guide cannula to maintain cannula patency. The presence of cerebrospinal fluid in the guide cannula was noted as verification of proper ventricular placement. The coordinates for implantation were (in mm relative to bregma) 0.5 posterior and 1.3 lateral (Paxinos and Watson, 1986). The cannula tip was lowered 4.5 mm below the surface of the dura. A CMA/11 microdialysis guide cannula (Bioanalytical Systems, West Lafayette, IN) was implanted with the tip directed toward the locus ceruleus. The coordinates for implantation were (in mm relative to bregma) 9.8 posterior and 1.1 lateral (Paxinos and Watson, 1986). The cannula tip was lowered 6.8 mm below the surface of the dura. Five stainless steel screws were secured to the skull, an aluminum protective cap was placed around each guide cannula and dental acrylic (Lang Dental, Wheeling, IL) was applied to anchor the assembly to the skull surface. Each rat received an s.c. injection of 150,000 units of procaine penicillin G immediately after surgical implantation of cannulae. One week was permitted for recovery from surgery before initiation of further experimental procedures. Each rat was killed after completion of an experimental protocol by injection of an overdose of Nembutal; the brain was removed and post-fixed by immersion in 10% formalin. The i.c.v. cannula track was verified visually by cutting vertically through the cannula mark on the surface of the cortex. The remaining brain fragment was frozen on dry ice, and 50-μm sections were cut through the pons using a microtome-cryostat (Ames Lab-Tek, Westmont, IL). Animals were included in statistical treatment of data only if probe placement within the locus ceruleus was histologically verified (fig. 1).

**Administration of drugs and induction of opioid dependence.** Each animal was reanesthetized with ether and prepared for implantation of an osmotic minipump (Alzet 2001; Alza, Palo Alto, CA), filled with sterile solutions of butorphanol (26 nmol/μl), morphine (26 nmol/μl) or saline vehicle. Dependence was produced by i.c.v. infusion (26 nmol/μl/hr for 3 days) of butorphanol or morphine. Saline vehicle rats received a similar volume rate of 0.9% NaCl (1 μl/hr) over the 3-day infusion period. Both the infusion period and dose paradigm have been used in studies from this laboratory (Feng et al., 1996; Horan and Ho, 1991). Osmotic minipumps were implanted s.c. between the scapulae with the animals under ether anesthesia. A 4-cm piece of Tygon tubing (0.38 mm inner diameter; Cole-Palmer, Chicago, IL) was used to connect the outlet of the minipump to a piece of L-shaped stainless steel injector tubing (32 gauge, 30 mm in length), which was placed into the i.c.v. guide cannula. Butorphanol, morphine or saline vehicle solutions were passed through a 0.2-μm sterile Acrodisk filter (Gelman Sciences, Ann Arbor, MI) before they were introduced into the minipump reservoirs. Minipumps were primed overnight at room temperature in normal saline so the nominal flow rate (1 μl/hr) was attained before implantation.

**Measurement of behavioral signs during opioid withdrawal.** Ten distinct behaviors (teeth-chattering, wet-dog shakes, rearing, locomotion, stretching, scratching, ptosis, yawning, forepaw-tremor and penis-licking) were scored as behavioral signs of withdrawal during the 30-min period after nor-binaltorphimine injection (i.c.v.). The reactions of each animal were evaluated by an independent investigator who did not have knowledge of the nature of the treatment received. The frequency of occurrence of each sign during the 30-min observation period was used to compare responses between the saline group and the nor-binaltorphimine-precipitated withdrawal groups for statistical purposes.

**Microdialysis sampling.** The microdialysis probes (CMA/11, 2 mm tip) and guide cannulae were purchased from Bioanalytical Systems (West Lafayette, IN) and used within 3 months. The dialysis membrane tip of the probe has an outer diameter of 240 μm and an inner diameter of 210 μm, a dead volume of 1 μl and a molecular weight cutoff of 20,000 Da. The in vitro recoveries of glutamate and aspartate were determined by immersion of probes in Ringer’s solution containing 100 μM each of glutamate and aspartate at room temperature.
temperature. Probes were perfused with Ringer’s solution at a rate of 2 μl/min, samples were collected and dialyzed samples were subsequently analyzed by HPLC. The values from three or four consecutive samples were averaged to determine the average recovery values for each probe. The averaged recovery values of glutamate and aspartate were 7.51 ± 0.89% and 5.43 ± 0.66%, respectively, of the external concentrations of each amino acid for 16 probes. Due to the variability of probe recovery, the extracellular levels of each amino acid were calculated individually for each animal.

**Analysis of amino acids**. The method of Ellison et al. (1987) was used, with minor modification, for measurement of amino acids. Briefly, the measurements were performed on an HPLC (BAS 200; Bioanalytical Systems) with electrochemical detection. A Rainin C18 column (150 × 4.6 mm I.D., 5 μm, 100 Å) was used. The mobile phase consisted of 0.1 M sodium phosphate (mono and dibasic)/methanol buffer (63:37, v/v; pH 5.2). The derivatizing agent consisted of o-phthaldialdehyde (50 mg), 2-mercaptoethanol (40 μl), absolute ethanol (0.9 ml) and 0.1 M borate buffer to make a final volume of 10 ml. The glutamate peak was verified by retention time, peak shape and comparison of samples with a standard consisting of eight amino acids (aspartate, glutamate, glutamine, histidine, arginine, glycine, taurine, and alanine; each at a concentration of 5 μM).

**General experimental procedures**. One week after stereotaxic surgery, each rat was anesthetized with ether, and an osmotic minipump was implanted. An i.c.v. infusion of butorphanol, morphine or saline was initiated and maintained for 3 days as described above. On the third day after initiation of i.c.v. infusion, each rat was placed in an individual stainless steel wire-bottom cage, and a plastic harness was secured loosely around the chest. This harness was tethered to a microdialysis counterbalance arm, and a freshly calibrated microdialysis probe was placed into the locus ceruleus guide cannula. The probe was perfused for the initial 18 hr at a low flow rate (0.2 μl/min) using a CMA/100 microdialysis infusion pump. Each experimental sequence began with disconnection of the tubing between the osmotic minipump and the i.c.v. inlet cannula. The infusion rate through the probe was increased to 2 μl/min for an equilibration period of 2 to 3 hr. Thereafter, a series of four or five sequential 15-min samples were collected for determination of basal concentrations of glutamate and aspartate. Nor-binaltorphimine was injected i.c.v. (48 nmol/5 μl) over a 5-min period using a hand-held microliter syringe. Samples were collected during the hour immediately after nor-binaltorphimine injection. This dose of nor-binaltorphimine was chosen based on the results of prior studies (Jaw et al., 1993, 1994). In particular, the dose was determined from the study of Jaw et al. (1994), who compared the effects of a range of nor-binaltorphimine doses (12–100 nmol i.c.v.) with those of naloxone in the elicitation of behavioral signs of acute withdrawal in butorphanol-dependent rats.

**Statistics**. The Student’s t test was used to test for differences between the basal level of an amino acid and the maximal value obtained after nor-binaltorphimine challenge. One-way analysis of variance and the Newman-Keul test were used to test among the amino acid concentrations in the butorphanol, morphine and saline groups. Calculated values of P < 0.05 were considered statistically significant. Values for amino acid concentrations that are expressed as percentage change from basal values were calculated from a single basal value that represented the average of the four individual basal samples taken immediately before nor-binaltorphimine challenge. The frequencies of occurrence of each behavioral sign were analyzed by one-way analysis of variance, and the Bonferroni test was used to differentiate among the mean values of these three groups. Mean ± S.E.M. values are reported.

**Results**

Basal levels of glutamate and aspartate did not differ (P > 0.05) among saline-treated (n = 4; 10.75 ± 1.22 and 8.16 ± 0.66 μM) and butorphanol-dependent (n = 6; 8.04 ± 1.58 and 6.76 ± 0.78 μM) or morphine-dependent (n = 6; 9.62 ± 1.76 and 8.29 ± 1.64 μM) groups, respectively. Precipitation of acute withdrawal after i.c.v. injection of nor-binaltorphimine produced significant increases in the concentration of glutamate in butorphanol-dependent, but not in morphine-dependent or saline-treated rats, within the locus ceruleus (fig. 2). A maximal increase in glutamate concentration of 226.5% above the basal level was noted in the initial 15-min sample after nor-binaltorphimine challenge; the absolute value for the glutamate concentration in that sample was 18.24 ± 4.08 μM. The glutamate concentration remained elevated in the second 15-min sample, as well, before returning to levels not statistically different from the basal level in the third 15-min sample. The glutamate levels in the initial samples from the groups receiving morphine (10.65 ± 1.23 μM) or saline (10.23 ± 1.04 μM) were not significantly different from basal values in the respective group. Similarly, a significant increase in the locus ceruleus aspartate concentration was noted in the initial 15-min sample after nor-binaltorphimine challenge in the butorphanol-dependent group (11.42 ± 1.17 μM; 158.3% of the basal value; fig. 3). The aspartate concentrations from the initial 15-min sample did not differ from basal values in the groups receiving either morphine (7.47 ± 1.62 μM) or saline (7.71 ± 0.63 μM). Although aspartate concentrations were not elevated to the same degree as those of glutamate, aspartate levels were remained elevated for a longer period. Significant increases above basal levels were noted in aspartate in the first three 15-min samples after nor-binaltorphimine challenge.

Behavioral evidence of withdrawal, as identified by the incidence of 10 specific signs in the 30-min period after nor-binaltorphimine challenge, was present only in butorphanol-dependent rats (table 1). Significantly higher frequencies of 5 of the 10 signs (wet-dog shakes, teeth chattering, stretching, scratching and fore-paw tremor) were noted in the butorphanol-dependent rats compared with morphine-dependent rats.
TABLE 1
Frequencies of withdrawal signs precipitated by i.c.v. injection of the kappa opioid receptor antagonist non-binaltorphimine in butorphanol- or morphine-dependent rats

<table>
<thead>
<tr>
<th></th>
<th>Butorphanol (n = 6)</th>
<th>Morphine (n = 6)</th>
<th>Saline (n = 4)</th>
</tr>
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<tbody>
<tr>
<td>Wet-dog shakes</td>
<td>10.67 ± 3.00</td>
<td>1.33 ± 0.80b</td>
<td>1.50 ± 0.86a</td>
</tr>
<tr>
<td>Teeth chattering</td>
<td>9.50 ± 2.14</td>
<td>0.17 ± 0.17b</td>
<td>0.50 ± 0.50</td>
</tr>
<tr>
<td>Rearing</td>
<td>1.16 ± 0.40</td>
<td>0</td>
<td>0.50 ± 0.50</td>
</tr>
<tr>
<td>Locomotion</td>
<td>2.67 ± 1.20</td>
<td>0.17 ± 0.17</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>Stretching</td>
<td>5.83 ± 1.57</td>
<td>0b</td>
<td>0</td>
</tr>
<tr>
<td>Penis-licking</td>
<td>1.50 ± 0.80</td>
<td>0.17 ± 0.17</td>
<td>0</td>
</tr>
<tr>
<td>Scratching</td>
<td>1.50 ± 0.50</td>
<td>0.17 ± 0.17</td>
<td>0</td>
</tr>
<tr>
<td>Ptosis</td>
<td>1.00 ± 0.44</td>
<td>0.17 ± 0.17</td>
<td>0</td>
</tr>
<tr>
<td>Yawning</td>
<td>3.00 ± 0.08</td>
<td>0.16 ± 0.16</td>
<td>0</td>
</tr>
<tr>
<td>Forepaw-tremor</td>
<td>3.17 ± 1.08</td>
<td>0b</td>
<td>0</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01 (analysis of variance test), withdrawal score of butorphanol group vs. morphine or saline group.
© 0, no positive signs detected.

Discussion

The relative involvement of opioid receptor subtypes in the development of dependence on, and withdrawal from, butorphanol differs from that observed with the prototype opioid analgesic morphine. The results of the present study extend the body of evidence that supports this conclusion by documenting that the kappa opioid receptor plays a role in neurochemical and behavioral indices of withdrawal in butorphanol, but not morphine, dependent rats. This is demonstrated by the finding that acute precipitation of withdrawal from butorphanol, but not morphine, dependence can be elicited by i.c.v. injection of the selective kappa opioid receptor antagonist nor-binaltorphimine. The results indicate further that excitatory amino acid neurotransmission within the pontine locus ceruleus is altered during withdrawal from dependence on butorphanol.

Morphine has been demonstrated to activate primarily the mu opioid receptor (Gulya et al., 1988), with possible agonistic actions also at the delta opioid receptor subtype (Abdelhammad et al., 1991; Martin et al., 1976; Miyamoto et al., 1993). In contrast, butorphanol is an agonist not only at the mu and delta opioid receptors but also at the kappa opioid receptor (Horan and Ho, 1989b), with binding affinity ratios to mu, delta and kappa opioid receptors of 1:4:25 (Chang et al., 1983; Lahti et al., 1985). The characteristics of action of butorphanol at the mu opioid receptor are such that it is capable of precipitating withdrawal in rats made dependent on morphine (Horan and Ho, 1989a; Piccio et al., 1976) and can substitute for agonists at the mu opioid receptor in drug discrimination paradigms (Picker and Dykstra, 1989). However, butorphanol has been shown to be self-administered to a lesser degree than was the mu opioid receptor agonist codeine and capable of producing antagonism of the analgesic effects of high-efficacy mu opioid receptor agonists (Woods and Gmerek, 1985). Butorphanol exerts a kappa opioid receptor agonist-like dependence profile (Woods and Gmerek, 1985) and has been demonstrated to cause increases in urinary output, although not to the extent as that produced by brexazoicine and other high-efficacy kappa opioid receptor agonists. In addition, butorphanol has been reported to blunt the urinary output (Leander, 1983a, 1983b) and analgesic responses (Piccio et al., 1976) to administration of high-efficiency kappa opioid receptor agonists. These observations have led to the classification of butorphanol as a mu and kappa opioid receptor agonist with intermediate efficacy (Leander, 1983b; Picker and Dykstra, 1989).

An earlier report from this laboratory found that morphine and butorphanol were equipotent in the induction of dependence, as demonstrated by the behavioral signs elicited after acute precipitation of withdrawal by challenge with the nonselective opioid antagonist naloxone (Horan and Ho, 1991). It is also of note that the general pattern for the behavioral response to precipitated withdrawal of morphine and butorphanol is similar (Jaw et al., 1994). The development of tolerance has been demonstrated in both the tail-flick and acetic acid writhing tests of analgesia after 1 to 3 days of the i.c.v. infusion of butorphanol (Feng et al., 1994b). In addition, cross-tolerance between butorphanol and morphine could be produced through i.c.v. infusion of the two agents (Feng et al., 1994a). In the latter study, chronic i.c.v. administration of butorphanol produced similar rightward shifts of the analgesic response to morphine in both the tail-flick and acetic acid writhing tests (Feng et al., 1994a). A recent investigation from this laboratory, using a protocol similar to that used in the present study, examined the ability of selective antagonists at mu and delta opioid receptors to precipitate withdrawal in rats rendered dependent on butorphanol or morphine. The i.c.v. injections of the selective mu opioid receptor antagonist CTOP and the delta opioid receptor antagonist naltrindole elicited equivalent behavioral signs of withdrawal and increases in locus ceruleus concentrations of glutamate and aspartate in butorphanol- and morphine-dependent rats (Feng et al., 1994c, 1996). The results have been interpreted to indicate that dependence on either butorpha-
nol or morphine involves roughly equivalent actions at mu and delta opioid receptors.

In contrast, nor-binaltorphimine challenge precipitates withdrawal selectively in butorphanol-dependent rats, which supports the hypothesis of kappa opioid receptor involvement in the development of dependence on butorphanol. Specifically, 48 nmol of nor-binaltorphimine was found to elevate locus ceruleus concentrations of both glutamate and aspartate in butorphanol- but not in morphine-dependent rats. For example, the degree of elevation in glutamate, above basal levels, was 227% in butorphanol-dependent rats. This compares with maximal elevations in glutamate of 253% and 150%, respectively, after precipitation of withdrawal from butorphanol dependence by i.c.v. injections of the selective mu and delta opioid receptor antagonists CTOP and naltrindole (Feng et al., 1996). Table 2 is provided to permit comparison of basal and maximal elevations in glutamate levels between this and our previous study (Feng et al., 1996). Although nor-binaltorphimine injection increased locus ceruleus glutamate levels only in butorphanol-dependent rats, CTOP and naltrindole elicited increases in glutamate in both morphine- and butorphanol-dependent rats (Feng et al., 1996). Finally, elicitation of the behavioral signs of withdrawal by nor-binaltorphimine was evident in butorphanol- but not in morphine-dependent rats. This latter result is meaningful because in a previous study, i.c.v. infusion of the agents CTOP and naltrindole elicited significant behavioral evidence of withdrawal in both morphine- and butorphanol-dependent animals (Feng et al., 1996). Although direct comparison of results from the present study with those of Feng et al. (1996) is not possible due to slight differences in the behavioral scoring protocol, the data support a significantly greater involvement of kappa opioid receptors in withdrawal from butorphanol dependence. Nor-binaltorphimine is a bivalent pharmacophore consisting of two molecules of naltrexone bound by a pyrrole ring, which possesses a 20-fold selectivity for kappa over mu opioid receptors in the guinea pig ileum and exerts a long-lasting antagonistic action (Portoghese et al., 1987). Moreover, after i.c.v. administration, nor-binaltorphimine exerts a 100-fold selectivity for kappa over mu opioid receptors when tested against the selective kappa opioid receptor agonist U-50,488 and the prototypical mu opioid receptor agonist morphine in the mouse writhing test for analgesic efficacy (Takemori et al., 1988). A 160-fold selectivity for kappa compared with mu and delta opioid receptors has been reported for nor-binaltorphimine in radioligand binding assays (Takemori et al., 1988). Naloxone differs from nor-binaltorphimine in that it can exert relatively nonselective antagonistic effects at mu and delta, as well as kappa, opioid receptors (Patterson et al., 1984). Both naloxone and nor-binaltorphimine challenge can precipitate behavioral signs of acute withdrawal in butorphanol-dependent rats (Jaw et al., 1994). However, protection of kappa opioid receptors by pretreatment with nor-binaltorphimine minimizes behavioral symptoms of acute, naloxone-precipitated withdrawal in butorphanol-dependent rats (Jaw et al., 1995). It has also been shown that continuous i.c.v. infusion of butorphanol for 3 days will induce cross-tolerance to the analgesic action of the selective kappa opioid receptor agonist U-50,488 as well as to morphine (Feng et al., 1994a). The results of the present study detail a kappa opioid receptor action to the actions of butorphanol, as demonstrated by the ability of nor-binaltorphimine to precipitate increases in locus ceruleus concentrations of glutamate and aspartate, as well as behavioral symptoms of opioid withdrawal in butorphanol- but not in morphine-dependent rats.

The contention that the locus ceruleus participates in morphine withdrawal is supported by an abundance of electrophysiological data (Rasmussen et al., 1990, 1991a; Rasmussen and Aghajanian, 1989). In this regard, the hallmark of withdrawal from morphine dependence is hyperactivity of noradrenergic neurons within the locus ceruleus (Aghajanian, 1978), a response that has been correlated with behavioral symptoms of opioid withdrawal (Gold et al., 1980; Redmond and Krystal, 1984). Indeed, the locus ceruleus has been identified as a principal site within the brain from which the behavioral signs of withdrawal from morphine dependence can be elicited by local tissue injections of the naloxone derivative methyl naloixonium (Maldonado et al., 1992). The issue addressed by the present study stems from the aforementioned body of data and is 2-fold. First, does a neurotransmitter system other than the opioid system or the alpha adrenoceptor system (Aghajanian, 1978) also contribute to the mediation of opioid withdrawal? Second, does withdrawal

### TABLE 2

**Comparison between concentrations of glutamate in extracellular fluid dialysate samples taken from the locus ceruleus of conscious rats in the present study with those from Feng et al. (1996)**

Values presented are from 15-min samples taken before (Basal) and from the initial 15 min samples taken immediately after i.c.v. injections of nor-binaltorphimine (48 nmol/5 µl) in rats rendered dependent on butorphanol or morphine and in rats treated with saline. The data are compared with previously published data (Feng et al., 1996) in which acute withdrawal was precipitated by i.c.v. injection of either CTOP (48 nmol/5 µl) or naltrindole (100 nmol/5 µl). Mean = ± S.E.M. values are given. The numbers in parentheses denote the number of animals tested in each group.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Glutamate concentration</th>
<th>Butorphanol</th>
<th>Morphine</th>
<th>Saline</th>
<th>Butorphanol</th>
<th>Morphine</th>
<th>Saline</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Initial 15-min sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Nor-binaltorphimine</td>
<td></td>
<td>Basal</td>
<td>Initial 15-min sample</td>
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<td></td>
</tr>
<tr>
<td>(48 nmol/5 µl)</td>
<td>8.04 ± 1.58</td>
<td>9.62 ± 1.76</td>
<td>10.75 ± 1.22</td>
<td>18.24 ± 4.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.65 ± 1.23</td>
<td>10.23 ± 1.05</td>
<td></td>
</tr>
<tr>
<td>CTOP†</td>
<td>10.17 ± 0.75</td>
<td>10.03 ± 1.12</td>
<td>9.99 ± 1.27</td>
<td>25.73 ± 1.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.17 ± 5.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.63 ± 1.59</td>
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<tr>
<td>(48 nmol/5 µl)</td>
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<tr>
<td>Naltrindole†</td>
<td>11.66 ± 1.19</td>
<td>12.68 ± 0.91</td>
<td>9.59 ± 1.27</td>
<td>17.47 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.56 ± 3.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.77 ± 1.60</td>
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<tr>
<td>(100 nmol/5 µl)</td>
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</table>

* Asterisks denote a statistical difference between an indicated and basal value for each group (<sup>a</sup> P < 0.05, <sup>b</sup> P < 0.01). Data from Feng et al. (1996) are reproduced by permission (†).
from butorphanol dependence involve such a system to a
greater extent than does withdrawal from morphine de-
pendence? Several lines of evidence are available that suggest
the participation of excitatory amino acids in both the hyper-
activity of locus ceruleus neurons and the behavioral symp-
toms that accompany acutely precipitated withdrawal from
opioid analogues. Specifically, the withdrawal-induced acti-
tivation of locus ceruleus neurons has been shown to be
blunted by i.c.v. pretreatment with kynurenic acid, a nonspe-
cific antagonist at excitatory amino acid receptors (Rasmus-
sten et al., 1991b; Tung et al., 1990). Additional investigations
by Akaoka and Aston-Jones (1991) determined that locus
ceruleus neuron hyperactivity could be suppressed, although
totally abolished, by local administration of kynurenic acid
or antagonists selective for NMDA and non-NMDA glu-
tamate receptor subtypes. Importantly, those investigations
established that local application of opioid antagonists into
the locus ceruleus was ineffective in precipitating neuron
hyperactivity, which indicated that locus ceruleus hyperac-
tivity does not result from local opioid-mediated events. How-
ever, it must be stated that withdrawal-induced hyperactiv-
ity of locus ceruleus neurons could not be blocked by
peripheral (s.c.) administration of MK-801 (10,11-dihydro-
5H-dibenzo[δc]cyclohepten-5,10-imine), a
noncompetitive NMDA receptor antagonist, in a similar
study, although the behavioral responses to withdrawal were
effectively inhibited (Rasmussen et al., 1991a). As these lat-
ter results suggest, the behavioral responses observed during
acutely precipitated withdrawal also appear to involve exci-
tatory amino acid mechanisms. For example, the s.c. admin-
istration of MK-801 effectively suppressed the behavioral
symptoms produced during acute withdrawal from morphine
dependence, not only in guinea pigs and mice (Tanganelli et al.,
1991) but also in rats (Rasmussen et al., 1991a). In
addition, administration of MK-801 has been demonstrated to
inhibit the development of both tolerance to and depend-
ence on morphine (Trujillo and Akil, 1991).

Microdialysis studies from the laboratories of Aghajanian
et al. (1994), as well as from our laboratories (Feng et al.,
1996; Zhang et al., 1994), have demonstrated that increased
extracellular fluid levels of excitatory amino acid neurotrans-
mitters within the locus ceruleus occur contemporaneously
with acutely precipitated withdrawal from opioid depend-
ence. However, until recently, only morphine had been used
to elicit dependence. The results of our studies (Feng et al.,
1995, 1996) have demonstrated that extracellular levels of
 glutamate are also elevated in the locus ceruleus during
naloxone-precipitated withdrawal from dependence on butor-
phanol. This provided direct evidence that increases in locus
ceruleus levels of excitatory amino acids may represent a
general phenomenon of opioid antagonist-precipitated with-
drawal from opioid dependence. This conclusion is supported
further by the results of our recent study (Feng et al., 1995,
1996) that demonstrated that withdrawal from butorphanol
elicits effects similar to those seen in morphine withdrawal.

Nevertheless, the mechanism that underlies the association
between opioid withdrawal and increased locus ceruleus lev-
els of excitatory amino acids remains unclear.

Glutamatergic projections to the locus ceruleus are known
to originate from the nucleus paragigantocellularis of the
rostral medulla oblongata (Aston-Jones et al., 1986; Ennis
and Aston-Jones, 1986, 1988). Lesions of the nucleus paragi-
gantocellularis have been shown to attenuate the hyperac-
tivity of locus cereuleus neurons associated with opioid with-
drawal (Rasmussen and Aghajanian, 1989), a response that
is believed to be mediated by an excitatory amino acid path-
way from the nucleus paragigantocellularis to the locus cer-
uleus. These data have led investigators to suggest that the
source of the increased glutamate originates from activation
of glutamatergic nerve projections from the nucleus paragi-
gantocellularis to the locus ceruleus (Ennis et al., 1992;
Zhang et al., 1994). No direct evidence is available concerning
the source of withdrawal-associated glutamate overflow from
the locus ceruleus. However, behavioral signs that mimic
those evoked during precipitated withdrawal from depend-
ence on butorphanol can be elicited after electrical stimula-
tion of the nucleus paragigantocellularis in conscious rats
(Liu et al., 1997). This strengthens an argument for involve-
ment of the nucleus paragigantocellularis in excitatory
events related to opioid withdrawal. Additional studies to
examine glutamate efflux from the locus ceruleus after elec-
trical stimulation of the nucleus paragigantocellularis are in
progress.

McFadzean et al. (1987) noted that administration of the
selective kappa opioid receptor agonist U 50488 to rat brain
slice preparations could depress excitatory synaptic trans-
mision to locus cereuleus neurons. This raises the possibility
that the increases noted in glutamate in the present study
were the result of blockade, by nor-binaltorphimine, of this
previously identified, presynaptic mechanism. However, the
i.c.v. administration of butorphanol to naive rats does not
alter locus cereuleus levels of glutamate (Feng et al., 1995;
Hoshi et al., 1996), as would be expected if the presynaptic
pathway were the principal mechanism underlying a selec-
tive kappa opioid receptor mediation of the observed phenom-
ena. One potential explanation for this apparent discrepancy
lies in the observation of McFadzean et al. (1987) that only a
modest percentage (38%) of the excitatory synaptic potential
within the locus cereuleus could be depressed by U-50488.
They suggested that kappa opioid receptors might inhibit
presynaptic glutamate release only on specific neuronal af-
erents to the locus cereuleus. In the naive rat, the depression
produced by butorphanol may not sufficiently reduce extra-
cellular concentrations of glutamate to be detected by in vivo
microdialysis techniques.

The results of the present study are significant in that they
demonstrate that excitatory amino acid levels within the
locus cereuleus can be increased and behavioral symptoms of
withdrawal can be elicited selectively by nor-binaltorphimine
in butorphanol-dependent rats. Thus, the physical depend-
ence produced by chronic administration of butorphanol can
be attributed to interaction with the kappa opioid receptor, a
phenomenon that does not occur when dependence is pro-
duced by similar administration regimens of morphine. Ad-
ditional investigations will need to be conducted to determine
the site of the opioid receptors on which butorphanol acts to
mediate withdrawal.

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