Brain Research, 289 (1983) 223–234
Elsevier

Chronic Naltrexone Increases Opiate Binding in Brain and Produces Supersensitivity to Morphine in the Locus Coeruleus of the Rat

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(Accepted May 3rd, 1983)

Key words: naltrexone — morphine — opiate receptor — α-adrenergic receptor — locus coeruleus — receptor supersensitivity

Rats were implanted subcutaneously for 2–4 weeks with slow-release pellets of naltrexone (10 mg) or placebo and then the pellets were removed. One day after removal of the pellet, animals were either (1) sacrificed and various CNS regions examined for specific binding of [^{3}H]naloxone, [^{3}H]etorphine or [^{3}H]rauwolscine or (2) they were anesthetized and prepared acutely for assessing morphine-induced changes in the spontaneous activity of neurons in the locus coeruleus (LC). Naltrexone treatment significantly increased the number of specific binding sites for opiates, but not for α_2-adrenergic antagonists, in spinal cord, hypothalamus, striatum and cortex. Specific binding of [^{3}H]naloxone was also increased in the LC. The spontaneous activity of neurons in the LC was reduced by the chronic naltrexone treatment, suggesting that these neurons became supersensitive to the tonic inhibitory effect of endogenous opioid peptides. Moreover, neurons in the LC of chronic naltrexone-treated rats exhibited an enhanced response to the inhibitory effects of morphine administered systemically. These results demonstrate that chronic opiate receptor blockade increases the number of receptor sites for morphine and that this increase in receptors is accompanied by a neuronal supersensitivity in the LC to morphine which can be assessed electrophysiologically.

INTRODUCTION

The chronic administration of drugs which block the action of a neurotransmitter may produce an increase in the number of postsynaptic receptor sites for that neurotransmitter. This increase in receptors is often accompanied by an enhanced response of the postsynaptic neuron to the neurotransmitter (i.e. supersensitivity). Increase in the number of receptor sites following the chronic administration of receptor antagonists have been demonstrated for a variety of classical neurotransmitter systems, including dopaminergic[11,34,46], noradrenergic[63] and cholinergic[8,32,48] systems. In general, the increase in these neurotransmitter receptor sites is accompanied by a supersensitivity to the neurotransmitter which is assessable electrophysiologically[10].

Recent evidence indicates that opioid neuronal systems also may adapt similarly to the chronic administration of opiate antagonists. The chronic administration of naloxone produces an increase in the number of opiate receptors which preferentially bind morphine and naloxone (i.e. μ-type opiate receptors) in the brains of infant[5,7] and adult[29,45,66] animals. However, it is not known presently whether this increase in opiate receptors is also accompanied by a neuronal supersensitivity to opiate agonists.

The brainstem locus coeruleus (LC) offers a potentially useful neuronal system for assessing the effects of chronic opiate blockade on subsequent electrophysiologic responses to opiates. The LC is involved functionally in a wide range of physiologic and behavioral processes[2] and is thought to have a modulatory influence on brainstem neurons which are involved directly in nociceptive and antinociceptive mechanisms[20]. There is a dense population of opiate receptors located in the LC[3,6,39,40] and the norepinephrine-containing cell bodies and fibers within this nucleus are innervated by enkephalinergic and endophinergic terminals[44,49,53,59]. Although the response

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of neurons to opiates may be characterized as heterogenous[20,27], neurons within the LC respond consistently to opiates and opioid peptides by decreasing their spontaneous firing rate[1,9,26,28,65]. Thus, opiate-induced depression of neuronal activity in the LC might be potentiated following the chronic administration of opiate antagonists.

In the present report, we examined the effect of chronic naltrexone treatment on the specific binding of opiates to various CNS regions and on the response of neurons in the LC to systemically administered morphine. The results presented here demonstrate that an increase in the number of opiate receptors (μ-type) following chronic naltrexone blockade is accompanied also by a neuronal supersensitivity in the LC to morphine assessed electrophysiologically.

METHODS

Animals

The animals were adult male Sprague–Dawley rats (Harlan, Madison, WI) weighing between 200 and 250 g at the start of the experiments. Food and water were available continuously under group housing conditions.

Naltrexone pellets

Long-acting naltrexone pellets were manufactured by the method of Misra and Pontani[33]. Briefly, naltrexone free base (620 mg) was mixed with cholesterol (2230 mg), glyceryltristerate (248 mg) and $CHCl_3$ (62 ml) in a round-bottom flask. The mixture was evaporated to dryness in a flash evaporator and powdered with mortar and pestle. Portions (50 mg) of the powder were then pressed into flat-face pellets (7 mm diameter, 1 mm thick) using a Carver press. Thus, each 50 mg pellet contained 10 mg of naltrexone as base. Placebo pellets were manufactured as above, except that naltrexone was omitted from the mixture.

Each animal was anesthetized lightly with ether and a single pellet of either naltrexone or placebo was implanted subcutaneously in the mid-scapular region. At 2 or 4 weeks after implantation, the pellet was removed and, one day later, each animal was prepared for assessing either opiate binding or supersensitivity to morphine electrophysiologically. A preliminary experiment confirmed the results of Misra and Pontani[33]; we found that the naltrexone pellets antagonized completely the analgetic effect of morphine (10 mg/kg) in the hot plate test for at least 20 days and that this antagonist action was dissipated completely within 24 h after removal of the pellet.

Opiate and α_2 receptor assays

One day after pellet removal, some animals were decapitated and their brains and spinal cords removed rapidly. Brains were dissected on a cold plate (4 °C) into hypothalamus, striatum and frontal cortex according to the method of Glowinski and Iversen[21]. Tissue was frozen immediately on dry ice and stored at —80 °C for 1–3 months. Following storage, the tissue was prepared for assaying the specific binding of either the opiate antagonist [^{3}H]naloxone (New England Nuclear, 50 Ci/mmol), the opiate agonist [^{3}H]etorphine (Amersham, 51 Ci/mmol) or the α_2-noradrenergic antagonist [^{3}H]rauwolscine (New England Nuclear, 88 Ci/mmol). For determining the specific binding of [^{3}H]naloxone, tissue was homogenized (Polytron, setting 7, 10 s) in 200 vols. of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. Portions (0.95 ml) of the tissue homogenate were incubated at 0 °C for 180 min with 1 nM [^{3}H]naloxone in the presence or absence of 100 nM levallorphan tartrate (Hoffman-La Roche). The final incubation volume was 1 ml. For Scatchard analyses, tissue was pooled from 3–4 animals and incubated with varying concentrations of [^{3}H]naloxone (0.1–4.0 nM). Incubation was terminated by filtration under vacuum pressure over Whatman glass fiber circles (GF/B) and washed twice with 5 ml vols. of ice-cold Tris buffer. The filters with washed tissue fragments were soaked in 8 ml Aquasol-2 (New-England Nuclear) and the radioactivity was determined by liquid scintillation spectrometry. All samples were assayed in duplicate. The specific binding of [^{3}H]naloxone was calculated as the radioactivity obtained in the absence of levallorphan (total binding) minus the radioactivity obtained in the presence of levallorphan (non-specific binding). The receptor binding assays for [^{3}H]etorphine and [^{3}H]rauwolscine were similar to that described for [^{3}H]naloxone. In determining the specific binding of [^{3}H]etorphine, the Tris buffer did not contain NaCl and the opiate agonist levorphanol tartrate (Hoffman-LaRoche) was substituted for the antagonist levallorphan; for determin-

ing the specific binding of [3H]rauwolscine, 50 mM Na,K-phosphate buffer (pH 7.4) was substituted for Tris buffer and phentolamine hydrochloride (Ciba-Geigy) was substituted for levallorphan.

In another experiment, the specific binding of 1 nM [3H]naloxone in the LC was determined in tissue microdissected according to the method of Palkovits[35,36]. The brainstem was frozen, mounted and sliced in a freezer-microtome (American Optical, Model 840C). Each brain slice (300 μm thickness) was mounted on a cold glass slide and microdissected at —10 °C using a stainless steel needle (800 μm internal diameter) which was kept on dry ice. Bilateral punches were made on each of 3 serial slices through the LC. The tissue was pooled from two animals, homogenized by sonication in 4.5 ml of ice-cold Tris buffer, and assayed for [3H]naloxone binding. With the microdissection procedure, the tissue was primarily, but not solely, from within the anatomical borders of the LC. The glass fiber filters used in the assay procedure (Whatman GF/C) were prewashed in water saturated with amyl alcohol in order to reduce the binding of [3H]naloxone to the filter[6]. Protein concentrations were determined by the method of Lowry[31].

Electrophysiologic recording

One day after pellet removal, some animals were prepared acutely for assessing the effect of opiates on extracellular single-unit activity within the LC. Each animal was anesthetized with chloral hydrate (400 mg/kg, i.p.) and the femoral vein was cannulated with polyethylene tubing (PE-10) attached to a two-way valve which allowed for delivery of drugs intravenously. A glass microelectrode (1–2 μm tip, 1–5 MΩ impedance at 1000 Hz) filled with 3 M NaCl/Fast Green dye was placed stereotaxically in the LC with the incisor bar at 0 mm. The coordinates were 1.1–1.3 mm lateral to lambda, 1.2–1.6 mm posterior to lambda and 5.8–6.8 mm below the dura. We used the caudal electrode approach described by Guyenet[25] in order to spare the transverse sinus dorsal to the LC, as severe blood loss can alter the firing rate of neurons in the LC[55]. A large portion of the skull was removed so that the dura posterior to the sinus could be slit open laterally with a small knife. The electrode was lowered 4.5 mm through the dural opening and then slowly moved anterior to the appropriate AP coordinate. With this caudal approach, we found that all electrode tracts in the AP plane were confined to the cerebellum and that, with experience, the transverse sinus was not damaged. After the electrode was in place, neuronal activity was amplified, monitored oscillographically, and discriminated using conventional techniques. The output of the window discriminator was counted and the data preserved on a chart recorder. The spontaneous firing rate was recorded, counted and averaged for 5 min. Morphine sulfate (Merck) was subsequently administered in incremental doses (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/kg, i.v.), followed by naloxone hydrochloride (Dupont) given also in incremental doses (0.01, 0.02, 0.04 and 0.06 mg/kg, i.v.). Each dose was given at 2 min intervals and each was followed by 0.10 ml saline to insure that the drug was flushed entirely from the i.v. femoral cannula. Only one unit was recorded from each animal. At the end of each recording session, Fast Green dye was iontophoresed (40 μA, 10 min) at the recording site and the animals were perfused transcardially with 10% buffered formalin. The brain was then removed, sectioned on a freezer-microtome and stained with cresyl violet, in order to verify electrode tip placement.

Statistical analyses

All data were analyzed with unbalanced factorial and split-plot analyses of variance, except for the data obtained from the receptor binding saturation curves[12]. The saturation curves were subjected to Scatchard analyses, using linear regression plots to estimate receptor affinity (K_d), receptor number (B_{max}) and strength of correlation (r^2).

RESULTS

Opiate and α_2-binding

The specific binding of [3H]naloxone (1 nM) was increased significantly in each CNS region examined following 2 weeks of chronic naltrexone blockade (Fig. 1). The increase in opiate binding was 59% in spinal cord, 42% in hypothalamus, 39% in striatum and 51% in frontal cortex. There was no further increase in specific binding of [3H]naloxone when the naltrexone pellet was implanted for 4 weeks rather than 2 weeks. Although chronic naltrexone blockade clearly increased specific opiate binding, spe-

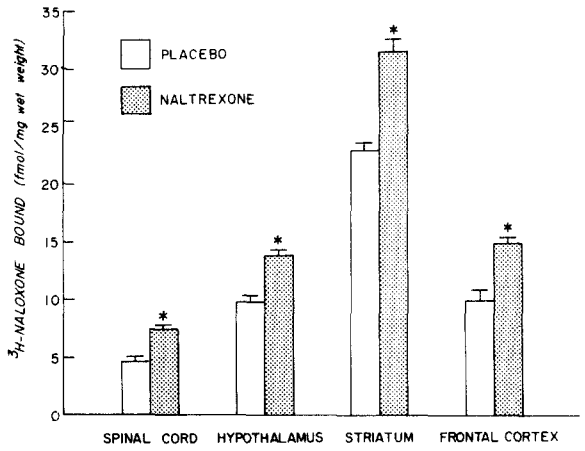

Fig. 1. Specific binding of [^{3}H]naloxone in the CNS in animals implanted with either a placebo or a naltrexone pellet for 2 weeks. Values reflect binding 1 day after removal of the pellets. Each mean and S.E.M. is based on 8 rats. Asterisks represent significant differences from the placebo control group, $P < 0.001$.

cific binding of the α_2-adrenergic antagonist [^{3}H]-rauwolscine was not changed by the chronic naltrexone in any of the CNS regions examined (data not shown).

Scatchard analyses of the specific binding of [^{3}H]naloxone (0.1–4.0 nM) revealed that the increase in ligand binding reflected largely an increase in the number of binding sites for opiates rather than a change in receptor affinity for opiates (Fig. 2). The B_{max} values, which reflect the number of binding sites, were increased significantly by 64% in spinal cord, 41% in hypothalamus, 28% in striatum and 68% in frontal cortex (P values < 0.05). In addition to a change in B_{max}, chronic naltrexone treatment also tended to produce a slight increase in K_d values in each CNS region examined (5% in spinal cord, 17% in hypothalamus, 12% in striatum and 6% in frontal cortex), indicating that receptor affinity was slightly

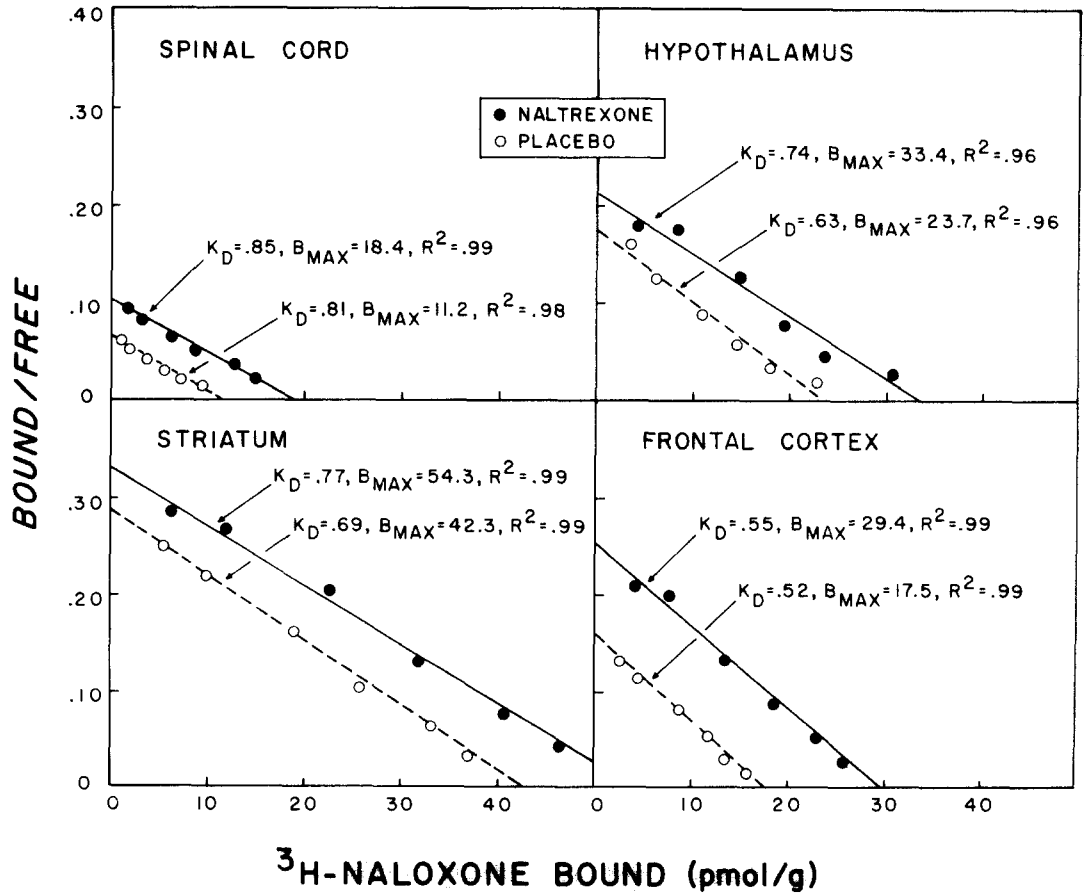

Fig. 2. Scatchard plots of specific [^{3}H]naloxone bound in the CNS of animals implanted with either a placebo or a naltrexone pellet for 2 weeks. Each plot was derived by pooling tissue from 3–4 animals. Values reflect binding 1 day after removal of the pellets. The K_d values are expressed in nM and the B_{max} values are expressed in pmol/g wet weight tissue.

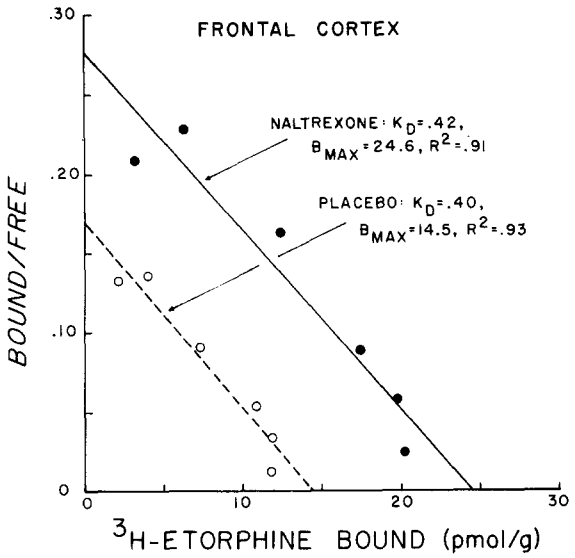

Fig. 3. Scatchard plots of specific [^{3}H]etorphine bound in frontal cortex of animals implanted with a placebo or a naltrexone pellet for 2 weeks. Values reflect binding 1 day after removal of the pellets. Each plot was derived by pooling tissue from 3–4 animals. The K_d values are expressed in nM and the B_{max} values are expressed in pmol/g wet weight tissue.

reduced. However, these differences in K_d between naltrexone- and placebo-treated groups were not statistically significant (P values > 0.05). As was obtained with [^{3}H]naloxone, the specific binding of the opiate agonist [^{3}H]etorphine (0.1–4.0 nM) was increased significantly following 2 weeks of chronic opiate blockade (Fig. 3). This increase in [^{3}H]etorphine binding also reflected an increase in B_{max}, rather than a change in K_d.

Chronic naltrexone treatment for 2 weeks also increased the specific binding of [^{3}H]naloxone in locus coeruleus micropunches (Table I). Although there was a 45% mean increase in DPMs per assay tube produced by naltrexone, this effect was not statistically significant because the variances within each treatment group were relatively large. However, when the data were expressed as fmol [^{3}H]naloxone bound/mg protein, the increase reached statistical significance. There were no significant differences in protein concentration in LC micropunches from naltrexone- and placebo-treated animals.

Electrophysiologic recording

All data reported are from recording sites (n = 23 units) subsequently determined histologically to have been within the LC (e.g. Fig. 4). In placebo-treated animals anesthetized with chloral hydrate, single units in the LC were of relatively large amplitude, with a steady, spontaneous firing rate. A noxious pinch to the contralateral hindpaw typically increased their firing rate and this pinch-induced excitation was usually, but not always, followed by a period of quiescence (Fig. 5A). The units were unaffected following the administration of dextrorphan, the d-isomer of levorphanol which is relatively devoid of analgetic action (Fig. 5B). In contrast to dextrorphan, morphine consistently produced a dose-dependent inhibition of unit activity in the LC which was rapidly and reliably reversed by naloxone (Fig. 5C).

Chronic naltrexone treatment (4 weeks) significantly lowered the spontaneous firing rate of units in the LC. One day after removal of the naltrexone pellet, the mean spontaneous firing rate was reduced by 44%, from 2.36 to 1.32 spikes/s (Table II). There was no significant difference in the spontaneous unit activities in the LC between control (placebo) animals and animals whose naltrexone pellet was implanted for 1 week and left intact during the recording session.

Chronic naltrexone treatment also increased the

TABLE I

Specific binding of [^{3}H]naloxone in the locus coeruleus following 2 weeks of chronic naltrexone blockade

Values reflect opiate binding 24 h after removal of pellets.

Treatment	*Tissue pools (n)*	*Mean ± S.E.M.*		
		μg protein/tube	*dpm/tube*	*fmol bound/mg protein*
Placebo	7	27.0 ± 1.8	225 ± 42	74.6 ± 12.0
Naltrexone	7	24.6 ± 2.1	326 ± 22	129.2 ± 21.4*

* Significantly different from placebo control group, $P < 0.05$.

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