Regional Fos Expression Induced by Morphine Withdrawal in the 7-Day-Old Rat

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Abstract

Human infants are often exposed to opiates chronically but the mechanisms by which opiates induce dependence in the infant are not well studied. In the adult the brain regions involved in the physical signs of opiate withdrawal include the periaqueductal gray area, the locus coeruleus, amygdala, ventral tegmental area, nucleus accumbens, hypothalamus, and spinal cord. Microinjection studies show that many of these brain regions are involved in opiate withdrawal in the infant rat. Our goal here was to determine if these regions become metabolically active during physical withdrawal from morphine in the infant rat as they do in the adult. Following chronic morphine or saline treatment, withdrawal was precipitated in 7-day-old pups with the opiate antagonist naltrexone. Cells positive for Fos-like immunoreactivity were quantified within select brain regions. Increased Fos-like labeled cells were found in the periaqueductal gray, nucleus accumbens, locus coeruleus, and spinal cord. These are consistent with other studies showing that the neural circuits underlying the physical signs of opiate withdrawal are similar in the infant and adult.

INTRODUCTION

Large numbers of seriously ill infant patients are treated with opiates to alleviate pain; other human infants are exposed in utero to illicit opiate drugs such as heroin and to prescribed opiates such as methadone. It is therefore important to understand the effects of acute and chronic exposure to drugs such as these on the immature organism. In the adult prolonged exposure to opiates results in distinct biochemical and physiological changes in individual

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neurons and in entire interacting circuits of neurons, which in turn can result in long-term alterations in cellular communication (Carlezon et al., 1997; Ingram, Vaughan, Bagley, Connor, & Christie, 1998; Koob & Bloom, 1988; Redmond & Krystal, 1984). These neural changes form the basis of the psychological and physical dependence that is seen after chronic exposure to the opiate. Yet it is not known if the same mechanisms mediate withdrawal in the infant.

Animal models of opiate dependence have defined the neuroanatomical regions that are involved in the physical signs of opiate withdrawal in the adult. In an early study injection of ^{3}H-naloxone into the lateral ventricle and basal cisterns of morphine dependent rats, produced strong withdrawal behaviors as a result of its spread to the periaqueductal gray (PAG) of the midbrain, the locus coeruleus (LC), and the periventricular gray substance (Laschka, Teschemacher, Mehraein, & Herz, 1976). More recent studies have shown that physical withdrawal can be induced by the microinjection of opiate antagonists into the amygdala, nucleus accumbens (NAcc), medial thalamus, preoptic hypothalamus, PAG, LC, and nucleus raphe magnus (Druhan, Walters, & Aston-Jones, 2000; Maldonado, Fournié-Zaluski, & Roques, 1992). Affective signs of withdrawal can be induced by the microinjection of antagonists into the NAcc and amygdala (Frenois, Stinus, Di Blasi, Cador, & Le Moine, 2005; Stinus, Le Moal, & Koob, 1990).

Opiate withdrawal increases regional cerebral glucose utilization or activation of c-fos in the amygdala, hypothalamus, LC, NAcc, medial and lateral preoptic areas, thalamus, and spinal cord (Adams & Wooten, 1990; Chieng, Keay, & Christie, 1995; Geary & Wooten, 1985; Hayward, Duman, & Nestler, 1990; Kimes & London, 1989; Rohde, Detweiler, & Basbaum, 1996a; Stornetta, Norton, & Guyenet, 1993; Veinante & Freund-Mercier, 2003). When these studies are taken together, a circuit evolves for the regulation of physical dependence in the adult rodent whose major components include (but are not limited to) the locus coeruleus, periaqueductal gray, amygdala, spinal cord, hypothalamus, thalamus, and nucleus accumbens.

As with adult human and rats, human infants and rat pups display physical withdrawal behaviors when long-term opiate exposure is terminated. These behaviors in human infants include altered sleep patterns, high-pitched crying, respiratory and gastrointestinal dysfunction, irritability, and tremors (Anand & Arnold, 1994; Finnegan, 1985; Franck, Vilardi, Durand, & Powers, 1998). Treatment options in the human infant in withdrawal are limited and include tapering with opiates such as methadone, opium or more recently buprenorphine or adjuvant treatment with barbiturates or benzodiazepines (Kraft et al., 2008; Osborn, Cole, & Jeffery, 2002; Osborn, Jeffery, & Cole, 2002).

In the rodent model there are qualitative changes in physical withdrawal behaviors that are seen throughout development. For instance, common behaviors seen in adult rats include wet dog shakes, teeth chattering, ptosis, piloerection, and jumping (Blasig, Herz, Reinhold, & Zieglgansberger, 1973; Buckett, 1964; Maldonado, Stinus, Gold, & Koob, 1992); behaviors that are unique to the 7-day-old rat include head movements, paw movements, twisting, stretching, rolling, and increased ultrasonic vocalizations (Barr & Wang, 1992; Jones & Barr, 1995; Thornton, Wang, & Smith, 1997; Windh, Little, & Kuhn, 1995). One

explanation for the different behavioral and physiological changes that are seen during ontogeny may be the relative immaturity of the neurons that comprise the individual brain loci and/or the pathways that interconnect these loci.

Little is known of the regions involved in opiate withdrawal in the infant rodent. There are dramatic increases in levels of c-fos mRNA in the olfactory bulb and small increases in the hypothalamus and medulla of 7-day-old morphine dependent rats after naloxone-induced withdrawal (Maeda et al., 2002), but more regional specificity is not known. In addition, whole brain and spinal cord show changes in c-fos mRNA (Akbarian et al., 2002; Jones et al., 2002) in an acute opiate withdrawal model. These and other loci are also associated with withdrawal when microinjected with an opiate antagonist (Jones & Barr, 2001) and with conditioned place preference when microinjected with morphine (Barr & Rossi, 1992).

Thus although withdrawal can be precipitated by antagonist injection into similar brain regions in the infant and adult, there are no data on specific brain loci that are activated by withdrawal in the infant. It is our general hypothesis that the neural circuitry of withdrawal in adults and infants is concordant despite differences in withdrawal behavior. Here we hypothesized that despite the relative immaturity of these same regions, areas that show activity during opiate withdrawal in the adult rat would show activity in the young rat. Thus Fos, a marker of cellular activity, the expression of which is rapidly activated in response to neuronal stimulation, was quantified. The presence of the protein Fos and its mRNA has been used successfully as a measure of neuronal activation throughout the CNS of adult (Le Guen, Catheline, & Besson, 1999; Morgan, Cohen, Hempstead, & Curran, 1987) and developing rats (Andersen, LeBlanc, & Lyss, 2001; Boucher, Jennings, & Fitzgerald, 1998; Joyce & Barr, 1995; Wiedenmayer & Barr, 2001a; Williams, Evan, & Hunt, 1990; Yi & Barr, 1995). Therefore, for the current experiment we precipitated physical withdrawal, using the opiate antagonist naltrex-one, in morphine dependent 7-day-old rat pups and quantified the number of Fos-like immunoreactive (Fos-LIR) cells in brain and spinal cord.

GENERAL METHODS

Subjects

Subjects were offspring of Long-Evans Hooded rats (Harlan Sprague Dawley) mated in our colony room. The parents (and offspring) were housed in plastic tubs under standard conditions. Tubs were checked twice daily (≈09:00 and 17:00 hr) and any new pups found at either time were recorded as 0 days old. The experiment began on postnatal Day 1 (PD-1), and at that time the litter was culled to eight pups, without regard to sex.

Induction of Morphine Dependence

All procedures for this study included adequate measures to minimize pain or discomfort and were conducted in accordance with the guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory animals and approved by the internal IACUC of Hunter College. At PD-1 pups were removed from the dam and transferred to a smaller plastic container, containing beta chips from the home cage. Using India ink, each pup was permanently tattooed on the ventral surface of the paws (Geller & Geller, 1966). All

pups from a litter were weighed and given an intraperitoneal (i.p.) injection with either the vehicle (saline) or the opiate agonist morphine sulfate (Henry Schein, Indianapolis, IN; 10 mg/kg, 0.01 ml/g). After injection of the final animal, the entire litter was placed in an incubator (maintained at 32 C) for 30 min before being returned to the dam. The weighing was repeated once a day (each morning) for 7 days. The saline or morphine injections were given twice daily (≈8:00 and 17:00) for 6 days and one more time on the morning of the 7th day. This regimen and dose has been shown to consistently produce morphine dependence in the 7-day-old rat pup (Jones & Barr, 1995; McPhie & Barr, 2000).

Precipitation of Withdrawal

Four hours after the final morning injection on the 7th day, all pups were removed from the dam and placed in a plastic container in the testing room. One pup was randomly removed from its littermates. It was weighed, sexed and injected (i.p.) with either saline or the long-acting opiate antagonist naltrexone hydrochloride (Sigma, St. Louis, MO; 1 mg/kg, 0.01 ml/g). The pup was then returned to the huddle. A second pup was treated similarly with the other drug/vehicle. The drugs were assigned randomly and the experimenter was blind to the drug each pup received. The presence or absence of withdrawal behaviors was noted and all pups in the morphine-naltrexone group exhibited classic neonatal withdrawal signs (Jones & Barr, 1995). None of the control groups expressed withdrawal. No more than one pup per litter was included in any experimental group; data for males and females were combined and not analyzed separately.

Fixation of Tissue/Perfusion

After 2 hr, pups were sacrificed with an i.p. injection of a lethal dose of sodium pentobarbital and perfused with 4% paraformaldehyde (Morgan et al., 1987; Wiedenmayer & Barr, 2001b; Yi & Barr, 1997). The brains and spinal cords were frozen and coronally sectioned (30 μm for spinal cord and locus coeruleus, 50 μm for periaqueductal gray and nucleus accumbens) on a cryostat. Sections were floated in PBS in isolated wells of a plastic container. One of the floated sections was stained for cresyl violet to locate anatomical regions; the other floated section was stained for Fos. The third cut section was stored at −80°C.

Immunohistochemistry

Sections were processed using a modified protocol of the avidin-biotin-peroxidase system of Hsu, Raine, and Fanger (1981). The sections were then incubated for 48 hr at 4°C on a shaker in the primary antibody, rabbit anti-Fos (Ab-5, Oncogene Science, Union, NY) diluted 1:2,000 in PBS with Triton-X and 1% goat serum. Sections were stained using diaminobenzidine peroxidase substrate tablets (Sigma). Assay controls included tissue without the primary antibody; no staining was seen. In prior experiments preabsorption of the Fos antibody with excess of the Fos protein abolished staining (Yi & Barr, 1995). Tissue from control pups and treated pups were processed together and the experimenter was blind to the treatment of each pup.

Fos Cell Quantitation

Fos-like immunoreactive (LIR) positive cells were visualized using a microscope (Nikon-Optiphot) equipped with a drawing tube. A person unaware of the chronic and acute treatment of the animal counted all Fos-LIR cells, regardless of the density of the staining (as long as it was distinct from the background; Fig. 1). For each animal, the mean number of Fos-LIR cells per brain area was calculated by averaging counts from all sections.

RESULTS

All sections containing the regions of interest were counted and averaged. There were 2–5 sections for the nucleus accumbens; 4–8 sections for the periaqueductal gray and locus coeruleus; and 2–5 sections in the lumbar enlargement of the spinal cord. Preliminary analyses showed that there were no significant differences between the left and right sides in any of the regions so the values were pooled together for subsequent analysis.

A factorial analysis of variance (ANOVA) was performed for each of the brain regions and spinal cord. The experimental group was (1) chronic morphine + acute naltrexone (M–N). The three controls were: (1) chronic morphine + acute saline (M-S); (2) chronic saline acute naltrexone (S-N); and (3) chronic saline acute + saline (S-S). The controls did not differ from each +other and were combined to form a single control group for analysis. However, for clarity the data from the controls are displayed as separate groups in the figures.

Nucleus Accumbens

There were significantly higher levels of Fos-LIR in the entire nucleus accumbens of the M-N group compared to all the controls, $F(1,9) = 9.66$, $P < 0.01$ (Fig. 2). In addition, the shell showed higher levels of Fos-LIR than the core in the treated group, $F(1,8) = 8.24$, $P < 0.05$.

Periaqueductal Gray Area

Significantly higher levels of Fos-LIR were found in the entire periaqueductal gray of the M-N animals compared to the controls, $F(1,8) = 9.94$, $P < 0.05$ (Fig. 3). There were no significant differences in Fos-LIR levels between the dorsal and ventrolateral regions of the periaqueductal gray (data not shown).

Spinal Cord

There were significant increases in the levels of Fos-LIR in the M-N group compared to the control, $F(1,7) = 42.3$, $P < 0.01$. *Note*: these data include only a M-S control because prior work had shown no or minimal Fos expression in pups treated with chronic saline (Fig. 4). The changes occurred largely in Lamina I and II (or dorsal surface) of the dorsal horn.

Locus Coeruleus

There were significantly higher levels of Fos-LIR in the M-N treated group compared to the controls, $F(1,12) = 6.73$, $P < 0.05$ (Fig. 5).

Other Regions

Although we did not quantify other brain regions, we noted substantially increased staining in chronic morphine treated animals in withdrawal in a variety of areas, all of which have been described previously in adults. These include the hypothalamus, including the paraventricular, dorsomedial, medial preoptic, and anterior nuclei. In addition there was substantial Fos staining in the midline thalamic nuclei, in particular the paraventricular nucleus, and the arcuate of the hypothalamus that was dense in morphine treated animals regardless of whether or not they received naltrexone. In addition, there was inconsistent and sporadic staining in the anterior dorsal thalamus, dorsal interpeduncular nucleus, and medial and central nuclei of the amygdala.

DISCUSSION

Withdrawal in the 7-day-old morphine dependent rat pup, precipitated by the long-acting opiate antagonist naltrexone, increased Fos-like immunoreactivity in several brain regions. These included the periaqueductal gray area, the nucleus accumbens, the dorsal horn of the spinal cord, and the locus coeruleus. The assumption of this and most studies with Fos is that the increased transcription of Fos indicates increased cellular activity.

The increased levels of Fos activity in the nucleus accumbens following opiate withdrawal was comparable to adult studies where behavioral withdrawal precipitated by the injection of opiate antagonists into the NAcc caused an increase in levels of the c-fos protein (Hayward et al., 1990), c-fos mRNA (Hayward et al., 1990), and glucose utilization (Geary & Wooten, 1985, 1986). In addition, the systemic administration of naltrexone caused increased Fos-related antigen immunoreactivity in chronically morphine treated rats (Walters, Aston-Jones, & Druhan, 2000) and increased CREB-mediated transcription in chronically morphine treated mice (Shaw-Lutchman et al., 2002).

The nucleus accumbens is part of the mesocorticolim-bic-dopamine pathway and a critical site for the reinforcing properties of opiates (Dworkin, Guerin, Goeders, & Smith, 1988; Koob, Wall, & Bloom, 1989; Shaw-Lutchman et al., 2002; Wise, 1987). Lever pressing in morphine dependent adult rats can be significantly reduced when the opiate antagonist methylnaloxonium is microinjected into the nucleus accumbens (Koob et al., 1989; Stinus et al., 1990). Place aversions cannot be conditioned in rats at PD-7 but can at PD-14 (Barr & Goodwin, 1997) and separation-induced ultrasonic vocalizations (USVs) (Barr & Wang, 1992) can be elicited in withdrawing rat pups younger than PD-3. Therefore the increased levels of Fos-LIR elicited in this current study may represent the cellular parallel of the increased ultrasonic vocalizations.

In addition to the nucleus accumbens, there were increased levels of Fos-LIR in the periaqueductal gray area of the 7-day-old rat, consistent with findings in the adult animal (Chieng et al., 1995). There were no differences between its dorsal and ventrolateral subregions. The periaqueductal gray contains a large number of opiate receptors in the adult rodent brain (Vaughan & Christie, 1997) and is therefore sensitive to the administration of opiate antagonists and agonists. The direct injection of methylnaloxonium into the ventral periaque-ductal gray elicits withdrawal behaviors similar to those seen after the systemic

injection of naltrexone in the infant and adult animal (Aghajanian, 1978; Jones & Barr, 2001; Laschka et al., 1976; Maldonado, Negus, & Koob, 1992). When combined with the current data, there is strong evidence that the periaqueductal gray becomes increasingly active during opiate withdrawal in the 7-day-old rat, as in the adult rat.

The dorsal horn of the spinal cord also showed significantly increased levels of Fos-LIR upon the precipitation of morphine withdrawal in the 7-day-old rat. As the initial CNS structure to receive afferent information, the spinal cord plays an important regulatory role and modulates noxious input. Moreover, it has a significant role in opiate dependence and physical withdrawal in the adult rodent. For instance, pretreatment with an intrathecal administration of an opiate antagonist blocks the development of dependence (Delander & Takemori, 1983) and the precipitation of withdrawal increases levels of Fos-LIR within the dorsal horn of the spinal cord (Rohde, Detweiler, & Basbaum, 1996b). In the infant rat, there is evidence for the role of the spinal cord in opiate tolerance and withdrawal. In an isolated spinal cord preparation from 7-day-old rats that had been chronically treated with morphine, the addition of naltrexone to the bath increased the magnitude of the electrically evoked slow ventral root potentials (Zhu & Barr, 2003, 2004).

The final brain structure quantified here where levels of Fos-LIR that were significantly increased during precipitated withdrawal was the locus coeruleus. This finding was expected considering the vast amount of published research that has focused on the locus coeruleus as a model for the long-term changes resulting from opiate exposure. Microinjections of opiate antagonists into the locus coeruleus of morphine dependent adult (Maldonado, Negus et al., 1992; Maldonado, Stinus et al., 1992) and infant rats (Jones & Barr, 2001) cause withdrawal signs similar to that seen by systemic injections.

Human infants are exposed to opiates either when the fetus is exposed passively to opiates used by the mother, or when premature infants (as early as 22–23 weeks of gestation) are treated for pain. We chose 7 days of age because we wanted to model exposure within the early human infant age range. It is not possible to draw direct equivalents between developmental stages across species because different systems have different developmental trajectories, although that effort has been attempted since the rat was first used as a developmental model (e.g., Donaldson, 1918). More recent attempts have examined specific nervous system regions and suggest that humans at birth are approximately equivalent to 10- and 14- day old rat pups (Porterfield & Hendrich, 1993; Romijn, Hofman, & Gramsbergen, 1991). In the most ambitious effort to date, Clancy developed a complex model to provide equivalents across species (Clancy, Darlington, & Finlay, 2001). In her model, there are clear regional differences but birth in the rat is roughly equal to a 4- to 6-month-old human fetus. On non-neural measures, the rat is far more altricial; thermoregulation, eye opening, and visual processing, pinna detachment, all present in the human newborn, do not happen until late in the second week of a rat's life. Therefore, to the extent comparisons are possible, we would expect similar neural mechanisms mediating withdrawal in the human fetus and human adult. And although it is likely true that human mothers do not use opiates only in the second half of pregnancy, it is equally true that human opiate abusers almost never use only a single drug; nor do they have the adequate nutrition that laboratory rats receive. Thus

although this is clearly not a model of what happens in the human condition, it is a reasonable model to understand mechanisms of withdrawal during early development.

Although decades of research have gone into understanding underlying mechanisms of opiate withdrawal, little is still known about infants and opiate dependence. Are the events that unfold in the infant rodent (and human) during chronic exposure to opiates qualitatively similar, somewhere in between, or completely different from those seen in adult rodents? We show here that, as in the adult, there is increased cellular activity (as measured by levels of Fos-LIR) following precipitated withdrawal in the locus coeruleus, nucleus accumbens, periaqueductal gray, and spinal cord. Mechanisms mediating that increase might include activation of nitric oxide synthesis (Adams, Kalicki, Meyer, & Cicero, 1993; Bhargava & Thorat, 1996; Buccafusco, Terry, & Shuster, 1995; Kimes, Vaupel, & London, 1993; Zhu & Barr, 2000), and activation of PKC, PKA, and ERK kinases. Given that withdrawal can be precipitated by injections of antagonist in the same regions of the adult and infant brain, it appears that some of the neural circuitry, and perhaps some intracellular signaling paths, that mediate withdrawal in adult and infant are similar. Nonetheless there are differences that lie in the ability of chronic opiates, but not acute opiates, to engage mechanisms that include, but may not be limited to NMDA glutamate receptors (Jones et al., 2002; Zhu & Barr, 2000, 2001, 2003, 2004). Furthermore, although the use of an opiate antagonist is standard in the animal studies to understand mechanisms of withdrawal, in humans withdrawal is more gradual. We know of no developmental work on the effects of spontaneous withdrawal in animal models. Understanding the similarities and differences in mechanisms between infants and adults is necessary for the development of more rational treatment of opiate exposed infants.

Acknowledgments

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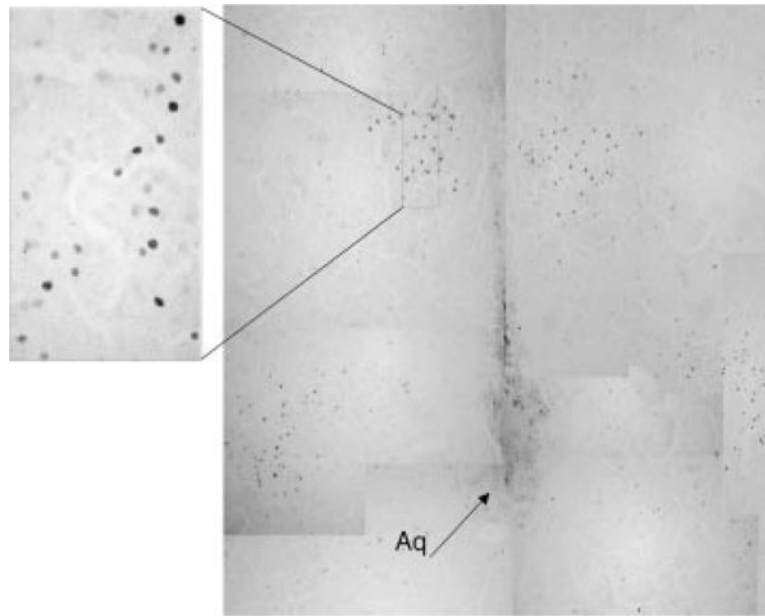
FIGURE 1.
Photomicrograph of Fos-like immunoreactive cells in the periaqueductal gray area of 7-day-old rats that were treated chronically with morphine and given an acute injection of naltrexone (M-N) to precipitate withdrawal.
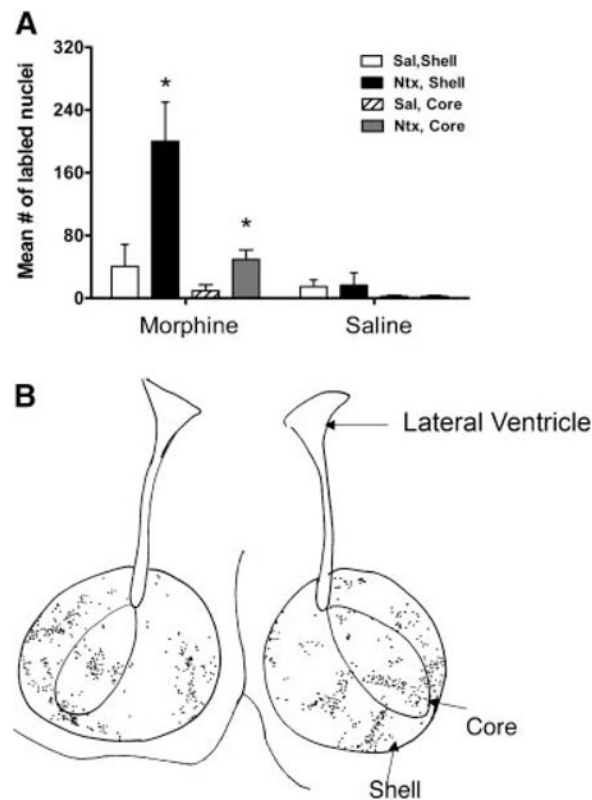
FIGURE 2.
(A) Mean number of Fos-like immunoreactive cells in the nucleus accumbens of 7-day-old rats that were chronically exposed to morphine (M) or saline (S) (labeled on the *X*-axis) and then given an acute injection of naltrexone (N) or saline (S) ($N = 5$/group; mean ± SE). Asterisk (*) denotes significance between the M-N group and the combined controls (M-S, S-S, and S-N). *Note*: The scale of this figure differs from that of Figures 3–5. (B) Camera lucida drawing of the shell and core of the nucleus accumbens. *Note*: The increased number of Fos labeled cells in the shell than the core.
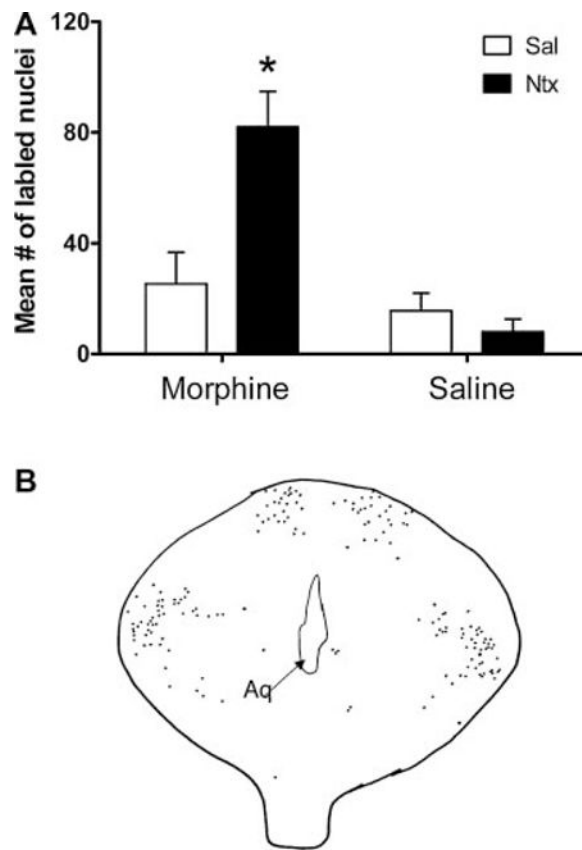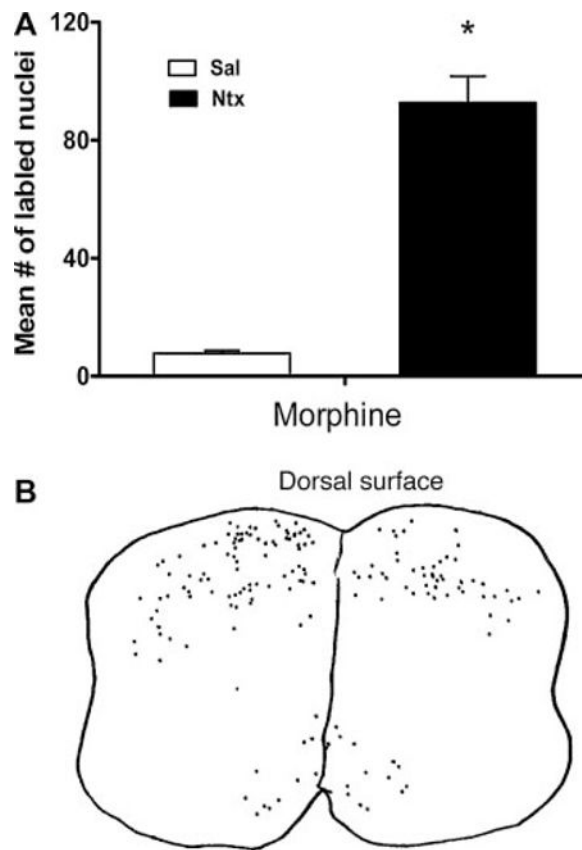
FIGURE 3.

(A) Mean number of Fos-like immunoreactive cells in the periaqueductal gray of 7-day-old rats that were chronically exposed to morphine (M) or saline (S) (labeled on the *X*-axis) and then given an acute injection of naltrexone (N) or saline (S) (*N* = 5/group; mean significance ± SE). Asterisk (*) denotes between the M-N group and the combined controls (M-S, S-S, and S-N). (B) Camera lucida drawing of the periaqueductal gray area. There were no differences between dorsal and ventrolateral regions.

FIGURE 4.

(A) Mean number of Fos-like immunoreactive cells in the dorsal horn of the spinal cord of 7-day-old rats that were chronically exposed to morphine (M) or saline (S) (labeled on the X-axis) and then given an acute injection of naltrexone (N) or saline (S) ($N = 8$/group; mean ± SE). Asterisk (*) denotes significance between the M-N group and the control group (M-S). (B) Camera lucida drawing of the spinal cord. More label cells can be seen in the dorsal horn (top of drawing).
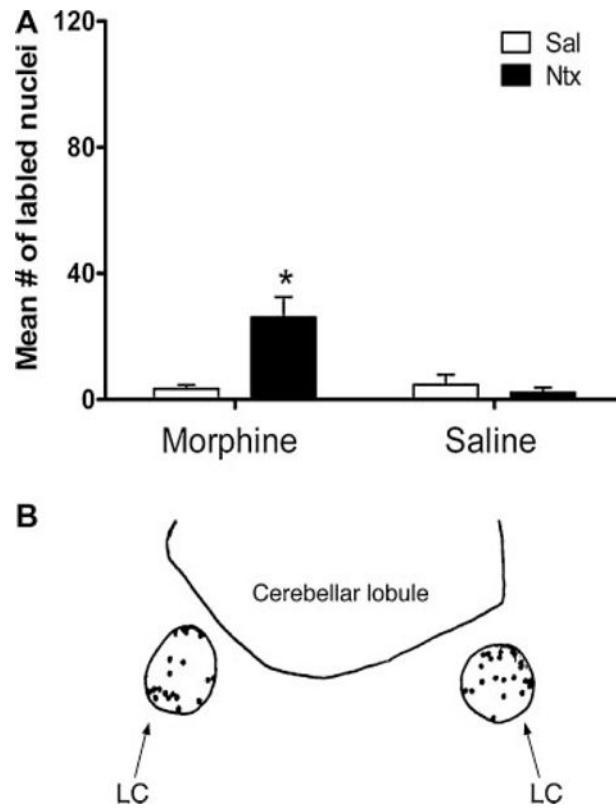
FIGURE 5.
(A) Mean number of Fos-like immunoreactive cells in the locus coeruleus of 7-day-old rats that were chronically exposed to morphine (M) or saline (S) (labeled on the X-axis) and then given an acute injection of naltrexone (N) or saline (S) ($N = 8$ for M-N and M-S, $N = 6$ for S-S and S-N; mean ± SE). Asterisk (*) denotes significance between the M-N group and the combined controls (M-S, S-S, and S-N). (B) Camera lucida drawing of the locus coeruleus.