Clinically available NMDA receptor antagonist dextromethorphan attenuates acute morphine withdrawal in the neonatal rat

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Abstract

The present study investigated the ability of dextromethorphan, a clinically available NMDA receptor antagonist, to attenuate the behaviors and the expression of *c-fos* mRNA associated with acute morphine withdrawal in the 7-day-old rat. Rat pups were given a single dose of morphine (10.0 mg/kg, s.c.) or saline. Two hours later, pups were removed from the dam and injected with either dextromethorphan (50.0 mg/kg, s.c.) or saline. Fifteen minutes later, acute morphine withdrawal was precipitated with naltrexone (10.0 mg/kg, s.c.) and behaviors were recorded every 15 seconds for the next 60 minutes. Immediately after the behavioral test, the brain was removed and assayed for *c-fos* mRNA by solution hybridization. The intensity of the morphine withdrawal syndrome was reduced in pups pre-treated with dextromethorphan. In addition, the acute morphine withdrawal-induced elevation in *c-fos* mRNA expression in the brain of the pups was attenuated by pre-treatment of dextromethorphan. These results demonstrate that the clinically available NMDA receptor antagonist dextromethorphan can attenuate both the behavioral and molecular manifestations of acute morphine withdrawal in the neonatal rat.

Theme: Neural Basis of Behavior
Topic: Drug of Abuse: Opioids and Others

Key words: *c-Fos* mRNA, Morphine withdrawal, Addiction, Solution hybridization, Dextromethorphan, NMDA receptor antagonist.

1. Introduction

In humans, exposure to opioids for either medical or nonmedical reasons leads to dramatic behavioral and neural changes resulting to physical dependence [19]. Although physical dependence is classically thought to develop after chronic exposure to opioids, the adaptational changes underlying physical dependence begin with the first exposure to an opioid [15,16,22]. Thus, acute opioid withdrawal refers to the withdrawal syndrome precipitated by administration of an opioid antagonist after either a single dose or a short-term infusion of opioid agonist [3,31].

Improvements in the short-term and long-term clinical outcomes of critically ill neonates have led to the widespread use of opioids for analgesia and sedation. Consequently, large numbers of neonates are now receiving progressively increasing amounts of opioids [38,41-43]. These clinical practices have led to an increasing incidence of opioid dependence during therapy, with consequent increases in the number of patients showing the clinical signs of opioid withdrawal when therapy is discontinued [41].

The acute effects of opioid withdrawal can adversely affect the infants' alertness, visual responsiveness, and sleep regulation [18]. In addition, because neonatal withdrawal occurs during the period when the maternal-infant interaction patterns are first established, withdrawal can induce far-reaching dysfunctional effects including not only attachment but also cognitive development [18]. Since physicians are now more likely to use opioids to manage pain in neonates and infants than in the past [41], the need is much greater for a clearer understanding of the mechanisms of acute opioid withdrawal in the developing organism.

There is increasing evidence that NMDA receptor antagonists inhibit opioid withdrawal syndrome in the mature organism [19,26,32,44]. Indeed, clinically available NMDA receptor antagonists such as memantine and dextromethorphan have been shown in animal models to attenuate opioid withdrawal [25,27,33,35-37]. Particular encouraging is that human subject clinical trials investigating the effect of clinically available NMDA receptor antagonists on opioid withdrawal have obtained quite positive results [4,5,23,24,40].

In contrast to the reports of the effectiveness of NMDA receptor antagonists in suppressing opioid withdrawal in the adult organism, there is little data in the infant.

Conclusions based on adult data often do not apply to the infant [47-49]. Indeed, the withdrawal repertoire is different in the infant than that in the adult [20,46]. The neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally [1,2,12,30]. Finally, the NMDA receptor, which is believed to play a crucial role in the establishment of opioid tolerance, undergoes qualitative and quantitative changes during development [9]. Thus, we previously examined the ability of LY235959, a competitive NMDA receptor antagonist, to attenuate acute morphine withdrawal in the infant rat [21]. The present study now investigates the ability of dextromethorphan, a clinically available NMDA receptor antagonist, to attenuate the behavioral and molecular manifestations associated with acute morphine withdrawal.

2. Materials and methods

2.1. Subjects

All animal procedures were in accordance with the “Principles of laboratory animal care" (NIH publication, 1996) and approved by the institutional animal care and use committee at the Weill Medical College of Cornell University. The subjects were the offspring of Sprague-Dawley rats. Pregnant dams were purchased on gestational day 19 or 20 (Charles River). Dams were individually housed in plastic tubs with wood chips in a colony room maintained at 22 to 24 ^{o}C on a 12-hour light/12-hour dark photocycle with light onset at 7 AM. The breeding colony existed in a separate room with minimal disturbances except for normal cleaning, feeding and record keeping. Dams had Purina Lab Chow (Purina 5012) and water available ad libitum. Pups were termed 0 days of age upon birth. After parturition, litters were culled to 6 male pups. Pups were tattooed with India ink [14], which was injected into one or two paws to label permanently individual pups in each litter.

2.2. Drug treatment and behavior testing

Morphine sulfate was obtained from the National Institute on Drug Abuse (Rockville, MD). Naltrexone and dextromethorphan were purchased from Sigma Chemical (St. Louis, MO).

In the afternoon of the 7th postnatal day, animals were transported from the animal facility to our laboratory in plastic tubs with wood chip bedding and placed in an observation chamber maintained at approximately 33 ^{o}C. Pups were weighed and assigned to 1 of 4 treatment groups (N = 15 for each group). Two groups received morphine (10 mg/kg, s.c.) and 2 groups received saline injection. Two hours later pups were injected with either dextromethorphan (50 mg/kg, s.c.) or saline. The order of treatment conditions was assigned randomly within each experiment. After the drug treatment, the pup was then placed back into the observation chamber with the remainder of the litter (without the dam). Fifteen minutes later, naltrexone (10.0 mg/kg, s.c.) was injected to precipitate withdrawal. The behavior of the pups was then observed for 60 minutes after the injection of naltrexone. Every 15 seconds, the behavior of the pups was identified and recorded on a checklist (see Table 1 for definitions of behavior included in

the checklist) by an observer who was blind to the drug and dose. The next pup was then tested until all treatment groups were completed.

Table 1 about here

2.3. Tissue collection and RNA extraction

Immediately after withdrawal behavior testing, animals were sacrificed by decapitation. Since *c-fos* mRNA production typically peaks approximately 15-45 minutes following a behavioral event [52], the 1 hour exposure of the rats to morphine withdrawal helped to insure a representative sampling of the withdrawal period.

Following CO_2 narcosis and decapitation, the brain (minus cerebellum) was removed. Each tissue was immediately homogenized and the RNA recovered using the Trizol reagent (Life Sciences, Giathersburg, MD). Each value represents the mean of 5 RNA extracts for each tissue (N = 5).

2.4. Solution hybridization for *c-fos* mRNA

c-Fos mRNA levels were determined in tissue extracts of total cellular RNA using a previously describe solution hybridization assay for *c-fos* mRNA [51]. A ^{32}P-labeled antisense riboprobe was prepared by in vitro transcription. The plasmid for the rat *c-fos* riboprobe contained a 970 bp BglII-ScaI fragment [51] obtained from a full length cDNA [8]. *c-Fos* riboprobe transcripts (specific activity = 6.5 X 10^8 dpm/μg) were applied to a CF11 column [50,51], washed to remove unincorporated label, and eluted with TSE: ethanol 78:22 (v/v) [TSE is 0.05 M Tris-HCl, 0.1 M sodium chloride and 0.001 M EDAT, pH 7.0)] to obtain a single stranded ribprobe fraction free of "snap back regions" which contribute to background in the solution hybridization assay [50]. Nonradiolabeled mRNA "sense" standards were prepared by in vitro transcription [50] using the full length cDNA described above [8].

Total cellular RNA was determined by UV absorbency at 260 nm. Duplicate aliquots of each RNA sample were dried under a vacuum in 1.5 ml eppendorf tubes and then resuspended in 30 μg of hybridization buffer (10 mM N-tris

[hydroxymethyl]methyl-2-amino-ethanesulfonic acid, 10 mM EDTA, 0.3 M NaCl, 0.5% SDS and pH 7.4) that also contained 150,000 dpm of riboprobe. Samples were covered with two drops of mineral oil and hybridized at 75 ^{o}C for 4 hours. After hybridization, 300 μl solution containing a high salt buffer (0.3 M NaCl, 5 mM EDTA, and 10 mM Tris-HCl, pH 7.5), 40 μg/ml RNase A (Worthington Biochemicals, Freehold, NJ), and 2 μg/ml RNase T1 (Calbiochem, San Diego, CA) was added and samples were incubated at 30 ^{o}C for 1 hour to digest unhybridized probe. The ribonuclease reaction was terminated with 1 ml of 5% trichloroacetic acid (TCA) and 0.75% sodium pyrophosphate. One drop of 0.5% BSA was added to aid precipitation. This solution was mixed and the TCA precipitable dpms were collected onto glass microfiber filter paper (Reeves Angel 934AH, Brandel, Gaithersburg, MD) using a 24 place cell harvester (Brandel, Gaithersburg, MD). The filter was washed three times with 5% TCA, dried under an infra-red light and counted by liquid scintillation in 5 ml hydrofluor scintillation solution (National Diagnostics, Manville, NJ).

The standard calibration curve for *c-fos* mRNA is linear from 1.95 to 250 pg of the full length *c-fos* sense transcript (i.e., *c-fos* mRNA) with a correlation coefficient of 0.997. In ten consecutive experiments the interassay coefficient of variation averaged 7.4% and the intraassay coefficient of variation average 3.8% for duplicate aliquots of 30 different extracts.

2.5. Statistics

Withdrawal was analyzed by separate one-way analysis of variances (ANOVA) for each withdrawal behavior followed by the Student-Newman-Keuls test for multiple comparison tests at the 0.05 level of significance.

All *c-fos* mRNA expression data points from the brain were analyzed with a one-way ANOVA with subsequent groups comparisons made using the Student-Newman-Keuls test at the 0.05 level of significance.

3. Results

3.1. Dextromethorphan attenuated acute morphine withdrawal behaviors

A single dose of morphine (10 mg/kg, s.c.) two hours later followed by a single injection of naltrexone (10 mg/kg, s.c.) induced a plethora of acute morphine withdrawal behaviors in the 7-day-old rat (Figure 1). Compared with the saline control group, the morphine treated animals displayed significantly increased withdrawal behaviors, including head moves, moving paws, rolling, vocalizations, walking and wall climbing. Thus, overall, pups experiencing acute morphine withdrawal remained less quiet than the controls. As reported earlier , the categories and intensity of these acute withdrawal behaviors are generally comparable to those seen in withdrawal after chronic morphine administration [20,21].

Figure 1 about here

Pre-treatment with 50 mg/kg dextromethorphan significantly reduced acute morphine withdrawal behaviors in the 7-day-old rat (Figure 1, Morphine + Saline group vs. Morphine + Dextromethorphan group). Compared with the control group that received morphine but no dextromethorphan prior to naltrexone challenge, all withdrawal behaviors in the dextromethorphan pre-treated pups were significantly attenuated and quiet behavior significantly increased. Withdrawal behaviors attenuated by pre-treatment of dextromethorphan included head moves, moving paws, rolling, vocalization, walking and wall climbing. In contrast, pre-treatment with dextromethorphan at 50 mg/kg did not cause any motor function impairment by itself, since pre-treatment of dextromethorphan at this dosage did not significantly change the occurrences of behaviors in rats that did not receive morphine treatment (Figure 1, Saline + Saline group vs. Saline + Dextromethorphan group). Therefore, dextromethorphan's effect in attenuating these behaviors is specific to acute morphine withdrawal rather than any non-specific motor effect of this compound.

3.2 Dextromethorphan attenuated acute morphine withdrawal evoked brain *c-fos* mRNA expression

In the 7-day-old rat, brain *c-fos* mRNA expression was significantly elevated during acute morphine withdrawal (Figure 2). This acute morphine withdrawal induced *c-fos* mRNA expression was significantly attenuated by the pre-treatment of dextromethorphan 15 minutes prior to naltrexone injection (Figure 2). Importantly, this attenuation was specific to the acute morphine withdrawal syndrome, since dextromethorphan alone did not significantly alter the expression of *c-fos* mRNA in the brain in 7-day-old rats that did not receive morphine treatment (Figure 2). However, although dextromethorphan significantly attenuated acute morphine withdrawal induced *c-fos* mRNA expression, *c-fos* mRNA expression did not return completely to baseline.

Figure 2 about here

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4. Discussion

Since physicians are now more likely to use opioids to manage pain in neonates and infants than in the past [41], there are increasing concerns about the adverse effects of acute opioid withdrawal in this population [41,42]. Due to the complexity of human setting, animal models are necessary to better study the mechanism and potential treatment of opioid withdrawal in human infant [1,18,49]

Recent studies by us and others have demonstrated that acute morphine dependence can be induced by a single injection of morphine in the neonatal rat [6,21]. The present study has further confirmed that acute morphine withdrawal can be observed after a single dose of morphine then followed by naltrexone challenge. In the 7-day-old rat, 2 hours after morphine injection, withdrawal behaviors including head moves, paw moves, rolling, vocalization, walking, and wall climbing were significantly increased, and quiet behavior was decreased by naltrexone challenge precipitated acute morphine withdrawal. In addition, *c-fos* mRNA expression levels were significantly increased in the brain. Thus, like in the adult rat, acute morphine withdrawal can be induced by a single injection of morphine in the infant rat. Based on the observations of previous reports [6,21] and the present study, we conclude that the neonatal rat can be used as a reliable model system of acute opioid withdrawal. The establishment of this model system provides a stepping stone for further study of the mechanism and potential treatment of acute opioid withdrawal in human infants.

The early discovery that NMDA receptor antagonists inhibit opioid tolerance and withdrawal in adult rodents [28,29,44] stimulated an explosion of research on the role of NMDA receptors in these phenomena in the following decade. As a result, the body of evidence accumulated over the past decade suggests that various NMDA receptor antagonists can attenuate opioid tolerance and withdrawal is overwhelming [17,26,45]. Recently, clinically available NMDA receptor antagonists such as memantine and dextromethorphan have examined in animal models of opioid tolerance and withdrawal [10,25,27,33,35-37]. Furthermore, human subject clinical trials investigating the effect of clinically available NMDA receptor antagonists (memantine and dextromethorphan) on opioid withdrawal have obtained quite positive results in both the United States [4,5,40] and other countries [23,24].

Dextromethorphan is a low affinity, use dependent open channel blocker [19,36]. Low affinity open channel blockers show much faster open channel/unblocking kinetics than high affinity ligands such as MK-801. Thus the low affinity open channel blockers can readily disassociate from channels activated by physiological concentrations of synaptic glutamate but the block the sustained depolarizations that occur during chronic excitotoxic conditions [7,34]. This differential effect in physiological versus pathological conditions appears to provide a mechanism for the well establish favorable safety profile of dextromethorphan.

The c-fos gene is expressed in the central nervous system (CNS) in response to neuronal stimuli [39]. Naltrexone increased c-fos mRNA levels in morphine dependent rats in several brain regions and this effect was blocked in the amygdala by pretreatment in with MK801 or LY274614 [39]. In adult mice the dextromethorphan blocks formalin-induced nociceptive behavior and the expression c-fos mRNA in the spinal cord [11].

Despite major progresses have been made toward the non-opioid treatment of opioid withdrawal in the mature organism, it is not known whether these data in adults apply to infants. The neonatal CNS is both structurally and functionally different from that of the adult, and significant changes in opioid actions occur both prenatally and postnatally [1,2,12,13,30]. In addition, the NMDA receptor undergoes major developmental changes during early life [9]. Thus, conclusions based on adult date often can not be directly applied to infants [47-49].

Therefore, using the neonatal rat as a model system, the present study examined the clinically available NMDA receptor antagonist dextromethorphan on acute morphine withdrawal in the developing organism. Our data clearly demonstrated that dextromethorphan was effective in suppressing behaviors associated with acute morphine withdrawal in the neonatal rat. Indeed, all withdrawal behaviors, including head moves, moving paws, rolling, vocalization, walking, and wall climbing are significantly, and in some case completely, suppressed by 50 mg/kg of dextromethorphan. Importantly, dextromethorphan's effect on acute morphine withdrawal is selective since in no case did this dose of dextromethorphan alter the normal motor behavior in the 7-day-old rat. Similarly, dextromethorphan selectively attenuated the elevation of brain c-fos mRNA expression associated with acute morphine withdrawal in the 7-day-old rat. Thus, the

results of the present study extended the available literature on dextromethorphan's ability in inhibiting acute morphine withdrawal to include the developing organism.

5. Conclusion

The neonatal rat can be used as a model system for the study of acute opioid withdrawal in the developing organism. Using this model system, we conclude that dextromethorphan, a clinically available NMDA receptor antagonist, can effectively attenuate both behavioral and biochemical manifestations of acute morphine withdrawal. As far as we know, the present report represents the first study of the pharmacological effects of a clinically available NMDA receptor antagonist on acute opioid withdrawal in the developing organism.

Table 1. Behavioral definitions.

Behavior	Definition
Head Moves	Lateral and rotary motions of the head
Moving Paws	Continuous movement of the hindpaws without walking
Quiet	Sedated appearance with no movement
Rolling	Turning the body over at least one full rotation
Vocalizations	Emitting an audible sound
Walking	Taking more than one step forward
Wall Climbing	Placing at least two forepaws on the wall of the observation chamber

Figure Legends:

Figure 1. The effect of pre-treatment with the NMDA receptor antagonist dextromethorphan (50 mg/kg, s.c.) on naltrexone precipitated acute morphine withdrawal behaviors in the 7-day-old rat. Ordinate: Mean occurrences (Mean ± one S.E.M.) in 60 minutes of opioid withdrawal behaviors (definition see Table 1). Abscissa: treatment conditions. Acute morphine withdrawal was precipitated by naltrexone (10 mg/kg, s.c.) in all groups. +, $p < 0.05$ compared to the saline plus saline control group. *, $p < 0.05$ compared to the morphine plus saline group. For all tested behaviors, no significant results were found between the two groups that did not receive morphine injection.

Figure 2. The effect of pre-treatment with the NMDA receptor antagonist dextromethorphan (50 mg/kg, s.c.) on brain *c-fos* mRNA expression evoked by naltrexone precipitated acute morphine withdrawal in the 7-day-old rat. Ordinate: Mean *c-fos* mRNA expression (pg/μg) in the brain (Mean ± one S.E.M.). Abscissa: treatment conditions. Acute morphine withdrawal was precipitated by naltrexone (10 mg/kg, s.c.) in all groups. +, $p < 0.05$ compared to the saline plus saline control group. *, $p < 0.05$ compared to the morphine plus saline group. The difference of c-fos mRNA levels between the two groups that did not receive morphine injection was not significant.

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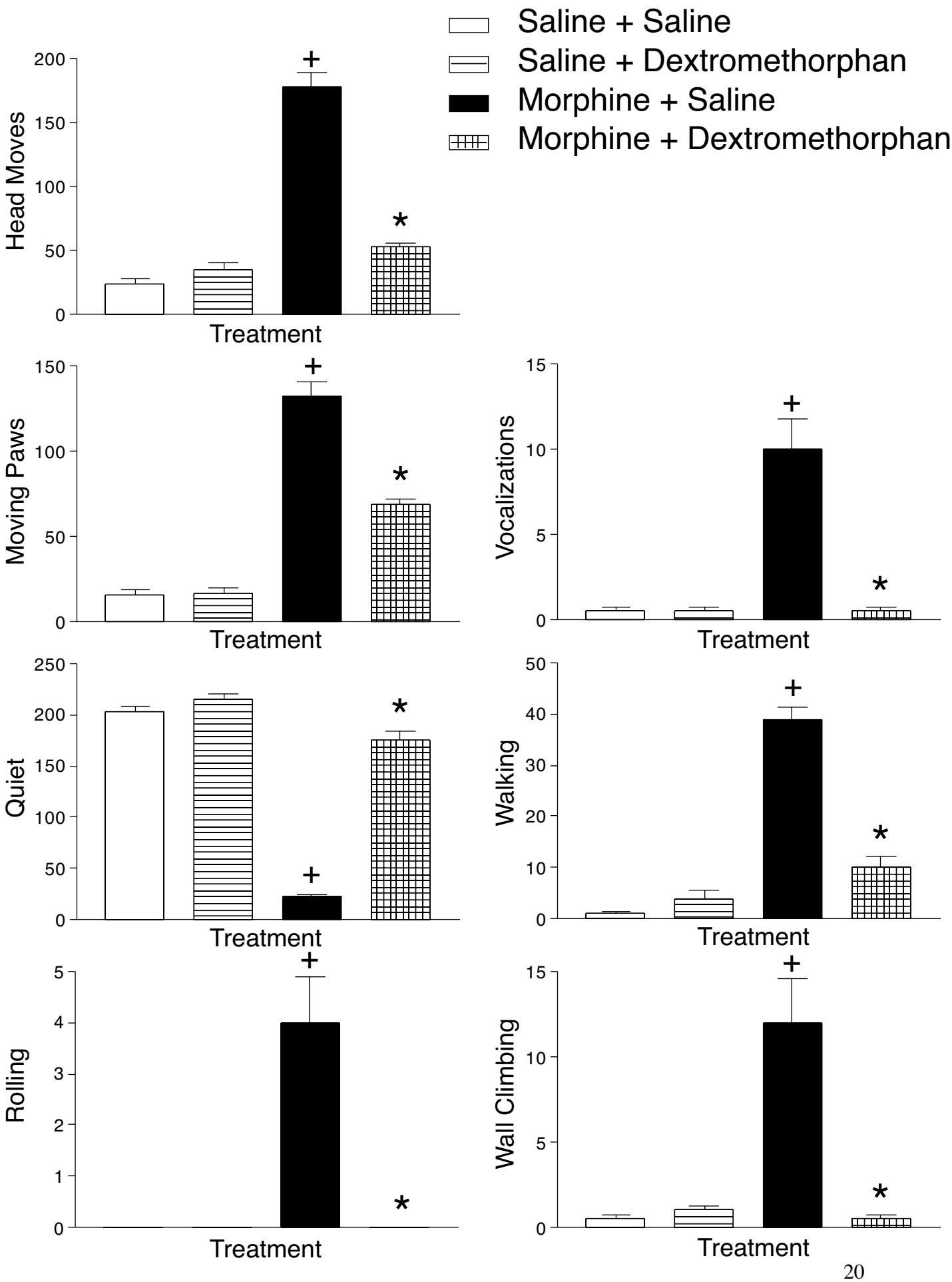
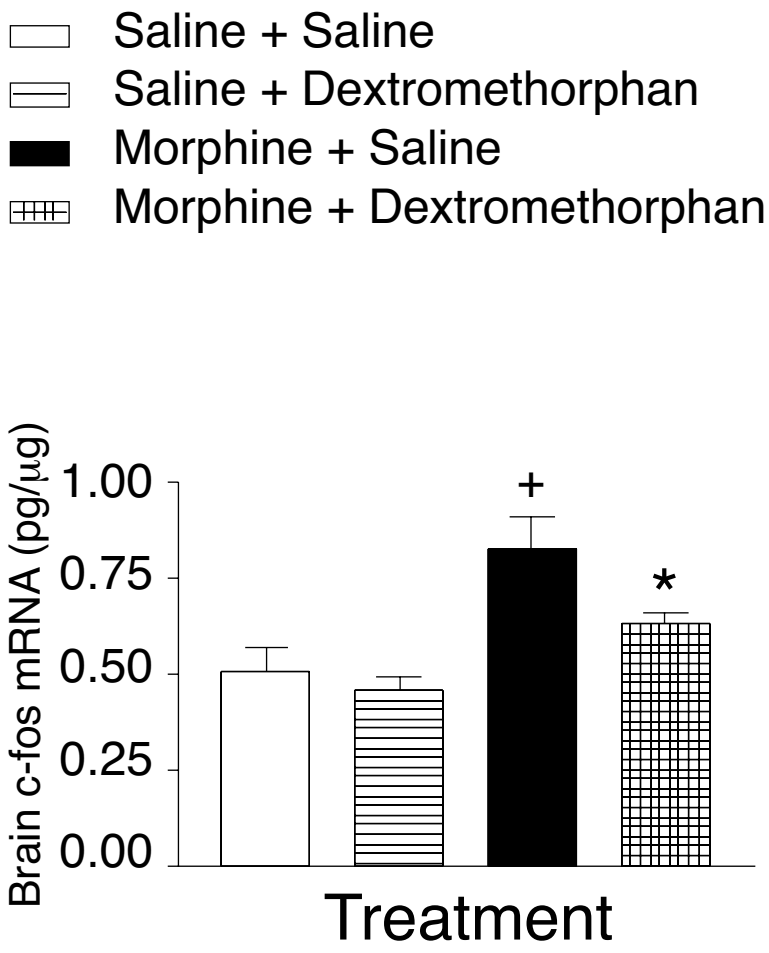

Saline + Saline
Saline + Dextromethorphan
Morphine + Saline
Morphine + Dextromethorphan
Head Moves
Moving Paws
Vocalizations
Quiet
Walking
Rolling
Wall Climbing
Treatment
+
*


Saline + Saline
Saline + Dextromethorphan
Morphine + Saline
Morphine + Dextromethorphan
Brain c-fos mRNA (pg/μg)
1.00
0.75
0.50
0.25
0.00
+
*
Treatment