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Long-Term Behavioral Effects in a Rat Model of Prolonged Postnatal Morphine Exposure

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Prolonged morphine treatment in neonatal pediatric populations is associated with a high incidence of opioid tolerance and dependence. Despite the clinical relevance of this problem, our knowledge of long-term consequences is sparse. The main objective of this study was to investigate whether prolonged morphine administration in a neonatal rat is associated with long-term behavioral changes in adulthood. Newborn animals received either morphine (10 mg/kg) or equal volume of saline subcutaneously twice daily for the first 2 weeks of life. Morphine-treated animals underwent 10 days of morphine weaning to reduce the potential for observable physical signs of withdrawal. Animals were subjected to nonstressful testing (locomotor activity recording and a novel-object recognition test) at a young age (Postnatal Days [PDs] 27–31) or later in adulthood (PDs 55–56), as well as stressful testing (calibrated forceps test, hot plate test, and forced swim test) only in adulthood. Analysis revealed that prolonged neonatal morphine exposure resulted in decreased thermal but not mechanical threshold. Importantly, no differences were found for total locomotor activity (proxy of drug reward/reinforcement behavior), individual forced swim test behaviors (proxy of affective processing), or novel-object recognition test. Performance on the novel-object recognition test was compromised in the morphine-treated group at the young age, but the effect disappeared in adulthood. These novel results provide insight into the long-term consequences of opioid treatment during an early developmental period and suggest long-term neuroplastic differences in sensory processing related to thermal stimuli.

Keywords: behavioral sensitization, hot plate, locomotor activity, novel-object recognition test, opioid

Pain management of infants and children has greatly evolved over the past 25 years, from no treatment at all to an emphasis on pain management. This was based on advances in our understanding of the neurobiology of sensory processing during development. As early as the 24th week of gestation, painful stimuli are associated with physiologic, hormonal, and metabolic markers of the stress response (Anand & Hickey, 1987; Lee, Ralston, Drey, Partridge, & Rosen, 2005). In addition to feeling pain, infants and children may have decreased pain thresholds and increased physiological responses to both noxious and innocuous stimuli as compared with older children and adults (Craig, Whitfield, Grunau, Linton, & Hadjistavropoulos, 1993; Grunau, Whitfield, Petrie, & Fryer, 1994; Johnston, Stevens, Yang, & Horton, 1995; Johnston, Stevens, Yang, & Horton, 1996). More important, several studies have demonstrated that untreated painful stimulation, especially in preterm infants, leads to long-term effects that may involve permanent changes in pain processing and impaired brain development (Brummelte et al., 2012; Fitzgerald & Walker, 2009; Johnston & Stevens, 1996; Vinall et al., 2012), including increased pain sensitivity and maladaptive behavior later in life (Anand & Scalzo, 2000; Taddio & Katz, 2005). Hence, due to the known negative implications of untreated pain in infants and children (Fitzgerald, 1991; Fitzgerald, Millard, & McIntosh, 1989; Fitzgerald & Walker, 2009), there has been an increase in the use of analgesic agents in the pediatric population.

Opioids have been shown to relieve acute pain in infants and have become the gold standard for pain treatment in procedural and perioperative settings (Anand & International Evidence-Based Group for Neonatal Pain, 2001). Even in the absence of surgical pain, critically ill neonates and children receive prolonged opioids for sedation to reduce anxiety, agitation, and stress responses and to facilitate ventilation (Anand & International Evidence-Based Group for Neonatal Pain, 2001; Berde & Sethna, 2002; Chambliss & Anand, 1997). Although the administration of opioids has been shown to improve behavioral measures of comfort in mechanically ventilated infants (Guinsburg et al., 1998; Saarenmaa, Huttunen, Leppäluoto, & Fellman, 1996), there is a gap in the literature regarding the effectiveness and hazards (benefit/risk ratio) of these agents. Moreover, such treatment is associated with markedly high

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incidence (35%–57%) of analgesic tolerance and opioid dependence (Anand et al., 2010; Fonsmark, Rasmussen, & Carl, 1999; Katz, Kelly, & Hsi, 1994).

The impact of chronic maternal opioid use on in utero brain development has been widely studied and is known to be associated with neurocognitive and motor impairments later in life (Hunt, Tzioumi, Collins, & Jeffery, 2008; McGlone, Mactier, & Weaver, 2009; van Baar & de Graaff, 1994). The added complexity of human studies makes it difficult to identify factors that are attributable to opiate use alone or are from confounding factors (e.g., the abuse of other drugs, poor prenatal care, poor nutrition). In contrast, the evidence for long-term neurodevelopmental delay following postnatal opioid exposure is limited (Bellù, de Waal, & Zanini, 2008, 2010; Ferguson, Ward, Paule, Hall, & Anand, 2012). It is possible that prolonged postnatal opioid treatment associated with the development of opioid dependence significantly alters neural pathways. Given that administration of opioids in both newborn and infant periods in the absence of pain (e.g., for sedation) is considered standard clinical care, the objective of our study was to address possible long-term behavioral sequelae of prolonged postnatal morphine exposure in a rodent model in the absence of nociception as opposed to prenatal rat models in which dams are treated. Both the maturation and function of pain pathways and the mechanisms of prolonged opioid effects in a rat model are age dependent. Specifically, increased excitability of nociceptive circuits peaks at Postnatal Day (PD) 6 and decreases to an adult-like level by PD 21 (Fitzgerald & Jennings, 1999; Jennings & Fitzgerald, 1998). Further, some of the mechanisms of opioid tolerance (Zhu & Barr, 2003) and dependence (Zhu & Barr, 2001a, 2001b) partially correspond to those of the adult rats at PD 14 and are equivalent to an adult at PD 21. Therefore, we decided to expose developing rat pups to prolonged morphine administration during this early period of the first 2 weeks of life (PDs 1–14), when different mechanisms of pain perception and opioid treatment are in effect in comparison with adult rats. Although exact equivalencies cannot be made to human developing age, there is a consensus that PDs 1–14 roughly extend from the last trimester of pregnancy up to the first few years of postnatal life in humans (Clancy, Darlington, & Finlay, 2001; Clancy, Finlay, Darlington, & Anand, 2007; Huttenlocher & Dabholkar, 1997). Specifically, we hypothesized that prolonged morphine administration in a modified neonatal rat model of antinociceptive tolerance and dependence (Bajic, Berde, & Commons, 2012; Jones & Barr, 1995; Zhu & Barr, 2003) would be associated with long-term behavioral changes in adulthood. To test our hypothesis, we investigated possible long-term influence on (a) sensory processing by measuring mechanical and thermal threshold; (b) sensitization by evaluating locomotor activation, a proxy of drug reward/reinforcement behavior; (c) stress/anxiety by using a forced swim test; and (d) short-term recognition memory using a novel-object recognition test.

Method

Animal Care and Use

The Institutional Animal Care and Use Committee at Boston Children's Hospital approved the experimental protocols for the use of vertebrate animals in this study. Experiments were conducted according to the U.S. Department of Health and Human Services "Public Health Service Policy on Humane Care and Use of Laboratory Animals" as a guide for the Care and Use of Laboratory Animals (NIH Publication No. 15-8013, revised 2015) prepared by the National Institute of Health Office of Laboratory Animal Welfare. All efforts were made to minimize the number of animals used and their discomfort. Pregnant rat dams (Sprague Dawley, Sasco; Charles River Laboratories International, Inc., Wilmington, MA) were received on Day 18 of gestation and were handled for several days before delivery. Cages with pregnant dams were checked at 9 a.m. and 5 p.m. daily, and pups found at either times were termed 0 days of age. The progeny from eight litters were used in this study. Each litter had between 9 and 12 pups that were randomly assigned to each of the pharmacological groups; this split-litter (within-litter) design represents all treatment groups within a single litter (Booze & Mactutus, 1985). We used balanced treatment distribution per litter (Festing, 2006). Pups from both sexes were included in the study. Animals were housed with their litters and were maintained on a 12-hr light/dark cycle, with food and water given ad libitum. Pups were weaned from dams at 3 weeks of age (PD 18).

Pharmacological Treatment

Barr's group originally described a method for prolonged morphine administration in rat pups that is associated with morphine dependence (Jones & Barr, 1995) and development of analgesic tolerance (Zhu & Barr, 2003) in newborn rats; the latter was also confirmed in our lab (Bajic et al., 2012). We used subcutaneous (SC) injections to minimize nociceptive experience from intraperitoneal drug administration, and, in this study, we extended the period of administration from 6½ (1 week) to 13½ days (2 weeks). The first day of injection always occurred at PD 1. Specifically, animals received twice-daily SC injections for 14 days (at 9 a.m. and 5 p.m.), from PD1 through PD14. All injections were done using either a 10- or 100-μl syringe (Hamilton Company, Reno, NV). Morphine sulfate (10 mg/kg; Baxter Health Care Corporation, Deerfield, IL) or an equal volume of saline was administered in the SC area of the upper or lower back. Experimental groups were (a) a control group that received saline injections and (b) a morphine group that received morphine injections.

Morphine Weaning and Quantification of Physical Signs of Morphine Withdrawal

Physical dependence is manifested indirectly as a myriad of physiological disturbances and physical symptoms of withdrawal that result from abrupt discontinuation of dosage reduction (spontaneous withdrawal). After the period of pharmacological treatment (PDs 1–14), pups injected with morphine underwent morphine weaning treatment for 10 days (PDs 15–24) to reduce the potential for observable physical signs of withdrawal. Specifically, pups received incrementally decreasing morphine dosages: 3 days of twice-daily 5 mg/kg, 3 days of twice-daily 2.5 mg/kg, 2 days of twice daily 1.25 mg/kg, and 2 days of once daily 1.25 mg/kg. To ensure that the weaning protocol was appropriate in preventing development of observable physical signs of withdrawal, we quantified the pups' daily behavior for 10 days of morphine weaning. We quantified the physical signs of withdrawal in progeny from the first two litters analyzed: eight pups (four female and four male) for the saline group and 10 pups (eight female and two male) for the morphine group. Specifically, a 10-min video was recorded

daily before morning injections. We selected this time point because it would reflect behavior following the longest time period since the last injection (14–16 hr). An observer blind to the treatment group manually scored the individual behaviors of the animals every 15 s. The scoring rubric was adapted from a previous study (Gellert & Holtzman, 1978). Briefly, 11 scored withdrawal behaviors corresponded to two different types of physical signs: checked signs and graded signs, with five and six behaviors in each category, respectively. *Checked signs* were behaviors for which only the absence or presence of the behavior was evaluated; these included diarrhea, facial fasciculation/teeth chattering, swallowing movements, profuse salivation, ptosis, and abnormal posture. *Graded signs* were scored on the basis of frequency (number of events) and included escape attempts, abdominal contractions, wet dog shakes, rearing, and grooming. Modifications of Gellert–Holtzmann method (Gellert & Holtzman, 1978) included the elimination of weight loss (as no animals lost weight during morphine weaning; see Figure 1B) and the addition of two graded behaviors (rearing and grooming) not previously analyzed. Withdrawal scores were expressed as individual sign means plus or minus a standard deviation (not shown) and the mean of the sum of all behavioral scores (total global score mean plus or minus a standard deviation; see Figure 1C) for the 10 min of observed behavior.

Behavioral Analyses

Following prolonged pharmacological treatment after birth (PDs 1–14) and morphine-weaning treatment (PDs 15–24), animals underwent stressful testing only one time in adulthood (PDs 55–56) for nociceptive and thermal threshold, as well as the forced swim test (saline group = 25 [12 female and 13 male rats from eight litters], morphine group = 24 [15 female and nine male from six litters]). Two nonstressful tests—locomotor activity recording and novel-object recognition—were performed at two different time points: at younger age (PD 30: saline group = 10 [five female and five male rats from four litters]; morphine group = 7 [four female and three male rats from four litters) and in adulthood (PDs 55–56: saline group = 17 [eight female and nine male rats from four litters]; morphine group = 21 [15 female and six male rats from four litters]). None of these animals underwent testing twice for the same test. When rats were tested in adulthood, tests were performed from the least to the most stimulating manipulations and proceeded as follows: (a) novel-object recognition test, (b) locomotor recording, (c) calibrated forceps, (d) hot plate test, and (e) forced swim test. An individual blinded to the pharmacological treatment performed all the tests.

Calibrated forceps testing. Mechanical threshold response was evaluated by using calibrated forceps (rodent pincher-analgesia meter; Bioseb In Vivo Research Instruments, Vitrolles, France). This algometer allows calibration of forceps to induce quantifiable mechanical stimulation in an animal on a linear scale. As described by Luis-Delgado et al. (2006), we recorded the effects of three repetitive measurements on each hind paw (a total of six measurements per individual rat) to provide a sensitive and reliable way of testing mechanical threshold. The force applied to the foot was increased incrementally at approximately 200 g every 3 s until the paw was withdrawn. A 5-min period was allowed between repeated testing. The maximum force applied to the paw at the time of withdrawal was recorded as displayed by the dynamometer in grams. Withdrawal latency was expressed as mean plus or minus a standard deviation per pharmacological group.

Hot plate test. The hot plate test was carried out to measure thermal withdrawal latency as an index of nociceptive threshold using previously described methods (Bannon & Malmberg, 2007; Eddy & Leimbach, 1953; Shi, Qi, Gao, Wang, & Luo, 2010). Briefly, rats were placed in a clear Plexiglas box standing on a hot plate (Cold Hot Plate Analgesia Meter; Bioseb In Vivo Research Instruments, Vitrolles, France) heated to 52.5° C (Allen & Yaksh, 2004). The latency time was defined as the period between the animal's initial placement on the hot plate surface and the time when the animal licked its paws, jumped to avoid thermal nociceptive stimulus, and/or urinated. To minimize tissue damage, a cutoff time of 40 s was adopted. We recorded the effects of three repetitive measures per individual rat performed at a minimum of 2 min apart. Thermal withdrawal time (in seconds) was expressed as mean plus or minus a standard deviation per pharmacological group.

Forced swim test. Adult animals underwent modified forced swim testing on PDs 55–56. The forced swim test—as originally reported by Porsolt, Le Pichon, and Jalfre (1977)—measures coping strategy in response to an acute stress and has become a widely used model for assessing depressant-/antidepressantlike activity in rats. We used a protocol that was identical to those previously described by others (Borsini, Lecci, Sessarego, Frassine, & Meli, 1989; Borsini & Meli, 1988; Detke, Rickels, & Lucki, 1995; Porsolt, Bertin, & Jalfre, 1977), except we increased a water depth from 15–30 cm. Briefly, rats were placed in a cylindrical glass tank (46 cm high × 20 cm diameter) filled with water (25±1° C) to a depth of 30 cm for 15 min. The 30-cm depth allowed rats to swim or float without their tails touching the bottom of the tank. Test sessions were video recorded (Flip Video UltraHD Video Camera; Cisco Systems, Inc., San Jose, CA) for later scoring. Immediately after the forced swim test, rats were removed from the tank, towel dried, and put in a warming cage (37° C) that contained a heating pad covered with towels for 15 min. Rats were then returned to their home cage. Videotaped sessions were reviewed and scored using a time-sampling technique wherein the predominant behavior over each 5-s period of the test was recorded. This contrasted with the original swim test, which used a cumulative timing measure in which the total amount of time was recorded for each behavior in seconds. Three types of predominant behavior (swimming, climbing, immobility; Slattery & Cryan, 2012) were recorded at the end of every 5-s period during the 15-min testing session. *Floating* was defined as rats keeping their heads above the water's surface with minimal body movements. *Climbing* was defined as vigorous movements of all four limbs, with the front paws breaking through the surface of the water against the wall of the tank. In contrast, during *swimming*, rats created coordinated and sustained movements with all four limbs, usually traveling around the interior of the cylinder but without breaking the surface of the water with their forelimbs. During diving, rats would submerge entirely beneath the water. Diving was counted as a swimming activity (Slattery & Cryan, 2012). An individual blinded to the treatment group scored videotaped tests. Frequency score measures for individual behaviors (swimming, climbing, immobility) were expressed as mean plus or minus a standard deviation per pharmacological group.

Locomotor activity Assay. Animals underwent locomotor testing either at a younger age (PD 27) or in adulthood (PDs 55–56). Locomotor activity was recorded in clear Plexiglas cages (10-in. × 19-in. × 8-in. high; Photobeam Activity System, San Diego Instruments, San Diego, CA) over 12 5-min intervals for 60 min. Considering that we used an unpaired paradigm (animals tested in an environment that differed [unpaired] from that used for injection), recorded locomotor activity can be considered context-independent sensitization (Vezina, Giovino, Wise, & Stewart, 1989). Total locomotor activity data (ambulatory, fine, and rearing movements) recorded over all 12 intervals (period of 1 hr) were expressed as mean plus or minus a standard deviation per pharmacological group.

Novel-object recognition Test. Animals underwent novel-object recognition testing either at a younger age (PD 31) or in adulthood (PDs 55–56). We used a protocol identical to those previously described (Bevins & Besheer, 2006). This test takes advantage of animals' tendency to approach and explore novelty (Berlyne, Koenig, & Hirota, 1966), does not require preliminary training, and has a high throughput potential given that it is conducted in one session. All testing was conducted in a nontransparent plastic chamber (10-in. × 10-in. × 10-in. for pups, 13-in. × 13-in. × 13-in. for adults). Briefly, to acclimate animals to the new environment, all rats were preexposed to the testing environment for 5 min the day before testing. Novel-object recognition testing consisted of two sessions (training and 1-hr testing), both of which were videotaped (Flip Video UltraHD Video Camera; Cisco Systems, Inc., San Jose, CA). In the first 10-min session (training), animals interacted with two identical (sample/familiar) objects that were placed in the back left and right corners of the box. Animals were always placed in the center of the box, facing the wall opposite the testing objects. This orientation prevented any unintentional bias toward one object/side of the box. The experimenters left the procedure area so as not to serve as a cue for the rats. After this initial session, rats were returned to their cage for a period of 1 hr (training-to-testing interval). Afterward, one of the sample objects was replaced with an object novel to the animals. The animals were then placed in the testing chamber again for 4 min (1-hr testing session). An experimenter blinded to the group treatment scored videotaped behaviors. The first 3 min of each video were analyzed. Two stopwatches were used to monitor the time an animal spent in *directed contact* with each object, which included

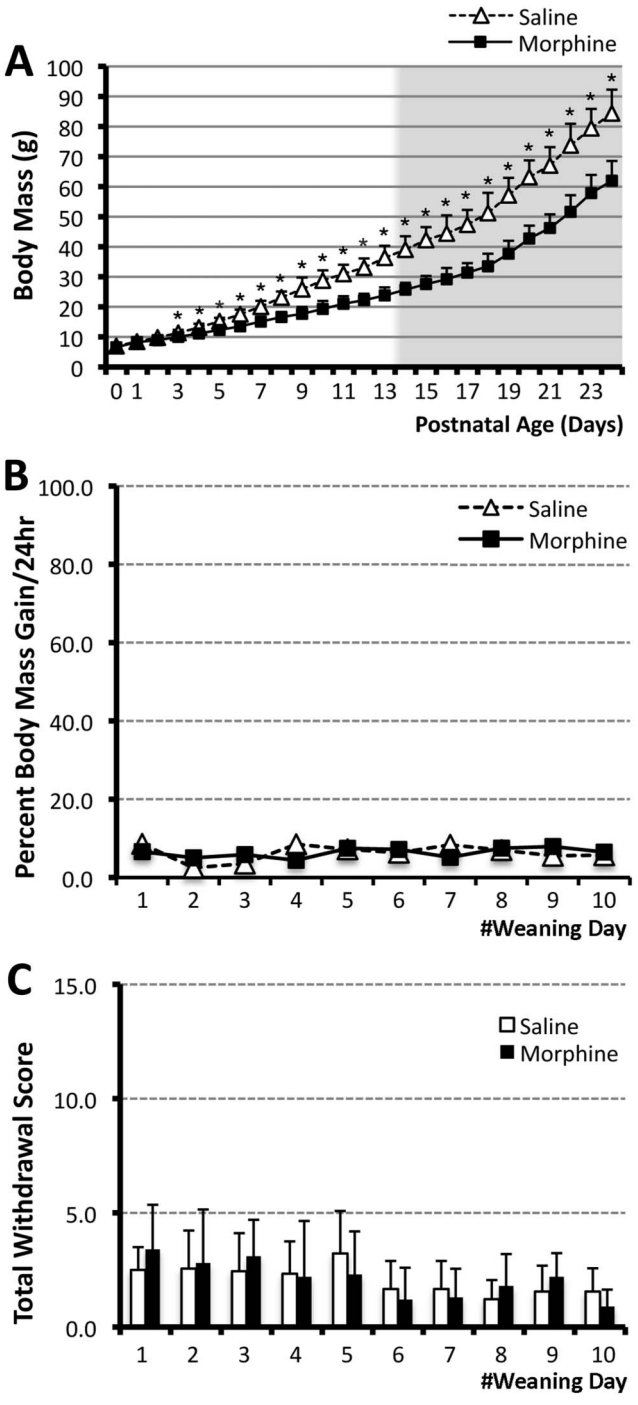

Figure 1 (opposite)

Figure 1. Prolonged morphine administration in newborn rats. A: The effect of repeated morphine treatment on the body mass of newborn rats (white background) in comparison to saline treated controls. Day of birth was considered Day 0 of life. Morphine sulfate (10 mg/kg subcutaneously twice daily) or equivalent volume of saline was injected for 14 days starting on postnatal day (PD) 1. After, animals underwent a 10-day weaning period (PDs 15–24 [gray background]). Progeny from four litters were used to analyze the weight. Systemic morphine exposure ($n = 21$; 15 female, six male) significantly decreased body mass (plus or minus a standard deviation) in newborn rats in comparison to saline control group ($n = 18$; eight female and 10 male) from PD 3. B and C: Analysis of physical signs of withdrawal that were analyzed in progeny of the first two litters (saline [$n = 8$] and morphine [$n = 10$] groups. Panel B illustrates percentage of body mass gain/24 hr (plus or minus a standard deviation) during the weaning period of 10 days. Consistent percentage of body mass gain was maintained through the period of morphine weaning, and no significant differences were observed between the morphine- and saline-treated animals at any of the days analyzed. Panel C shows analysis of total withdrawal score (plus or minus a standard deviation) for each day of the weaning period (PDs 15–24). There were no differences in any individual (not shown) or total daily withdrawal scores between morphine and saline treated animals.

contact of the mouth, nose, and/or paw with the object. It did not include accidental touches, like bumping or backing into the object. Also, standing or leaning on the object was not included in this definition of contact (Bevins & Besheer, 2006). In addition to mean interaction time (in seconds) plus or minus a standard deviation spent with objects per pharmacological group, we used the discrimination ratio as a measure of novel object recognition. The *discrimination ratio* is the time spent with the novel object divided by the total interaction time (time spent with both objects). Using this measure, a value of 0.5 indicates the same amount of time spent with both objects, whereas novel-object recognition is determined by a discrimination ratio greater than 0.5.

Statistical Analyses

Considering that we used split-litter (within-litter) design when individual pups within a single litter were subjected to different treatments, the analysis unit (n) was based on the number of individual pups in a treatment group (Festing, 2006). A statistical power analysis was performed for sample size estimation. The standardized effect size in this study was 1.00 (large; Hulley et al., 2001). With an alpha of .05 and power of .80, the projected sample size needed with this effect size was approximately 17. Thus, our adult group sizes (17–25) were more than adequate for detecting meaningful differences between the pharmacological groups. Given that the sample sizes for younger animals were smaller, the likelihood of Type II error was higher. No differences were found in any of the behavioral analyses between male and female rats (12 female and 13 male saline-treated animals, 15 female and 9 male morphine-treated animals [data not shown]). Because all tested behaviors were sex independent, data for each treatment group were collapsed for clarity. A two-tailed independent Student's t test was used to determine mean differences between pharmacological groups for each day of the morphine-weaning period (PDs 15–24), mechanical and thermal thresholds, and individual forced swim test behaviors (swimming, climbing, immobility). Further, we used one-way analysis of variance with Tukey's Honestly Significant Difference test to identify (a) mean differences with age and pharmacological treatment for nonrepeated-measures of locomotor activity data and (b) mean differences with pharmacological treatment and types of objects for interaction time and discrimination ratio of the novel-object recognition test. Significance was set at $p < .05$. All statistical analyses were done with VassarStats (http://vassarstats.net/), a website for statistical computation.

Results

Body Mass and Withdrawal Symptoms Evaluation in Newborn Period

We used a rat model of prolonged postnatal morphine exposure (PDs 1–14). Because we were interested in understanding the long-term behavioral effects of this prolonged and early morphine administration, it was important to avoid development of withdrawal symptoms, with weight loss being a cardinal symptom. Similar to our previous work (Bajic, Commons, & Soriano, 2013), prolonged morphine administration in neonatal rats (10 mg/kg twice daily) led to slower body mass gain (see Figure 1A; $n = 18$ for control and 21 for morphine group). This could be explained, in part, by the fact that morphine-treated animals were asleep after the injections, giving a nursing advantage to the saline-treated pups. However, morphine-treated animals did not show any loss of weight (on the basis of day-to-day measurements) either during the period of treatment (PDs 1–14) or during the period of morphine weaning (PDs 15–25). In fact, percentage of body mass gain was maintained through the period of morphine weaning and was not different from that in the saline group (see Figure 1B). Animals were separated from mothers on PD 18 (Day 4 of morphine weaning), which was not associated with change in growth. In addition, none of the individual physical signs of withdrawal (not shown) or total daily withdrawal scores were different between morphine- ($n = 10$) and saline-treated ($n = 8$) animals (see Figure 1C), confirming that animals did not exhibit any of the physical signs of withdrawal during the weaning period. In adulthood (PDs 55–56), male rats weighed more than female rats but did not differ between treatment groups (data not shown; 12 female and 13 male saline-treated rats, 15 female and 9 male morphine-treated rats), suggesting that during development, animals overcame the initial body mass differences.

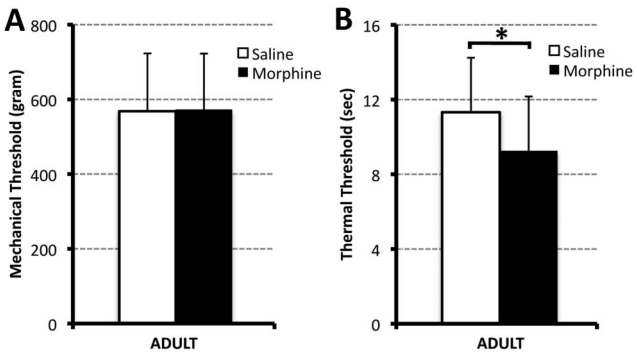

Figure 2. Average mechanical (A) and thermal (B) thresholds (plus or minus a standard deviation) in adult rats (Postnatal Days 55–56) that were exposed to different pharmacological treatment in the first 2 weeks of life. Morphine sulfate (10 mg/kg subcutaneously twice-daily [$n = 24$]) or equivalent volume of saline ($n = 25$) was injected for 14 days, starting on PD 1. Hind paw–withdrawal mechanical threshold (grams plus or minus a standard deviation) was measured using calibrated forceps. Postnatal morphine treatment did not change the mean mechanical threshold in comparison to controls in adulthood (A). Panel B illustrates average thermal thresholds (in seconds plus or minus a standard deviation), which were measured using the hot plate test (52.5° C). Prolonged postnatal morphine exposure is associated with long-term thermal hypersensitivity in the adulthood in comparison to saline treated group. * $p < .05$.

Long-Term Mechanical and Thermal Nociception

To examine the influence of prolonged postnatal morphine exposure on nociceptive pathways, both mechanical and thermal tests were performed when animals reached adulthood (PDs 55–56; 25 saline-group and 24 morphine-group rats). Average hind paw–withdrawal mechanical threshold (grams plus or minus a standard deviation) measured with calibrated forceps was not significantly different between pharmacological treatment groups (see Figure 2A). In other words, prolonged postnatal morphine treatment did not change the mean mechanical threshold (573.09 g

± 149.44) compared with that in the saline-treated animals (568.4 g ± 154.75), $t(47) = -0.11$, $p = .91$, in adulthood. This was in contrast to the average thermal threshold (seconds plus or minus a standard deviation) that was measured using the hot plate test (see Figure 2B). Prolonged postnatal morphine exposure was associated with long-term thermal hypersensitivity in adulthood (9.26 s ± 2.91) in comparison with the saline-treated group (11.32 s ± 2.92), $t(47) = 2.48$, $p = .02$.

Long-Term Forced Swim Test

The forced swim test is based on the assumption that animals try to escape from an aversive stimulus and is used to measure coping strategy in response to an acute stress (Borsini et al., 1989; Borsini & Meli, 1988; Detke et al., 1995; Porsolt, Anton, Blavet, & Jalfre, 1978; Porsolt, Bertin, & Jalfre, 1977). In this experiment, we quantified individual swim test behaviors (swimming, climbing, immobility) using a frequency score measure/15 min (plus or minus a standard deviation) in adult animals (PDs 55–56) following either prolonged postnatal morphine ($n = 24$) or saline treatment ($n = 25$). The pattern of individual swim behaviors was not different between treatment groups (see Figure 3). Specifically, no statistical differences between morphine- and saline-treated groups were found for swimming, $t(47) = -1.91$, $p = .06$; climbing, $t(47) = 0.16$, $p = .87$; or immobility behavior, $t(47) = -0.33$, $p = .74$.

Long-Term Locomotor Activity

Increased locomotor activity has been proposed to reflect neuroadaptations caused by a drug of abuse, such as heightened drug reward/reinforcement behavior, and it is a long-lasting phenomenon. Total locomotor activity data were recorded over 12 5-min intervals (total period = 1 hr; see Figure 4A). Animals did not receive any morphine pretreatment before the measurement of spontaneous locomotor behavior. We report no significant differences following prolonged postnatal morphine administration (PDs 1–14) in comparison with saline control with respect to average individual (ambulatory, fine, and rearing movements [not shown]) or total locomotor activity (plus or minus a standard deviation) at a young age (PD 27; saline group = 10, morphine group = 7) or over the long term (i.e, in adulthood; PDs 55–56 [saline group = 17, morphine group = 21]). Specifically, total average locomotor activity/animal/hr (plus or minus a standard deviation) either at a young age (1 week after treatment and 3 days after last injection during weaning) or in adulthood (8 weeks after treatment) did not differ with either treatment or age, $F(3, 44) = 2.51$, $p = .07$ (see Figure 4B).

Long-Term Novel-Object Recognition Test

Rodents' tendency to interact more with novel objects than with previously explored (familiar) objects (Berlyne et al., 1966) has been used to study memory in a form of a novel-object recognition test (Bevins & Besheer, 2006). In the first phase of the experiment (see Figures 5A and B, - TRAINING), animals were exposed to two identical objects (familiar objects). There were no differences in interaction time between either the left or the right familiar objects at a young age (PD 31; saline group = 10, morphine group = 7) or later in adulthood (PDs 55–56; saline group = 17, morphine group = 21). Analysis also showed that during training sessions, morphine-treated young animals spent more time with one of the objects in comparison with animals from the saline group, $F(3, 30) = 3.34$, $p = .03$, but this was not the case during adulhood, $F(3, 72) = 1.9$, $p = .14$. Equal time spent with both objects is also represented as a discrimination ratio (the time spent with one of the objects divided by the total interaction time) of 0.5 (see Figures 5A′ and B′). In the second phase of the experiment, when a novel object was introduced instead of a familiar one (see Figures 5A and B – 1-hr TESTING), animals spent more time interacting with the novel object. This significant increase in interaction time (plus or minus a standard deviation) was demonstrated for young, $F(3, 30) = 15.38$, $p < .001$, and adult animals, $F(3, 72) = 7.86$, $p < .001$. Specifically, at the young age, both saline ($p < .01$) and morphine ($p < .05$) groups interacted significantly longer with a novel object (see Figure 5A). In adulthood, although both saline and morphine groups interacted longer with a novel object, only morphine group reached significance ($p < .01$; see Figure 5B). However, we report a significant increase in the discrimination ratio at 1-hr testing in comparison with training session for both young (see Figure 5A'), $F(3, 30) = 27.72$, $p < .01$, and adult rats (see Figure 5B'), $F(3, 72) = 13.98$, $p < .01$. A discrimination ratio greater than 0.5 indicated that animals spent more time with the novel object. At a young age, the calculated discrimination ratio at 1-hr testing was significantly higher in saline than for morphine-treated animals (see Figure 5A′; $p < .05$). This difference disappeared in adulthood (see Figure 5B′).

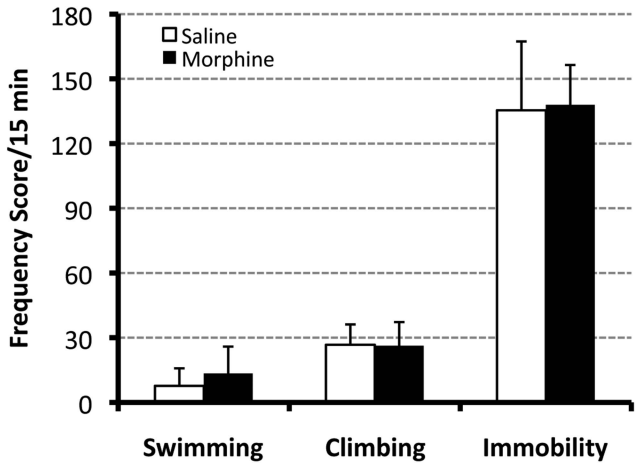

Figure 3. Mean frequency scores (plus or minus a standard deviation)/15 min for individual behaviors in the forced swim test. Adult animals (Postnatal Days [PDs] 55–56) were tested following either prolonged postnatal morphine exposure ($n = 24$) or saline control ($n = 25$). Specifically, subcutaneous morphine sulfate (10 mg/kg twice daily) or an equivalent volume of saline was injected from PD 1 to PD 14. We report no significant differences between two different pharmacological groups for any of the individual forced swim test behaviors.

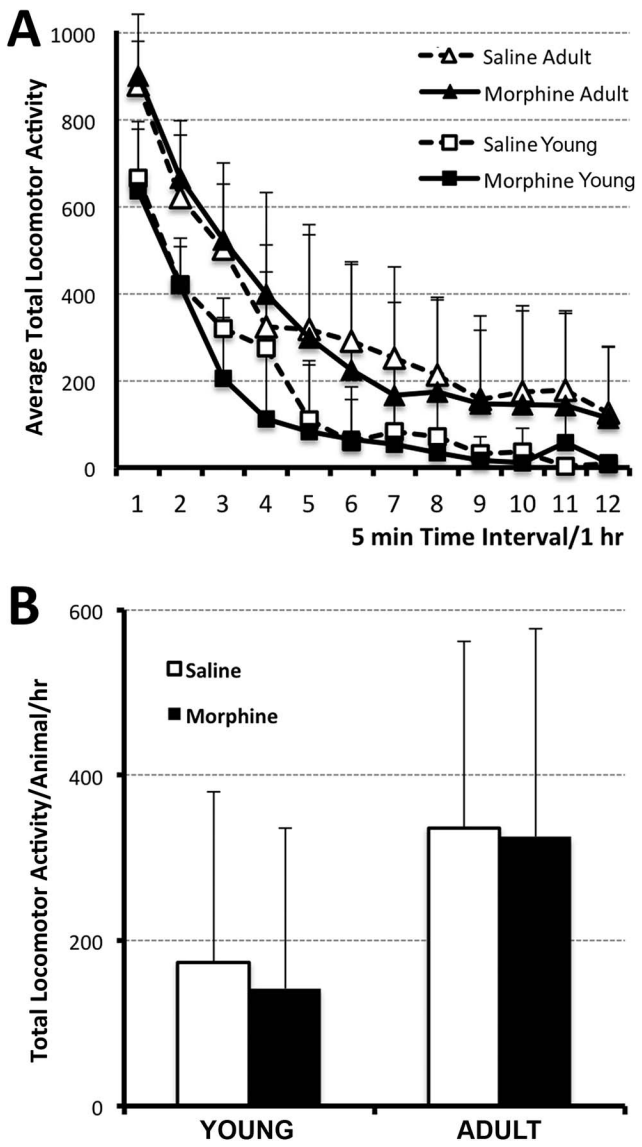

Figure 4. Total locomotor activity (ambulatory, fine, and rearing movements) plus or minus a standard deviation at two different ages: young age (1 week after the pharmacological treatment; Postnatal Day [PD] 27) or adulthood (8 weeks after treatment [PDs 55–56]). Morphine sulfate (10 mg/kg subcutaneously twice daily) or equivalent volume of saline was injected for 14 days starting on PD 1. A: Average total locomotor activity over a period of 1 hr (12 5-min intervals). Although the mean values are slightly lower for younger animals ($n = 10$ [saline group], $n = 7$ [morphine group]) in comparison with adults ($n = 17$ [saline group], $n = 21$ [morphine group]), there were no significant differences at any of the time points. B: Graph summarizes total average locomotor activity/animal/hr, which did not differ with either pharmacological treatment or age (one-way analyses of variance with Tukey's honestly significant difference test).

Discussion

Our novel findings show that prolonged morphine exposure in neonatal rats altered long-term nociceptive processing, as manifested by thermal hyperalgesia and not mechanical allodynia later in adulthood. Equally important, prolonged morphine exposure in neonatal rats was not associated with any long-term changes in locomotor sensitization (proxy of drug reward/reinforcement behavior in rats) or the forced swim test (proxy of coping behavior to an acute stress). Initially compromised novel-object recognition test performance (short-term recognition memory) at a younger age was not different from control in adulthood.

Long-Term Effects on Nociceptive Thresholds

Prolonged postnatal morphine exposure did not produce mechanical allodynia in adulthood. However, it was associated with long-term thermal hyperalgesia (see Figure 2B). These findings are in contrast to recently published work by Zhang and Sweitzer (2008), who reported that neither mechanical nor thermal nociception was significantly lower than control in adulthood. Several factors might explain the discrepancy with our study, including differences in (a) pharmacological treatment (3 mg/kg once daily from PDs 1–9 vs. 10 mg/kg twice daily from PDs 1–14, as used in our study); (b) differences in frequency of behavioral testing (total of eight testing sessions from PD 11 through 48 vs. only one in adulthood at PDs 55–56 in our study); (c) methodological differences (heat exposure of the single hind paw with Ugo Basile Plantar testing apparatus vs. exposure of all four limbs during the hot plate test in our study); and (d) justification of the absence of the withdrawal symptoms, as shown in the present report. Unlike Zhang and Sweitzer's (2008) model, we avoided repeated nociceptive testing, because it was reported to be associated with behavioral tolerance (Lane & Morgan, 2005). Despite differences in methodology between our study and that of Zhang and Sweitzer, thermal response latencies were within a similar range. It is possible that the higher morphine dose administered in our study had a long-term effect on thermal nociceptive processing. Future studies should investigate dose-dependent morphine effects on potential long-term behavioral sequelae on thermal threshold. Postnatal maturation that occurs during the first 3 postnatal weeks in rat involves dramatic changes in (a) opioid receptor expression, binding, and locations in the brain (Georges, Normand, Bloch, & Le Moine, 1998; Kent, Pert, & Herkenham, 1981; Kivell, Day, McDonald, & Miller, 2004; Petrillo, Tavani, Verotta, Robson, & Kosterlitz, 1987; Tong et al., 2000; Winzer-Serhan, Chen, & Leslie, 2003), and the spinal cord (Beland & Fitzgerald, 2001; Nandi et al., 2004; Nandi & Fitzgerald, 2005; Rahman, Dashwood, Fitzgerald, Aynsley-Green, & Dickenson, 1998; Rahman & Dickenson, 1999); (b) distribution of afferent fiber types (C, Aδ, and Aβ) in different laminae of the spinal cord dorsal horn (Fitzgerald, Butcher, & Shortland, 1994; Fitzgerald & Jennings, 1999); and (c) maturation of cortical (Colonnese, Phillips, Constantine-Paton, Kaila, & Jasanoff, 2008) and descending inhibitory mechanisms originating from the brainstem (Barr & Wang, 2013; Fitzgerald & Koltzenburg, 1986; Hathway, Koch, Low, & Fitzgerald, 2009; van Praag & Frenk, 1991). Therefore, additional studies should look into mechanistic elucidation of neuronal plasticity of both mechanical and thermal nociceptive circuitry at different levels of the neuroaxis.

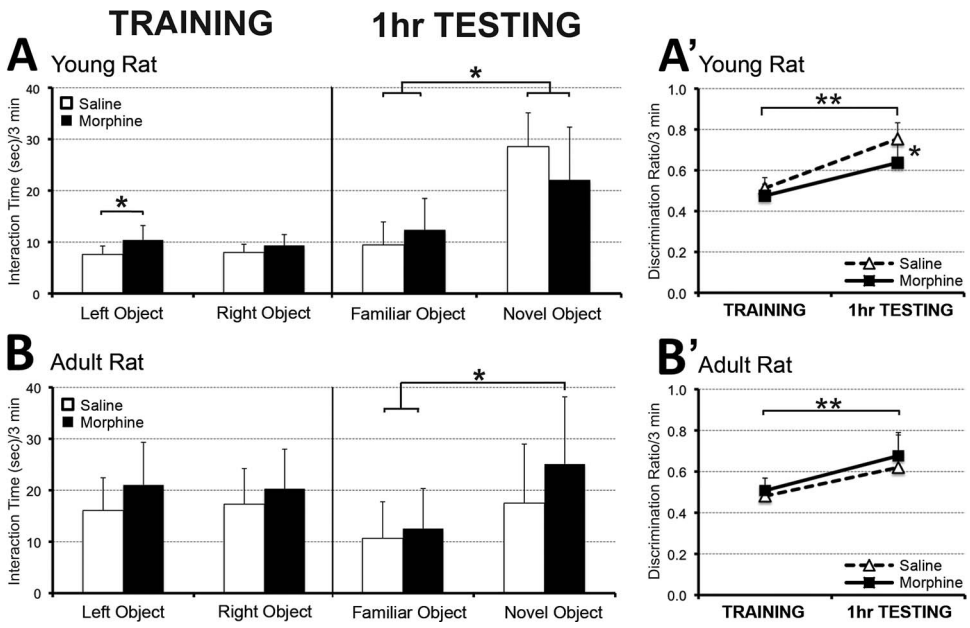

Figure 5. Interaction times (in seconds) during two phases of the novel-object recognition test performed at a younger age (Postnatal Day [PD] 31; A) and later in adulthood (PDs 55–56; B). Animals were initially treated with morphine sulfate (10 mg/kg subcutaneously twice daily) or equivalent volume of saline from PD 1 to PD 14. During the training session, animals interacted with each of the two identical objects (familiar objects) for equal amount of time both at the young age (PD 31; $n = 10$ for saline group, $n = 7$ for morphine group [Panel A]) and in adulthood (PDs 55-56; $n = 17$ for saline group; $n = 21$ for morphine group [Panel B]). In a second phase (testing session after 1 hr), one of the familiar objects was replaced by a novel object. At a young age, both saline- and morphine-treated animals spent significantly more time interacting with the novel object. In adulthood, althought both saline and morphine groups interacted longer with a novel object, only morphine group reached significance. Panels A′ and B′ illustrate the discrimination ratio (time spent with the novel object divided by total interaction time). During the training session, a discrimination ratio of 0.5 indicates that animals spent equal amounts of time with both objects, whereas values greater than 0.5 during the testing session after 1 hr indicate that animals interacted more with the novel object. We report significantly increased discrimination ratio for both young and adult animals. Although morphine-treated animals discriminated between the familiar and the novel object at both ages, the calculated discrimination ratio was significantly higher in saline-treated young animals. This difference disappeared in adulthood (one-way analysis of variance with Tukey's honestly significant difference test). $^{*}\ p < .05$. $^{**}\ p < .001$.

Long-Term Effect on Forced Swim Test

The behavioral pattern elicited by the swim stress test measures animals' coping strategy in response to an acute stress (Borsini et al., 1989; Borsini & Meli, 1988; Detke et al., 1995; Porsolt, Anton, et al., 1978; Porsolt, Bertin, & Jalfre, 1977). Our novel findings show that prolonged postnatal morphine exposure (PDs 1–14) has no long-term influence on forced swim test behavior later in adulthood (see Figure 3). Similar studies addressing the potential behavioral changes using the forced swim test following postnatal morphine exposure unfortunately are lacking. In contrast, a study that looked into perinatal morphine exposure (whole gestation and lactation period) reported enhanced depressive-like changes (as indicated by increased percentage of time spent in immobile/floating posture) both immediately following cessation of the treatment in young age (PD 25) and in adulthood (PD 56; Klausz et al., 2011). Further, our unpublished preliminary data at the young age (PD 16) showed no difference in immobility following 2 weeks of treatment (PDs 1–14) with either saline or morphine. It is possible that timing and/or the total opioid dose exposure might have influenced the neuronal adaptations that relate to increased risk of depressive-like behavior later in life. The serotonin system originating from the midbrain's dorsal raphe nucleus, implicated in mood and affective disorders, is dramatically affected by swim stress. It is generally believed that the neuronal circuits underlying behavioral responses of swim stress engage inputs to GABAergic neurons in the dorsolateral subregion of dorsal raphe nucleus, which, in turn, inhibit serotonergic neuronal activity and serotonin release in certain forebrain regions (Roche, Commons, Peoples, & Valentino, 2003), such as the lateral septum and amygdala (Kirby, Allen, & Lucki, 1995; Kirby, Chou-Green, Davis, & Lucki, 1997; Kirby & Lucki, 1997). Enhancement of serotonin neurotransmission may mediate swimming, whereas enhancement of another neurotransmitter, norepinephrine, has been implicated in mediation of climbing in the forced swim test (Detke et al., 1995). Given

this evidence, our results suggest that prolonged postnatal morphine exposure does not cause long-term changes of neurochemical substrates involved in control of serotonin and, possibly, norepinephrine.

Long-Term Effects on Locomotor Activity

Behavioral sensitization processes in rodent models are important for the incentive–motivational components of drug reward (see reviews: Robinson & Berridge, 1993; Wise & Bozarth, 1987). They are defined by the augmented motor–stimulant responses that occur with repeated, intermittent exposure to a specific drug and are long-lasting phenomena. Behavioral sensitization to the locomotor activating effects of morphine can be demonstrated without a period of withdrawal (Norwood, Al-Chaer, & Fantegrossi, 2014), which we have shown in previous work in adult rats (Bajic, Soiza-Reilly, Spalding, Berde, & Commons, 2015). Interestingly, our behavioral findings do not support the hypothesis that the period of the first 2 weeks of life (PDs 1–14) is crucial to long-term behavioral sensitization to prolonged morphine exposure 1 week after completion of treatment (3 days after the last injection during weaning; see Figure 4). This finding is consistent with reports that repeated psychostimulant treatment does not produce long-term sensitization in young rats (Fujiwara et al., 1987; Kolta et al., 1990; McDougall, Duke, Bolanos, & Crawford, 1994; Ujike et al., 1995), suggesting that the critical period for behavioral sensitization may be a late-developing effect, one that occurs after the 3rd week of rat postnatal life and corresponds to the period of presynaptic dopamine autoreceptor formation in the rat brain (Hedner & Lundborg, 1985; Murrin et al., 1985). This finding in rodent models is consistent with the generally held belief that prolonged psychostimulant administration does not increase the likelihood of current or future drug abuse among children and adolescents (Klein & Wender, 1995; Levin & Kleber, 1995; Spencer et al., 1996; St. Dennis & Synoground, 1996). In contrast, a study byMcDougall, Collins, Karper, Watson, and Crawford (1999) demonstrated that young rats are capable of exhibiting locomotor sensitization after an abstinence period from repeated administration of comparatively high doses of a potent dopamine reuptake blocker. We have also shown that the locomotor activation 8 weeks following the treatment was not evident in spontaneous activity (see Figure 4). Further, our unpublished preliminary data show that when exposed to repeated morphine administration in adulthood, animals showed no difference in locomotor sensitization whether they were exposed to morphine at an early age (as per protocol described in this article) or not. Taken together, these conflicting data indicate that the occurrence and persistence of behavioral sensitization in young animals may depend on both the amount and the type of psychostimulant. Indeed, dopaminergic neurons of the ventral tegmental area (Bozarth & Wise, 1981; Joyce & Iversen, 1979; Kalivas & Stewart, 1991; Spanagel & Shippenberg, 1993; Vezina & Stewart, 1984) and their terminal field in the nucleus accumbens are critical for locomotor sensitization by morphine (for reviews, see Koob & Le Moal, 2001; Steketee & Kalivas, 2011). Future studies should aim to examine critical time periods in ontogeny of the mesocorticolimbic dopaminergic system, which are known to be involved in reward and addiction of morphine (Bozarth & Wise, 1981).

Long-Term Effect on Novel-Object Recognition Testing

Modulation of learning and memory processes by morphine and other opioids has been demonstrated in adult animals (Bodnar & Klein, 2005; Canli, Cook, & Miczek, 1990). We used a novel-object recognition test as a useful tool for assessing the behavioral and neural processes mediating storage and subsequent recall of the features that compromise the familiar object (Berlyne et al., 1966) under normal, low-stress environmental conditions (Ennaceur & Delacour, 1988). Calculated discrimination ratio following novel-object recognition testing revealed that although compromised in the young age (see Figure 5A′), novel-object recognition showed no differences in adulthood between morphine-treated and control animals (see Figure 5B′). It was reported that chronic exposure to opioids in adult rats results in a residual long-term performance impairment on a memory task (Sala et al., 1994; Spain & Newsom, 1991). Further, because spontaneous morphine withdrawal gives rise to memory impairment, the test has been reported to elucidate memory deficits in morphine-withdrawal settings in the adult rodents (Mesripour, Hajhashemi, & Rabbani, 2008; Rabbani, Hajhashemi, & Mesripour, 2009; Vaseghi, Rabbani, & Hajhashemi, 2013). Our results are novel in assessing the long-term effects of prolonged postnatal morphine exposure in the absence of physical signs of withdrawal. These results suggest that possible neuronal maladaptations related to a novel object recognition at an early age are normalized by adulthood. This lack of discrimination at the younger age is unlikely to be explained by factors such as differences in locomotor efficiency (see Figure 4; locomotor activity test). However, future studies should include analyses that parametrically vary the retention interval given that a long retention interval may increase the subjective similarity between the stimuli (King, Jones, Pearlman, Tishman, & Felix, 2002). This should be evaluated along the stress reactivity (e.g., forced swim test), considering that we evaluated physical and not affective signs of withdrawal during our weaning period. Finally, future behavioral studies (e.g., radial arm maze, Morris water maze, fear conditioning) should be done to strengthen the present results, which are limited to nonspatial working memory.

Translational Aspects and Significance

The association of rat and human developmental stages depends on several endpoints, such as number of brain cells, degree of myelination, brain growth rate, synaptogenesis, and measures related to more contemporary neuroinformatics (Clancy et al., 2001, 2007). In rodents, this critical period of neuronal differentiation and synaptic development is limited to a time window up to a 4th postnatal week (PDs 1–28; De Felipe, Marco, Fairen, & Jones, 1997; Micheva & Beaulieu, 1996, 1997). In humans, the described brain growth spurt characterized with synaptogenesis and accompanied by dendritic and axonal growth, as well as myelination of the subcortical white matter, extends from the last trimester of pregnancy up to the first few years of postnatal life (Huttenlocher & Dabholkar, 1997). In fact, the newborn rat model at PD 7 and PD 14 has been extensively used in relation to early (premature, neonatal, and infant) and childhood development in humans, respectively (McCann, Bellinger, Davidson, & Soriano, 2009; Zhang & Sweitzer, 2008).

Consensus statements on the analgesic treatment of neonatal pain and discomfort suggest the use of prolonged opioid treatment for preterm and term neonates undergoing ventilatory support (Anand et al., 2006). However, our understanding of the effects of such treatment in the developing brain on possible long-term sequelae is limited. Results from the recent Neurological Outcomes and Preemptive Analgesia in Neonate (NEOPAN) trial (Ferguson et al., 2012) are strongly suggestive of long-lasting negative effects. Specifically, body weight (similar to our current animal study) and head circumference were still decreased in the morphine-treated group at 5–7 years of age. Morphine-treated children had more social problems and exhibited increased latencies to choice responses in the short-term memory task. Future studies are needed to translate significance of a novel-object recognition test used in a rat model (see Figure 5) to findings in NEOPAN study cohort of former preterm infants. Unfortunately, no clinical studies are available to provide insight into mechanical/thermal pain perception, addictive potential, or stress coping in the older age.

Conclusions

Administration of opioids for treatment of acute pain and prolonged sedation of newborns and infants is considered standard clinical care. Because this treatment is often associated with a high incidence of opioid tolerance and dependence (Anand et al., 2010), it is necessary to investigate the possible long-term influence of prolonged morphine administration during this early developmental period. Clinical observations of possible long-term effects of prolonged morphine treatment in infants are informed by the use of animal models that permit analysis of potentially neurotoxic effects, the importance of age, and the underlying mechanisms while controlling for handling and maternal separation. Using a rat model of prolonged postnatal morphine exposure, our novel behavioral results demonstrate selective long-term neuroplastic differences in thermal but not mechanical sensory processing. Importantly, our results also suggest a lack of long-term alterations on drug reward/reinforcement behavior, affective processing, and novel-object recognition. Future studies are needed to address the potential neuroplastic alterations at the molecular, cellular, and/or brain networks levels and the translational significance of the animal model findings to clinical sequelae in humans.

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