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Preemptive Morphine Analgesia Attenuates the Long-Term Consequences of Neonatal Inflammation in Male and Female Rats

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ABSTRACT: Despite mounting evidence on the importance of pain management in preterm infants, clinical use of analgesics in this population is limited. Our previous studies have shown that neonatal inflammation results in long-term alterations in adult somatosensory thresholds, characterized by decreased baseline nociceptive sensitivity, and enhanced hyperalgesia after a subsequent inflammatory insult. The present studies were conducted to determine whether preemptive morphine attenuates these negative consequences. At P0, pups received an injection of morphine sulfate before an intraplantar injection of 1% carrageenan. Control pups received either saline (SAL) followed by intraplantar carrageenan, morphine sulfate followed by intraplantar SAL, or SAL followed by intraplantar SAL. Preemptive morphine significantly attenuated neonatal injury-induced hypoalgesia in adolescence and adulthood. Similarly, morphine pretreated animals displayed significantly less hyperalgesia and recovered faster from a subsequent inflammatory insult compared with controls. Neonatal morphine had no significant effect on morphine analgesia in adulthood. Interestingly, neonatally injured animals that did not receive morphine displayed a significant rightward shift in the morphine dose-response cuve in the absence of peripheral inflammation. Together, these results demonstrate that preemptive morphine significantly attenuates the long-term behavioral impact of neonatal inflammatory injury. **(*Pediatr Res* 64: 625–630, 2008)**

Until the late 1980s, many in the medical community believed that neonates were incapable of experiencing pain (1). It is now well established, however, that premature infants are highly responsive to noxious stimulation (2,3) and generate developmentally specific and distinct responses to noxious stimuli (4,5). Nociceptive responses to noxious stimulation have been demonstrated in preterm neonates using an array of physiologic, biochemical, and behavioral measures (6,7). Cortical activation has also been reported in response to noxious stimulation in preterm neonates at 25 wk, suggesting the potential for higher level processing of pain (8,9).

Accumulating evidence indicates that exposure to invasive procedures during the neonatal period leads to both short- and long-term alterations in nociceptive processing (10–13). For example, a higher frequency of invasive procedures in preterm infants has been associated with dampened pain responses at 32 wk of age (14). Decreased facial responsiveness to immunization at 4 and 8 mo (15), and blunted pain sensitivity has also been reported in former preterm neonates (10).

Animal studies have also reported that neonatal injury induces long-term alterations in somatic and visceral sensitivity (12,13,16). In particular, animals that received intraplantar carrageenan (CGN) at birth display significant hypoalgesia in the previously injured and uninjured paws in adulthood (12,13). Similar changes in nociceptive sensitivity have also been reported using intraplantar formalin (17). Neonatal injury also results in enhanced hyperalgesia after a subsequent insult in adulthood and significantly decreases the rate of recovery (12,13).

The use of opioid analgesics in the treatment of neonatal pain is widely debated. Accumulating data, however, suggest that premature infants undergoing invasive procedures benefit from the use of opioid analgesics (18,19). For example, altered pain responses in former preterm neonates can be predicted by the number of previous painful procedures and are normalized by the use of morphine (14). Moreover, postoperative morphine in preterm and full-term infants reduces behavioral and hormonal stress responses (20,21), and is associated with decreased mortality (18).

There is evidence that opioid analgesics are efficacious in neonatal rodents (22). Few studies, however, have examined whether opioid analgesics can be used to prevent the consequences of neonatal injury (23). Therefore, the present studies were conducted to determine whether neonatal morphine 1) attenuates neonatal injury-induced hypoalgesia in adulthood; 2) reduces enhanced hyperalgesia observed after adult inflammation and impacts the rate of recovery; and 3) alters the response to morphine in adulthood.

METHODS

Animals. Time-pregnant Sprague-Dawley rats were obtained on the 14th day of gestation (E14) (Zivic Miller) and housed individually. Animals were maintained on a 12:12 h light:dark cycle, with food and water available *ad libitum*. Offspring from the litters were combined on the day of birth, divided up equally, and randomly assigned to a neonatal treatment condition and dam. All litters were reared identically, weaned at P21, and housed with same-sex littermates in groups of 2–3. All experiments were approved by the Georgia State University Animal

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Abbreviations: CFA, Complete Freund's adjuvant; **CGN,** carrageenan; **MDS,** mean difference score; **MOR,** mu opioid receptor; **MOR-LI,** mu opioid receptor-like immunoreactivity; **MS,** morphine sulfate; **P,** postnatal day; **PWL,** paw withdrawal latency; **SAL,** saline

Care and Use Committee and were conducted in compliance with the guidelines established by the International Association for the Study of Pain.

Early life manipulations. On the day of birth (P0), male and female rat pups received MS (MS; 2 mg/kg, i.p.) or SAL 15 min before a unilateral intraplantar injection of 1% CGN (5 μL) or SAL. This dose of morphine was chosen based on previous studies (22,24). In preliminary studies, we noted that paw inflammation peaked at 5 h post-CGN. Therefore, at 5 h post-CGN or SAL, a second dose of either MS or SAL was administered. This resulted in a total of four groups: MS+SAL, MS+CGN, SAL+CGN, and SAL+SAL. All pups within a litter received the same neonatal treatment.

Maternal behavior. Maternal behavior was observed daily at 18:00 h for 60 min from P0–P7. Maternal observations were conducted by both direct observations and by videotape for later offline analysis. Specific maternal behaviors were recorded including pup licking/grooming, nursing posture (crouching), pup retrieval, nest construction, eating/drinking, exploring, inactive/napping, and self-grooming. Observations were conducted by an individual blind to the neonatal group assignment.

Baseline nociceptive behavior. On postnatal days 40 and 60 (P40 and P60), baseline paw withdrawal (PWL) and tail flick (TFL) latencies in response to noxious thermal and mechanical stimuli were determined. Thermal testing was conducted using the Paw Thermal Stimulator (UCSD, CA). In this test, animals were placed in a clear plastic testing chamber on a glass surface and allowed to acclimate for 30 min before testing. A radiant beam of light beneath the glass base was directed at the plantar surface of the each hindpaw or 1.5 inches from the distal end of the tail, and the withdrawal latency was electronically measured (25). To avoid potential tissue damage, a 20-s automatic termination of the heat stimulus was imposed if withdrawal did not occur. Mechanical testing was conducted using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Animals were placed in Plexiglas chambers above a wire mesh, and allowed to acclimate for 30 min before testing. A metal probe was directed at the hind paw and the force applied electronically increased until PWL occurred. Both time and force were recorded, with an automatic cutoff of 60 s and 50 g, respectively. Male and female rats were tested separately. For both tests, the average response of three trials was taken; all trials were separated by a 5-min intertrial interval.

Nociceptive behavior after re-inflammation. After baseline PWL determination at P60, animals received an intraplantar injection of Complete Freund's adjuvant (CFA; 1:1 CFA: SAL solution; 200 μL; Sigma Chemical Co.) into the neonatally injured hindpaw. CFA was used for re-injury as neonatally injured animals may potentially develop antibodies against CGN, thereby limiting its potency for use as an inflammatory agent. Twenty four hours after CFA, paw diameter and PWLs were tested as aforementioned.

Recovery after re-inflammation. Paw diameter and PWLs were tested at 7, 14, and 21 d after CFA re-inflammation (P60) to determine the impact of morphine pretreatment on the response to recovery from a subsequent insult in adulthood.

Response to morphine in adulthood after neonatal morphine exposure. The effect of neonatal morphine on adult morphine responses was tested in the absence of peripheral inflammation. After baseline PWL determination, animals were administered increasing cumulative doses of morphine (1.8, 1.4, 2.4, 2.4, 2.0, and 8.0 mg/kg s.c. resulting in quarter log doses of 1.8, 3.2, 5.6, 8.0, 10.0, and 18.0 mg/kg; Sigma Chemical Co.) (26). PWLs were determined 15 min after each injection, followed by administration of the subsequent injection 5 min later.

Mu opioid receptor western blot analysis. Western blot analysis was conducted to confirm that mu opioid receptors (MOR) are present centrally and peripherally on P0. Five hours after birth, rat pups were rapidly decapitated and brain, spinal cord, and paw tissue were removed and collected on dry ice. Dams were decapitated and maternal blood samples taken. Tissue and blood were stored at −80°. Tissue samples were homogenized in Homogenization Buffer (50 mM HEPES pH 7.4, 1 mM EDTA, and 0.001% Protease Inhibitor Cocktail; Sigma Chemical Co., USA) on ice. The samples underwent freeze/thawing three times on dry ice and 37°C waterbath, respectively, and were stored at −80°C. Sample protein concentrations were determined with a BCA Protein Assay Kit (Pierce) against a BSA standard curve. Next, 20 μg of each sample was run on 12% acrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). PVDF membranes were blocked in 5% nonfat dry milk in TTBS (0.1% Tween in 20 mM Tris-HCl; pH 7.5, 175 mM NaCl) overnight at 4°C. PVDF membranes were washed three times with TTBS, 10 min each, then incubated with rabbit anti-MOR (1:50,000; Sigma Chemical Co.) and rabbit anti-GAPDH (1:300,000; Covance) in 2% nonfat dry milk in TTBS overnight at 4°C. Membranes were washed three times with TTBS, 10 min each, and incubated with anti-rabbit HRP conjugated antibodies (1:3000; Cell Signaling) for 1 h. Membranes were subsequently washed with TTBS three times, 10 min each, and washed in TBS (20 mM Tris-HCl pH 7.5, 175 mM NaCl) for 30 min. Bands were visualized *via* chemiluminescence using LumiGLO (KPL). To confirm that the MOR protein observed in the tissue samples was not the result of mu receptors located on maternal immune cells, western blot analysis was also conducted on maternal blood samples.

Statistical analysis. Data are expressed as either raw withdrawal latencies or mean difference scores (MDS). All values are reported as Mean ± SEM. Data were analyzed for significant main effects of neonatal treatment and sex using ANOVA and repeated-measures ANOVA was used to analyze recovery post-CFA data; $p < 0.05$ was considered statistically significant. *A priori* specified *post hoc* tests were conducted using the method of Sheffe as warranted to determine significant mean differences. Where multiple comparisons were made, p values were adjusted accordingly using the Bonferroni method. Morphine dose-response curves were plotted and the half maximal effective doses (ED50) were calculated using percent maximal possible effect (%MPE) data, defined as [(Dose mg/kg-baseline PWL)/(20.0-baseline PWL)]100 (GraphPad, Prism). Analysis of variance was used to compare differences between ED50 values.

RESULTS

Neonatal morphine attenuates hypoalgesia in adulthood. Preemptive morphine significantly attenuated both thermal (Fig. 1) and mechanical (Fig. 2) hypoalgesia induced by neonatal inflammation at P60. Animals that received SAL+CGN displayed significant thermal hypoalgesia in both the injured and uninjured paws, with females displaying PWLs of approximately 14 s and males 11.5 s ($p < 0.05$ treatment; $p < 0.05$ sex), *versus* PWLs of 10 s in SAL+SAL animals. In contrast, morphine pretreated animals (MS+CGN) were not significantly different from uninjured controls. Consistent with our previous study (13), a significant

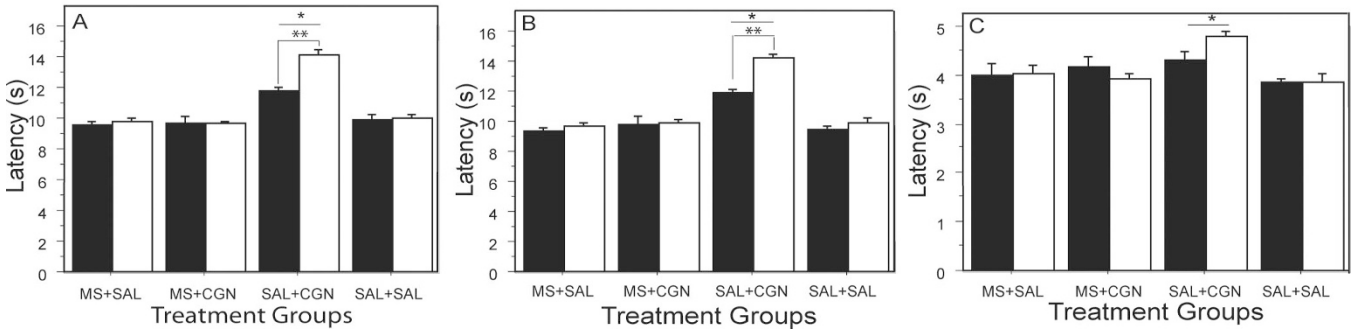

Figure 1. Morphine attenuates thermal hypoalgesia in adulthood. MS+CGN animals displayed significantly shorter PWLs compared with SAL+CGN in response to thermal stimulation applied to the (*A*) uninjured paw [$F(3,73) = 61.330$, $p < 0.0001$]; (*B*) injured paw [$F(3,73) = 80.066$, $p < 0.0001$]. (*C*) MS+CGN animals displayed significantly reduced TFLs compared with SAL+CGN animals [$F(3,73) = 5.879$, $p = 0.0012$]. Neonatally injured females displayed significantly longer PWLs compared with males; uninjured paw [$F(1,73) = 8.912$, $p = 0.0039$] and injured paw [$F(1,73) = 16.914$, $p < 0.0001$]. $n = 5$–15 rats per group/per sex; *significant main effect of treatment; **significant main effect of sex; male: ■; female: □.

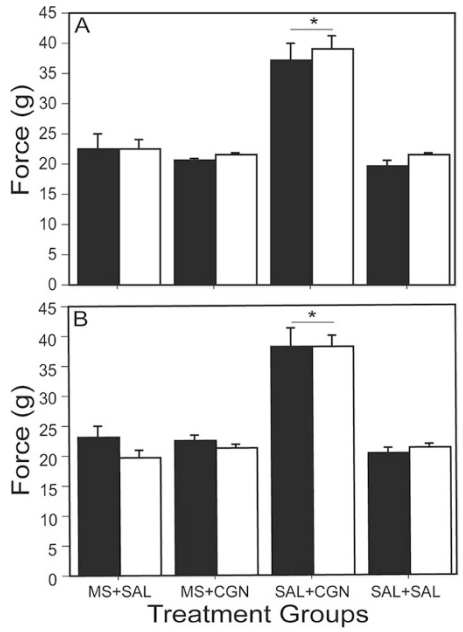

Figure 2. Morphine attenuates mechanical hypoalgesia in adulthood. MS+CGN displayed significantly reduced force (*g*) to withdrawal compared with SAL+CGN in response to mechanical stimulation applied to the (*A*) uninjured paw [$F(3,40) = 50.604$, $p < 0.0001$]; (*B*) injured paw [$F(3,40) = 56.898$, $p < 0.0001$]. $n = 5$–15 rats per group/per sex; *significant main effect of treatment; male: ■; female: □.

main effect of sex on injury-induced thermal hypoalgesia was noted (Fig. 1*A* and *B*). Morphine also reduced the injury-induced hypoalgesia present in the tail at P60 (Fig. 1*C*) ($p < 0.05$). Furthermore, a significant reduction in the mechanical force threshold was present in morphine pretreated animals in both paws in adulthood (P60) (Fig. 2*A* and *B*) ($p < 0.05$). Significant morphine attenuation of the inflammation-induced hypoalgesia was also observed at P40 (data not shown).

Neonatal morphine alters the response to re-injury and recovery. Neonatal morphine significantly attenuated CFA-induced hyperalgesia at 24 h compared with controls (Fig. 3*A*) ($p < 0.05$). At 24 h post-CFA, PWLs for SAL+CGN animals were approximately 2 s in females (MDS, of 12 s), and approximately 3.5 s in males (MDS 8.5 s). Neonatally injured females displayed significantly enhanced thermal hyperalgesia (*i.e.*, greater MDS) at 24 h after intraplantar CFA compared with males as previously reported ($p < 0.05$; Fig. 3*A*) (13). The rate of recovery was also significantly reduced in morphine pretreated compared with control animals (Fig. 3*B*). SAL+CGN animals continued to show signs of hyperalgesia at 14 d ($p < 0.05$; MDS of 1 s in males and 3 s in females). In contrast, MS+CGN animals had completely recovered at this time point (MDS of 0), similar to control animals ($p < 0.05$). Furthermore, a significantly decreased rate of recovery was noted in injured females compared with injured males; where females displayed greater MDS at 7 and 14 d post-CFA ($p < 0.05$).

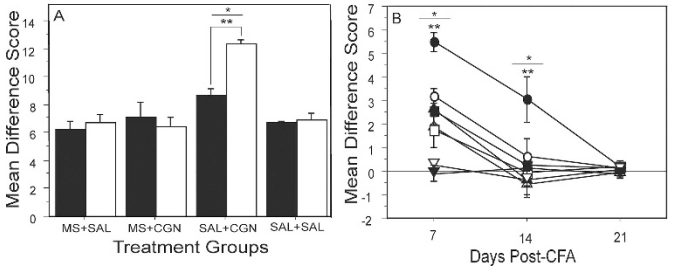

Figure 3. Morphine attenuates the hyperalgesia after re-inflammation and increases the rate of recovery. MS+CGN animals displayed significantly lower MDS (*i.e.*, reduced hyperalgesia) compared with SAL+CGN animals (*A*) 24-h post-CFA [$F(3,36) = 22.524$, $p < 0.0001$]; male: ■; female: □. (*B*) At 7, 14 and 21 d post-CFA, SAL+CGN animals displayed a significantly higher MDS (*i.e.*, reduced rate of recovery) compared with controls [$F(6,76) = 10.644$, $p < 0.0001$]. Females displayed significantly higher MDS (*i.e.*, recovered slower) compared with males [$F(2,76) = 3.240$, $p < 0.05$]. $n = 5$–7 rats per group/per sex; *significant main effect of treatment; **significant main effect of sex; male: ■; female: □ (MS+SAL-Δ; MS+CGN-□; SAL+CGN-○; SAL+SAL-▽).

Neonatal morphine does not affect morphine analgesia in adulthood. Neonatal morphine did not significantly alter morphine's antinociceptive effects in adulthood in males or females (Fig. 4*A* and *B*). As summarized in Table 1, morphine pretreated females (MS+CGN) displayed similar ED50 values as morphine (MS+SAL) and saline (SAL+SAL) control females. Interestingly, a significant shift in ED50 was noted in neonatally injured females (SAL+CGN) that did not receive morphine ($p < 0.05$). Similarly, neonatal morphine in injured males did not alter adult morphine responses (Fig. 4*B*). Nevertheless, SAL+CGN males also displayed a significant shift in ED50 compared with MS+CGN and control males. Finally, females had significantly higher ED50 values compared with males ($p < 0.05$; Table 1), as previously reported (27).

Mu opioid receptors are present at birth. As shown in Figure 5, MOR-like immunoreactivity (LI) is present on P0 in the brain, spinal cord, and right hindpaw tissue. To control

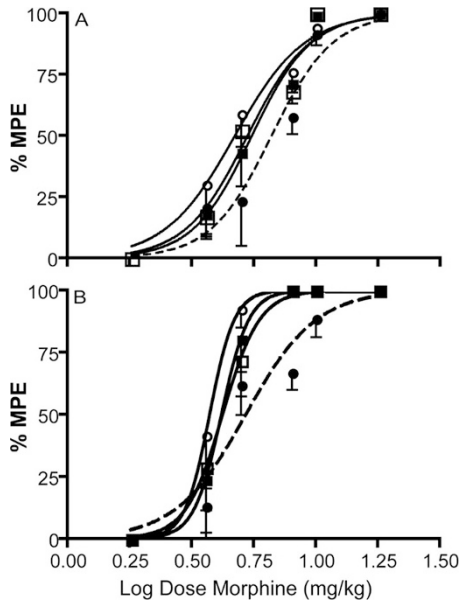

Figure 4. Neonatal morphine has no effect on morphine analgesia. Neonatal morphine had no effect on morphine potency in (*A*) females; or (*B*) males in adulthood. Neonatal injury (SAL+CGN) (- - -) produced a significant shift in the morphine dose-response curve in (*A*) females [$F(3,250) = 2.982$, $p = 0.03$]; and (*B*) males [$F(3,208) = 2.979$, $p = 0.03$] compared with control animals (——). $n = 6$–13 rats per group/per sex; MS+CGN-■; SAL+SAL-○; MS+SAL-□; SAL+CGN-●.

Table 1. *Morphine ED50 values*

Treatment	Female	95% C.I.	Male	95% C.I.
MS+/CGN	5.49	4.82–6.26	4.17*	3.70–4.71
SAL+SAL	4.69	4.16–5.29	3.74*	3.41–4.11
MS+SAL	5.28	4.77–5.84	4.22*	3.76–4.74
SAL+CGN	6.60†	5.54–7.86	5.24*†	4.35–6.32

*$p < 0.05$ significant effect of sex.
† $p < 0.05$ significant effect of treatment.
C.I., confidence interval.

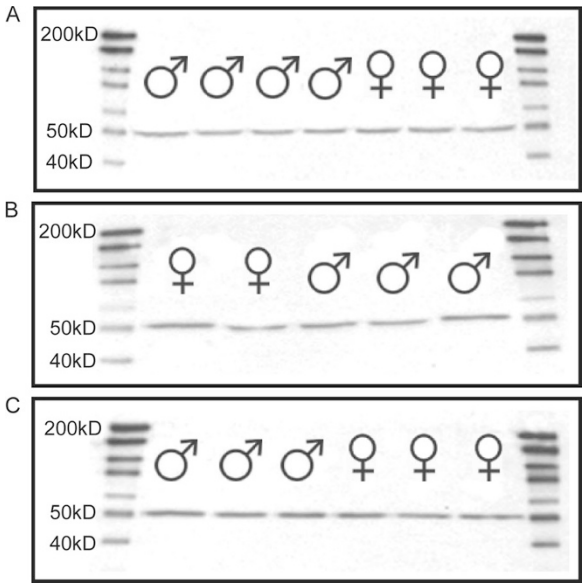

Figure 5. MOR is present centrally and peripherally on P0. After western blot analysis, MOR protein was present in the (*A*) brain; (*B*) spinal cord; (*C*) hind paw on P0.

for the presence of MOR on maternal immune cells, we assessed the presence of MOR-LI on maternal blood samples taken at 5 h postparturition. MOR-LI was undetectable in maternal whole blood or serum (data not shown).

Neonatal injury has no impact on maternal care. There were no significant differences in maternal behavior between morphine pretreated and control animals in the amount of time the dam spent on/with pups (Fig. 6). Similarly, no differences were noted in the amount of time the dam spent off/without pups or in the amount of time spent licking/grooming pups (Fig. 6).

DISCUSSION

Our principal findings are as follows: 1) morphine pretreatment attenuates neonatal injury-induced hypoalgesia in adolescent and adult rats; 2) neonatal morphine reduces hyperalgesia after a subsequent inflammation in adulthood; 3) neonatal morphine increases the rate of recovery; 4) neonatal morphine does not affect morphine analgesia in adulthood; 5) neonatal injury significantly reduces morphine potency.

Neonatal morphine attenuates the consequences of neonatal injury. The present studies are the first to demonstrate that preemptive morphine attenuates the long-term behavioral consequences associated with neonatal intraplantar CGN in rodents. Morphine blocked thermal and mechanical hypoalgesia in the injured and uninjured paws in adolescence and adulthood in both sexes. This is consistent with previous studies in rodents that report daily morphine administration before intraplantar formalin during the first week of life significantly reduces the long-term effects of repetitive pain (22). Similarly, previous studies in humans have reported that morphine ameliorates the effects of early repetitive noxious stimuli in preterm infants at 4 mo of age (14). Moreover, children who had minor neonatal operations and received preemptive analgesia responded to immunization pain in a similar manner as controls (28).

There is ample evidence that the hypoalgesia observed after neonatal inflammation might be mediated by a potentiation in descending endogenous opioid tone. Studies have shown that the functional activity of endogenous opioid systems is enhanced after noxious inflammatory stimulation (29,30). Furthermore, increased endogenous opioid peptide expression and release in the periaqueductal gray after peripheral inflammation has been reported, and is associated with decreased nociceptive sensitivity (31). Moreover, we have reported that systemic naloxone attenuates behavioral deficits in pain responsiveness associated with neonatal inflammation, suggesting that alterations in opioid tone may underlie the observed hypoalgesia (13). Our recent studies have demonstrated that injury-induced alterations in opioid tone primarily involve mu and delta opioid receptors in the periaqueductal gray (LaPrairie J 2007 SFN poster). These results are consistent with findings in former preterm infants (32,33) and rodents treated neonatally with capsaicin (34) that early life trauma may impair the proper development of endogenous inhibitory systems in

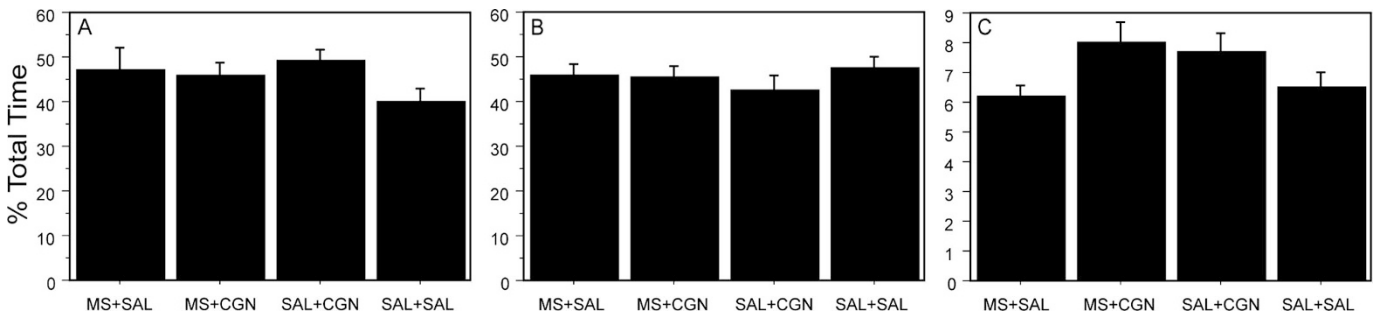

Figure 6. Neonatal injury has no impact on maternal care. Neonatal injury had no effect on the duration of time the dam spent (*A*) on/with her litter $F(3,13) = 0.734$, $p = 0.5503$, (*B*) away from her litter $F(3,13) = 0.393$, $p = 0.7600$, (*C*) licking and grooming pups $F(3,13) = 1.883$, $p = 0.1822$. $n = 3–6$ litters per group.

the CNS. In the present study, the ability of preemptive morphine to block the hypoalgesia may indeed occur through direct modulation of primary afferent drive into the spinal cord, thereby inhibiting the central relay of inflammatory pain and preventing the subsequent increase in descending endogenous opioid tone.

We cannot rule out alterations in the HPA axis as a contributing factor to the observed hypoalgesia. Nociception is one element in a broader context of stress reactivity (35,36), and experimental studies have shown that exposure to early life stressors can permanently increase nociceptive thresholds and decrease the behavioral and physiologic responses to stress in adult rodents (36–38). Data from human preterm infants also suggest that neonatal exposure to noxious stimuli may alter future pain responses. Ex-preterm infants exposed to 4 wk of NICU care display reduced pain responsiveness after heel lance (39). In addition, preterm birth is associated with increased salivary cortisol in response to vaccination at 6 mo of age (40).

Neonatal morphine reduces inflammatory hyperalgesia and increases the rate of recovery after re-injury. In the present study, morphine pretreatment significantly attenuated CFA-induced hyperalgesia and increased the rate of recovery, such that both males and females recovered 7 d faster than nonpretreated animals. Interestingly, preliminary anatomical studies in our laboratory suggest that neonatal inflammation results in increased primary afferent innervation of the dorsal horn reflected by increased expression of calcitonin gene-related peptide (CGRP) and substance P immunoreactivity (LaPrairie JL, Murphy AZ, SfN abstract, 2005), which may account for our observed hyperalgesia. Our working hypothesis is that re-inflammation in adulthood results in an enhanced dorsal horn release of CGRP and/or substance P because of increased primary afferent input. Increased release of these pro-nociceptive peptides would be predicted to result in an enhanced hyperalgesic response. Clinical reports demonstrate that at 32 wk, preterm infants experience a reduced rate of recovery to skin breaking procedures (41), and exhibit subtle differences in ability to recover from finger lance at 4 mo compared with controls (15). There are no reports on the impact of preemptive morphine on recovery rates in premature neonates; however, our data suggest that morphine analgesia may in fact significantly increase the rate of recovery after procedural pain in NICU infants. Neonatal pretreatment with morphine may prevent the injury-induced increase in primary afferent innervation in the spinal cord, thereby blocking the susceptibility of re-inflammation to produce enhanced hyperalgesia and increasing the rate of recovery.

Neonatal morphine does not affect morphine analgesia in adulthood. Our original hypothesis was that exposure to morphine during neonatal development may alter opioid receptor number and/or affinity, thereby altering morphine's antinociceptive effects in adulthood. This hypothesis was based on previous studies that report a 60-fold shift in the morphine dose-response cuve in adult rats that received repeated neonatal morphine in the absence of pain (17). Nevertheless, we show that neonatal morphine did not affect morphine analgesia in adult animals (*i.e.*, no significant shift in ED50 values). In contrast to the previous reports, however, neonatal morphine administration was associated with peripheral inflammation and was only provided on P0 in the present study.

Interestingly, neonatal injury alone resulted in a significant decrease in morphine potency in both males and females in adulthood. These results have serious clinical implications. Previous studies have reported that preterm infants that experience surgery during the first 3 mo of life have significantly higher peri- and postoperative analgesic requirements in response to surgery in the same or different dermatome compared with control infants (21,40). Similarly, mice exposed to chronic noxious stimulation display increased TFLs compared with controls, and a significant 2-fold increase in the ED50 of morphine in response to abdominal constriction (42). As noxious stimulation during the neonatal period leads to increased activation of opioid systems in a manner analogous to the repeated application of exogenous opiates, these studies provide evidence that neonatal injury produces cross-tolerance to the analgesic effects of morphine thereby decreasing the subsequent effectiveness of morphine (43,44). Again interestingly, exposure to *morphine* neonatally did not result in a significant shift in ED50 values. Therefore, we believe that opioid cross tolerance is associated with neonatal injury-induced chronic exposure to endogenous opioids resulting from a potentiation of the descending inhibitory circuit, and not a result of exposure to morphine on P0. Alternatively, neonatal stress associated with maternal separation and repeated handling is also associated with reduced opioid analgesia (36,37). This suggests that alterations in opioid analgesia may reflect a combined effect of neonatal nociceptive experience as well as early life stress, and therefore may involve altered responsiveness of endogenous analgesia circuits and the hypothalamic-pituitary-adrenal axis.

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