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A Single Neonatal Injury Induces Life-Long Deficits in Response to Stress

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Key Words
Early-life pain · Stress · Opioids · Met-enkephalin · Adult · Long-term consequences · Preterm infants

Abstract
Approximately 500,000 infants are born prematurely each year in the United States. These infants typically require an extensive stay in the neonatal intensive care unit (NICU), where they experience on average 14 painful and invasive procedures each day. These procedures, including repeated heel lance, insertion of intravenous lines, and respiratory and gastric suctioning, typically result in an inflammatory response, inducing pain and stress in the newborn. Remarkably, the majority of these procedures are performed in the complete absence of pre- or post-emptive analgesics. Recent clinical studies report that former NICU patients have increased thresholds for pain and stress later in life as compared with term-born infants. However, to date, the mechanisms whereby early-life inflammation alters later-life response to stress and pain are not known. The present studies were conducted to determine if neonatal injury impairs adult responses to anxiety- and stress-provoking stimuli. As we have previously reported that early-life pain results in a significant increase in opioid peptide expression within the midbrain periaqueductal gray, the role of endogenous opioids in our behavioral studies was also examined. Male and female rats received an intraplantar injection of the inflammatory agent carrageenan (1%) on the day of birth. In adulthood, animals were assessed for changes in response to anxiety- and stress-provoking stimuli using the open field and forced swim tests, respectively. Injury-induced changes in sucrose preference and stress-induced analgesia were also assessed. As adults, neonatally injured animals displayed a blunted response to both anxiety- and stress-provoking stimuli, as indicated by significantly more time spent in the inner area of the open field and a 2-fold increase in latency to immobility in the forced swim test as compared to controls. No change in sucrose preference was observed. Using in situ hybridization and immunohistochemistry, we observed a 2-fold increase in enkephalin mRNA and protein expression, respectively, in stress-related brain regions including the central amygdala and lateral septum. Administration of the opioid receptor antagonist naloxone reversed the attenuated responses to forced swim stress and stress-induced analgesia, suggesting the changes in stress-related behavior were opioid-dependent. Together, these data contribute to mounting evidence that neonatal injury in the absence of analgesics has adverse effects that are both long-term and polysystemic.

Introduction

Premature birth, defined as birth prior to 37 weeks of gestation, occurs at alarmingly high rates worldwide. According to the World Health Organization, 16.5% of all

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infants are born prematurely, with over 500,000 preterm babies born each year in the United States alone [1]. Preterm infants spend, on average, 25 days in the neonatal intensive care unit (NICU), where they undergo 10–18 invasive procedures each day, including repeated heel lance, endotracheal intubation, surgery, and respiratory and gastric suctioning (www.marchofdimes.com/peristats/) [2–4]. Despite strong evidence that pain and stress circuitry are established and functional in preterm infants, 65% of these procedures are performed in the complete absence of analgesics [3–7]. Unfortunately, it is becoming increasingly clear that pain experienced during the critical perinatal developmental period has long-lasting effects on adult responses to pain-, anxiety- and stress-provoking stimuli [8–10].

Clinical studies report that children with prior NICU experience display decreases in pain and stress sensitivity that persist long after discharge from the NICU [8, 11]. For example, former preterm infants have significantly decreased nociceptive sensitivity and blunted cortisol responses before and after immunization pain as compared with full-term controls [7, 8, 12, 13]. In middle school, former preterm infants are at least 28% more likely to suffer from disorders of externalization and internalization [14, 15]. At 20 years of age, parents and teachers of former NICU patients report significantly higher rates of neurobehavioral impairments relative to control counterparts [10, 15–17]. The mechanism(s) by which these long-term changes in pain and stress behavior occur in humans are not known.

While previous studies in rodents have reported decreased pain sensitivity following early-life injury, its impact on adult stress responsiveness is not known [18–22]. Therefore, the present studies were conducted to determine whether a single inflammatory insult on the day of birth impairs adult male and female responses to anxiety- and stress-provoking stimuli. We have previously reported that a *single* inflammatory insult (1% carrageenan; hind paw) administered on the day of birth (P0) significantly increases leu- and met-enkephalin protein levels in the ventrolateral periaqueductal gray (vlPAG) of adult rats [23]. Indeed, in both male and female rats, a 180% increase in met-enkephalin protein levels was observed. As enkephalin has been implicated previously in responses to anxiety and stress [24–26], the role of opioid peptides in mediating the effects of early-life pain was examined.

Using behavioral pharmacology, in situ hybridization and immunohistochemistry, we present evidence for the first time that one inflammatory insult experienced on the day of birth attenuates adult responses to stress through an opioid-dependent mechanism. Parallel changes in adult proenkephalin mRNA and met-enkephalin protein expression were observed in the central amygdala (CeA), lateral septum (LS) and vlPAG, regions previously implicated in pain-, anxiety- and stress-related behaviors [27–31].

Materials and Methods

Animals

Pregnant Sprague-Dawley rat dams were obtained on gestational day 14 (Charles River). Dams were housed individually under 12-hour light:12-hour dark cycle with ad libitum access to food and water. On the day of birth (P0), pups were sexed by examination of anogenital distance and subjected to neonatal treatment. All litters were reared identically, weaned on P21 and housed with same-sex littermates in groups of 2–3. Male and female rats were used in all experiments and tested on separate days [22]. All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and were approved by the Georgia State University Animal Care and Use Committee. Behavior experiments were conducted during the light phase (10:00–17:00), animal order was randomized and the experimenter was blinded to neonatal treatment. All experiments were conducted with different cohorts of neonatally injured and handled adults. To ensure participation in previous behavioral experiments did not influence enkephalin expression, separate cohorts of animals were used for the anatomical studies.

Neonatal Treatment

Acute neonatal inflammatory injury was induced as in our previous studies [22, 23]. Briefly, male and female rat pups were injected with 5 μl carrageenan (1% dissolved in saline; Sigma, USA) into the intraplantar surface of the right hind paw or handled identically within 24 h of birth on P0. This well-established model causes acute, local inflammatory pain that persists for 24–72 h and does not alter maternal behavior [20, 22]. Intraplantar saline control was not used, as we noted in our previous studies that this results in 24–48 h of inflammation [22]. Pups were separated from their dam for <20 min and returned to the home cage as a group. Each litter received a single treatment. Animals were largely undisturbed until adulthood (P60).

Test of Anxiety-Like Behavior

Adult anxiety-like behavior was assessed using the open field (OF), a well-established test sensitive to detecting the effects of early-life manipulations on anxiety [32]. Animals (P65–70; n = 6–8/treatment/sex) were habituated to the testing room daily for 60 min, 3 days before and on the day of testing. Adults were gently placed in the OF (gridded Plexiglas box 120 × 120 × 30 cm) facing the same direction. Testing occurred under red light. Each animal experienced the OF one time for 5 min. Behaviors were recorded digitally with Noldus Observer 5.0 (Noldus, USA) and observed remotely with a video monitor. The testing apparatus was cleaned thoroughly with 70% EtOH between each animal; va-

pors were allowed to evaporate completely before the next session commenced. Scoring of anxiogenic (duration in outer perimeter), anxiolytic behaviors (duration in inner area) and locomotor behavior (number of lines crossed) occurred post hoc by an experimenter blinded to neonatal treatment. Data were expressed as duration or frequency.

Tests of Stress-Related Behavior

Adult stress-related behavior was assessed with the modified forced swim test (FST), sucrose preference test and stress-induced analgesia test. Animals (P70–90) were habituated to the testing room daily for 60 min, 3 days before and on the day of testing. For stress-induced analgesia testing, animals were habituated to the paw thermal stimulator daily for 60 min 3 days before and on the day of testing. To avoid any carryover effects, different cohorts of neonatally injured and handled adults were used for each test (FST, sucrose preference test, stress-induced analgesia).

Forced Swim Test

Water was maintained at 25°C and filled to height such that animals could neither escape nor could the tail touch the bottom (63.5 cm) [33, 34]. On day 1 of the FST, adults (n = 8–9/treatment/sex) were placed in a circular swim tank (71.2 × 62.5 × 56 cm) for 5 min pre-swim to elicit 'behavioral despair' [35]. On day 2, animals were placed in the swim tank for a 5-min FST; all behaviors were digitally recorded. Following the FST, animals were dried with a clean towel and placed in clean cages. Fecal boluses were counted and removed from the tank between each test. The tank was cleaned with detergent and EtOH between tests. The following behaviors were scored post hoc: (1) latency to immobility (LTI), defined as the first cessation of swimming with arched-back floating [34, 40]; (2) duration of immobility, characterized by arched-backed floating and movement only necessary to keep the head above water or prevent drowning [34]. A separate cohort of animals (n = 8–9/treatment/sex) received naloxone HCl (1 mg/kg or 5 mg/kg; i.p; Sigma, USA) dissolved in saline (0.9%) or equivolume saline 15 min before testing. Data are expressed as frequency and duration.

Sucrose Preference Test

Animals (n = 9–16/treatment/sex) were singly housed for 7 days prior to testing. Separate water bottles were filled with 500 ml of tap water or sucrose solution (1%), weighed and placed in the cage in randomized order to control against place preference. During the 48-hour test, rats had ad libitum access to both bottles. After the first 24 h, bottles were removed and immediately replaced with new bottles oriented in the opposite order. After the second 24-hour period, bottles were removed. Final bottle weights (WT_F) were subtracted from initial weights (WT_I) yielding a difference score (D) for each animal. Percent sucrose preference was calculated using the following formula: [(WT_D sucrose bottle)/(WT_D sucrose bottle + WT_D H_2O bottle)] × 100, where WT_D = (day 1 $WT_I - WT_F$) + (day 2 $WT_I - WT_F$).

Stress-Induced Analgesia

Immediately before (pre-stress) and after (post-stress) 30 min of restraint in acrylic restraint cylinders, pain threshold was tested using a paw thermal stimulator (fig. 7) (UCSD, USA) [36, 37]. Animals (n = 6/treatment/sex) were placed in clear Plexiglas chambers mounted on the glass surface. A radiant beam of light was focused on the plantar surface of each hind paw and the latency for the animal to withdraw its paw in response to the noxious thermal stimulus (50–51°C) was recorded in seconds as the paw withdrawal latency (PWL) [38]. Average PWL for 3 trials was calculated for each animal. The paw thermal stimulator was set to produce latencies between 8 and 10 s and terminated after 20 s if no withdrawal occurred. Testing chambers and apparatus were cleaned thoroughly with 70% EtOH between sessions; vapors were allowed to evaporate completely before the next session commenced.

Twenty-four hours later, animals received naloxone (5 mg/kg; i.p.) or saline. Fifteen minutes after naloxone, PWL was measured immediately before (pre-stress) and after (post-stress) 30 min of restraint stress as stated. Data are presented in seconds.

Sample Collection

Behaviorally naïve neonatally injured and control animals (P75–80) underwent perfusion fixation for immunohistochemistry (n = 7/treatment/sex) or rapid decapitation for in situ hybridization (n = 5/treatment/sex). Tissue fixation occurred as reported previously [23]. Briefly, animals were given a euthanizing dose of sodium pentobarbital (160 mg/kg) intraperitoneally and perfused transcardially. Heparin sodium (0.1 ml) was injected into the heart to prevent blood coagulation. Blood was removed from the brain with 0.9% sodium chloride and 2% sodium nitrite solution (250 ml). Fixation was achieved using 4% paraformaldehyde in 1 M phosphate buffer containing 2.5% acrolein (350 ml; Polysciences, USA). A final wash of sodium chloride-sodium nitrite solution (250 ml) removed residual acrolein. Brains were stored in 30% sucrose at 4°C until sectioned. Alternatively, animals were placed in a decapicone (VWR, USA) and decapitated with a razor-sharp guillotine. Immediately thereafter, brains were extracted, flash frozen in 2-methylbutane (VWR, USA) chilled on dry ice and stored at –80°C until sectioned.

Immunohistochemistry

Perfusion-fixed brains were sectioned in 1:6 series at 25 μM through the rostrocaudal axis. Sections were removed from the cryoprotectant-antifreeze solution, rinsed extensively in potassium phosphate buffer (KPBS), and reacted for 20 min in 1% sodium borohydride to remove excess aldehydes. Sections were incubated in primary antibody solution directed against met-enkephalin in KPBS containing 1.0% Triton X for 1 h at room temperature, followed by 48 h at 4°C. Met-enkephalin (Lot No. 1004002) immunoreactivity was identified using polyclonal rabbit anti-met-enkephalin antibody at a concentration of 1:50,000 (Immunostar, USA). Staining was completely eliminated by pretreatment with 5 μg met-enkephalin per milliliter diluted antiserum, but not 5 μg leu-enkephalin per milliliter diluted antiserum (manufacturer technical information). For chromagen staining, tissue was rinsed in KPBS, incubated for 1 h in biotinylated goat-anti-rabbit IgG secondary antibody (Jackson Immunoresearch, USA; 1:600) solution containing KPBS and 0.4% Triton X, rinsed again, and incubated for 1 h in 0.009% avidin-biotin-peroxidase complex (ABC Elite Kit; Vector Labs, USA). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), antigens were visualized using nickel sulfate-intensified 3,3-diaminobenzidine solution containing 0.083% hydrogen peroxide in sodium acetate buffer. The reaction was terminated after 20–25 min by rinsing in sodium acetate buffer. Sections were mounted out of KPBS onto gelatin-subbed slides, air dried overnight, dehydrated in a series of graded alco-

hols, cleared in xylene, and coverslipped with Permount. For fluorescent staining, sections were washed in KPBS, incubated for 2.5 h at room temperature in goat-anti-rabbit IgG DyLight 488 secondary antibody (Jackson Immunoresearch, USA; 1:50). Tissue was rinsed in KPBS, mounted as above and immediately coverslipped with VectaShield Hardset (Vector Labs, USA).

Proenkephalin in situ Hybridization

Fresh-frozen brains were sectioned in 1:6 series at 20 μM and mounted on SuperFrost Plus slides (Fisher Scientific, USA). Sections were stored at –80°C until time of assay. Sense probe was hybridized to control for specific binding. Proenkephalin in situ hybridization was performed using oligonucleotide probe as reported previously [39]. Briefly, 50-base oligonucleotide (5′-TCA TCTGCATCCTTCTTCATGAAACCGCCATACC-TCTTGGCAAGGATCTC-3′), complementary to bases 715–764 of the rat proenkephalin mRNA (Genebank accession number NM_017139), was labeled with ^{35}S-dATP at the 3′ end using terminal deoxynucleotidyl transferase. Sections were fixed with 4% paraformaldehyde/PBS, acetylated, and hybridized with the antisense probe. After the hybridization, slides were washed, dried, and exposed to Kodak BioMax MR films (Kodak, USA).

Densitometry

Chromagen immunohistochemistry was quantified in pain- and stress-related regions with a high density of met-enkephalin protein expression according to the bregma and region size defined in Paxinos and Watson, 5th edition: vlPAG (bregma: rostral –6.72 to caudal –8.76), CeA (bregma: rostral –1.44 to caudal –3.24), LS (bregma: rostral 2.28 to caudal –0.48), paraventricular nucleus (PVN; bregma: rostral –0.84 to caudal –2.04), nucleus accumbens (NAcc; bregma: rostral 2.52 to caudal 0.84). For each region of interest (ROI), 12-bit grayscale images of each section were captured with a ×20 objective on a Nikon Eclipse E800 microscope using a QImaging Retiga EXi CCD camera and quantified with iVision Software (BD Biosciences, USA; Apple, USA). For each ROI, 3 sections per animal were sampled randomly. The mean grayscale pixel value was measured from a box of fixed size (vlPAG: 1.5 mm^2; CeA: 1.5 mm^2; LS 2.0 mm^2; PVN 1.0 mm^2; NAcc 2.0 mm^2) and recorded. Measures were corrected for nonspecific binding by subtracting background adjacent to the ROI that lacked immunoreactivity. Mean specific immunoreactivity was reported as the relative optical density. For in situ hybridization data, C-14 microscales (GE Healthcare Life Sciences, USA) were used to create standard curves ($R^2 > 0.99$) for each assay. For each ROI, sections were selected and captured using the above criteria with Scion Image (NIH and Scion Corp., USA), MTI CCD 72 camera and Northern Light box (Imaging Research, Inc., CN). The mean pixel value was recorded and measures were corrected for nonspecific binding by subtracting background adjacent to the ROI that lacked hybridization. Mean specific hybridization was reported as the disintegrations per minute per milligram of tissue (dpm/mg).

Statistical Analysis

Significant main effects of neonatal treatment and sex or neonatal treatment and drug were assessed using 2-way ANOVA or repeated-measures ANOVA. Where effects of sex were not observed, data are collapsed by treatment for simplicity. Student's unpaired or paired t tests were used for post hoc analyses to determine differences between groups. Where applicable, values ≥2 standard deviations from the mean were eliminated as outliers. All comparisons were a priori specified. Confidence was set to $p < 0.05$ and considered statistically significant.

Results

Neonatal Injury and Adult Affective Behaviors

To test the impact of neonatal injury on adult responses to anxiety-provoking stimuli, male and female rats were exposed to the OF. Significant main effects of treatment and sex were assessed using a 2-way ANOVA. Neonatally injured adults spent more time in the inner area than controls ($F_{1, 23} = 50.24$; $p < 0.0001$) independent of sex ($F_{1, 23} = 1.21$; $p = 0.28$) (fig. 1a). Neonatal treatment had no effect on the number of lines crossed ($F_{1, 23} = 1.13$; $p = 0.35$) (fig. 1b) indicating no effect of injury on locomotion. Together, these data suggest that early-life pain dampened behavioral responses to anxiety-provoking stimuli.

To test the effect of neonatal injury on adult stress-related behavior, rats were exposed to the FST. A 2-way ANOVA was used to test for significant main effects of treatment and sex. LTI was significantly increased in neonatally injured adults as compared with controls ($F_{1, 30} = 23.03$; $p < 0.0001$) (fig. 2a), suggesting reduced sensitivity to stress. No significant main effect of sex was observed ($F_{1, 30} < 1$; $p = 0.38$). Neonatally injured adults also excreted significantly less fecal boluses ($F_{1, 30} = 8.84$; $p = 0.0058$) (fig. 2b) consistent with the injury-induced decrease in sensitivity to stress. No significant change in the duration of immobility was observed ($F_{1, 30} < 1.0$; $p = 0.97$) (fig. 2c). Together, these data suggest that early-life pain dampened sensitivity to stress-provoking stimuli.

Neonatal Injury and Anhedonia

The sucrose preference test was used to assess for neonatal injury-induced changes in hedonic state. Significant main effects of treatment and sex were assessed using a 2-way ANOVA. Neonatal injury did not change adult preference for sucrose over water as compared with controls ($F_{1, 49} < 1$; $p = 0.98$). Similarly, no effect of sex was observed ($F_{1, 49} = 2.10$; $p = 0.15$) (fig. 2d). These results suggest that early-life pain had no impact on an animal's hedonic state.

Enkephalin mRNA and Protein Increase in Pain and Stress-Related Brain Regions

To test whether neonatal injury increased adult enkephalin mRNA in pain- and stress-related brain regions, density of in situ hybridized proenkephalin was measured

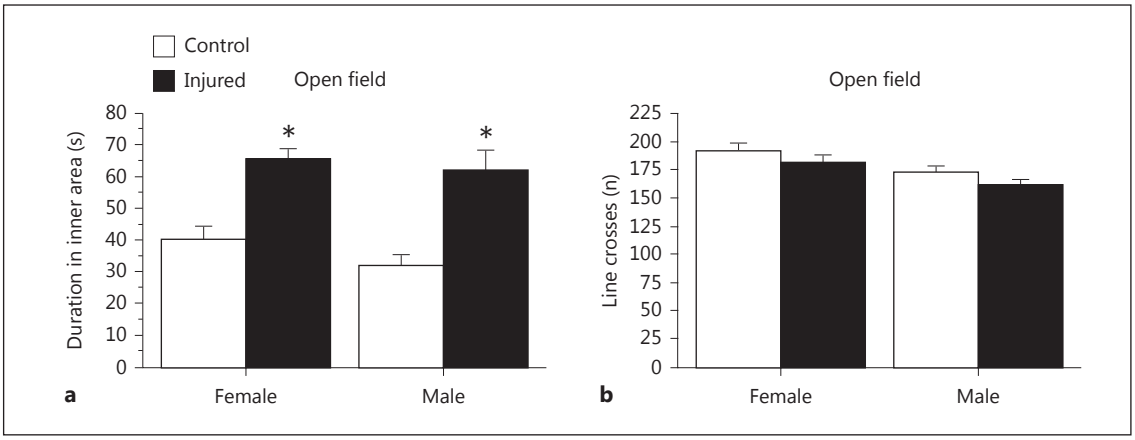

Fig. 1. Neonatal injury increases adult anxiolytic behaviors. **a** Duration spent in the inner area of the OF was significantly increased by neonatal injury for both females and males similarly. **b** Locomotor behavior, as measured by the number of lines crossed in the OF, was not affected by neonatal injury. Data are shown as 2-way ANOVA (mean ± SEM); n = 6–8 subjects per group. Significant main effect of treatment was observed in **a**. * $p < 0.05$: significant group differences as measured post hoc by Student's t test.

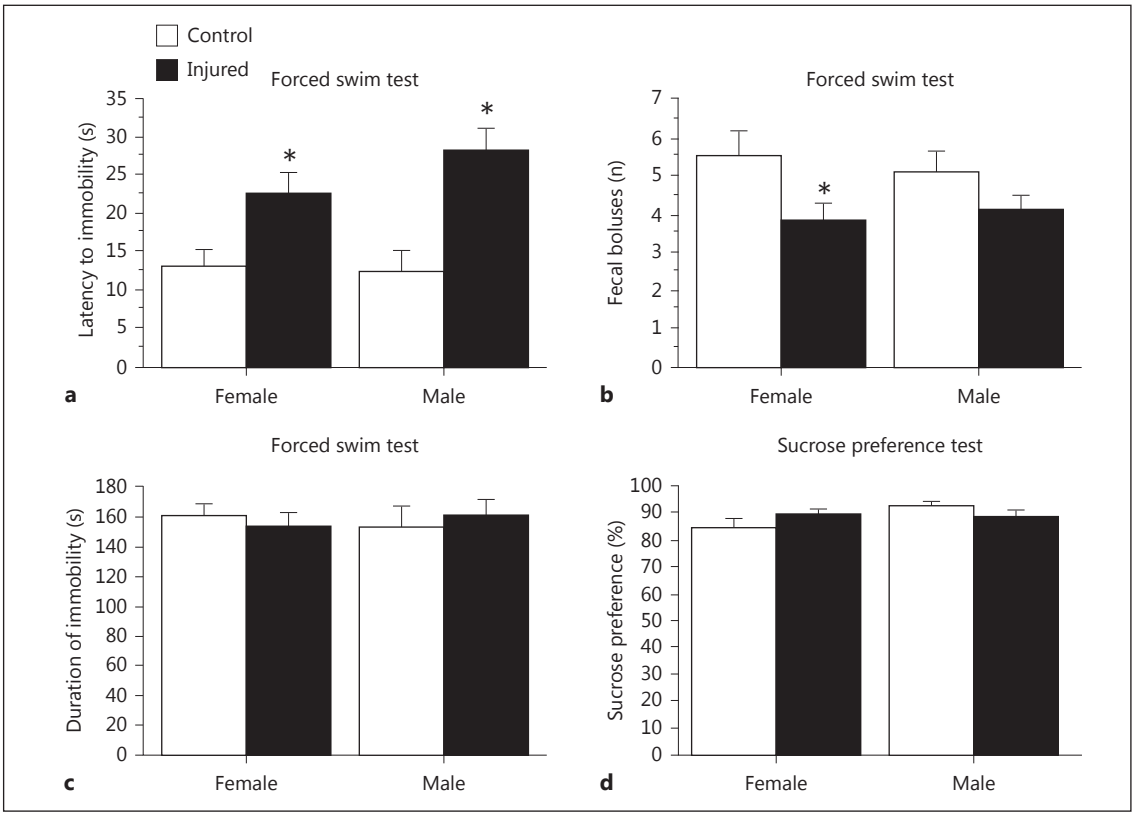

Fig. 2. Neonatal injury increases adult threshold for stress but does not produce anhedonia. **a** LTI, the first display of immobile behavior in the FST, was significantly increased by neonatal injury in females and males similarly, relative to handled controls. **b** Number of fecal boluses excreted during the 5-min FST were significantly reduced by neonatal injury. **c** Duration of immobility in the FST was not affected by neonatal injury. **d** Percent preference for 1% sucrose solution over a 48-hour period was not changed by neonatal injury. Data are shown as 2-way ANOVA (mean ± SEM); n = 8–9 subjects per group in FST; n = 9–16 subjects per group in the sucrose preference test. Significant main effect of treatment was observed in **a** and **b**. * $p < 0.05$: significant group differences as measured post hoc by Student's t test.

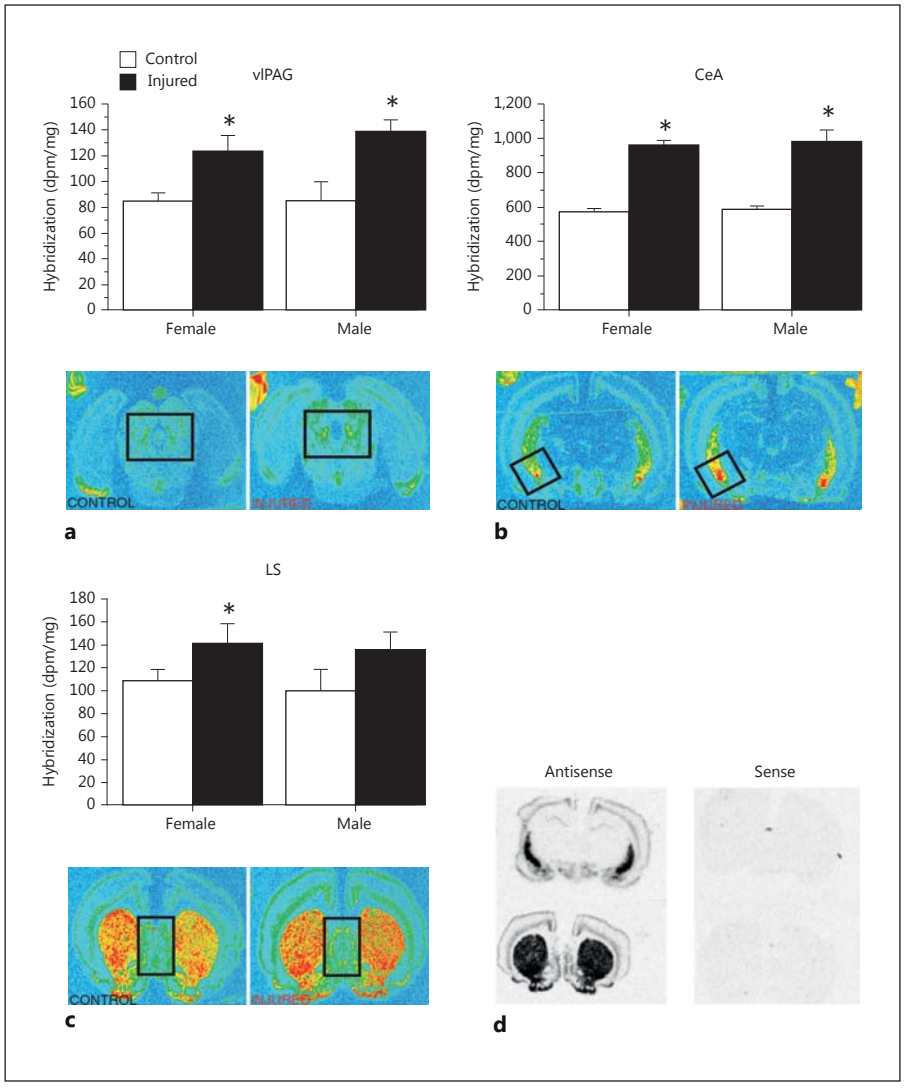

Fig. 3. Neonatal injury increases adult expression of proenkephalin mRNA in pain- and stress-related brain regions. Proenkephalin mRNA was visualized on film via in situ hybridization using an oligo-proenkephalin probe. Relative optical density of proenkephalin mRNA was significantly increased by neonatal injury in the mid through caudal vlPAG (**a**), CeA (**b**), and LS (**c**). **d** Specificity of oligo-antisense probe for proenkephalin sequence as demonstrated by lack of hybridization with sense control. ^{35}S disintegrations per minute per milligram tissue (dpm/mg). Data are shown as 2-way ANOVA (mean ± SEM); n = 5 subjects per group. Significant main effect of treatment was observed in **a–c**. * $p < 0.05$: significant group differences as measured post hoc by Student's t test.

in the vlPAG, CeA, LS, NAcc and PVN (fig. 3). These regions were selected as they contain high levels of enkephalin and have been previously implicated in an organism's response to stress [24, 27, 41]. Significant main effects of treatment and sex were assessed using a 2-way ANOVA. Neonatal injury significantly increased proenkephalin mRNA expression in the vlPAG (56%; $F_{1, 16} = 18.12$; $p < 0.0006$), CeA (66%; $F_{1, 16} = 96.80$; $p < 0.0001$) and LS (32%; $F_{1, 16} = 4.51$; $p = 0.049$) as compared with controls. No change in proenkephalin mRNA was observed in the NAcc ($F_{1, 16} < 1$; $p = 0.47$) or PVN ($F_{1, 16} = 3.33$; $p = 0.087$) (data not shown). Sex differences were not observed in any of the brain regions examined.

To test whether the injury-induced increase in proenkephalin mRNA expression increased met-enkephalin protein, density of protein immunoreactivity was measured in the above ROIs (fig. 4). Two-way ANOVA was used to test for significant main effects of treatment and sex. Neonatal injury significantly increased the expression of met-enkephalin protein in the vlPAG (138%; $F_{1, 24} = 180.81$; $p < 0.0001$), CeA (101%; $F_{1, 24} = 136.83$; $p < 0.0001$) and LS (55%; $F_{1, 24} = 34.78$; $p < 0.0001$) as compared to controls. No change in met-enkephalin protein was observed in the NAcc ($F_{1, 24} = 1.10$; $p = 0.30$) or PVN ($F_{1, 24} < 1$; $p = 0.68$) (data not shown). Consistent with proenkephalin mRNA expression, sex differences in met-enkephalin protein expression were not observed in any of the brain regions examined.

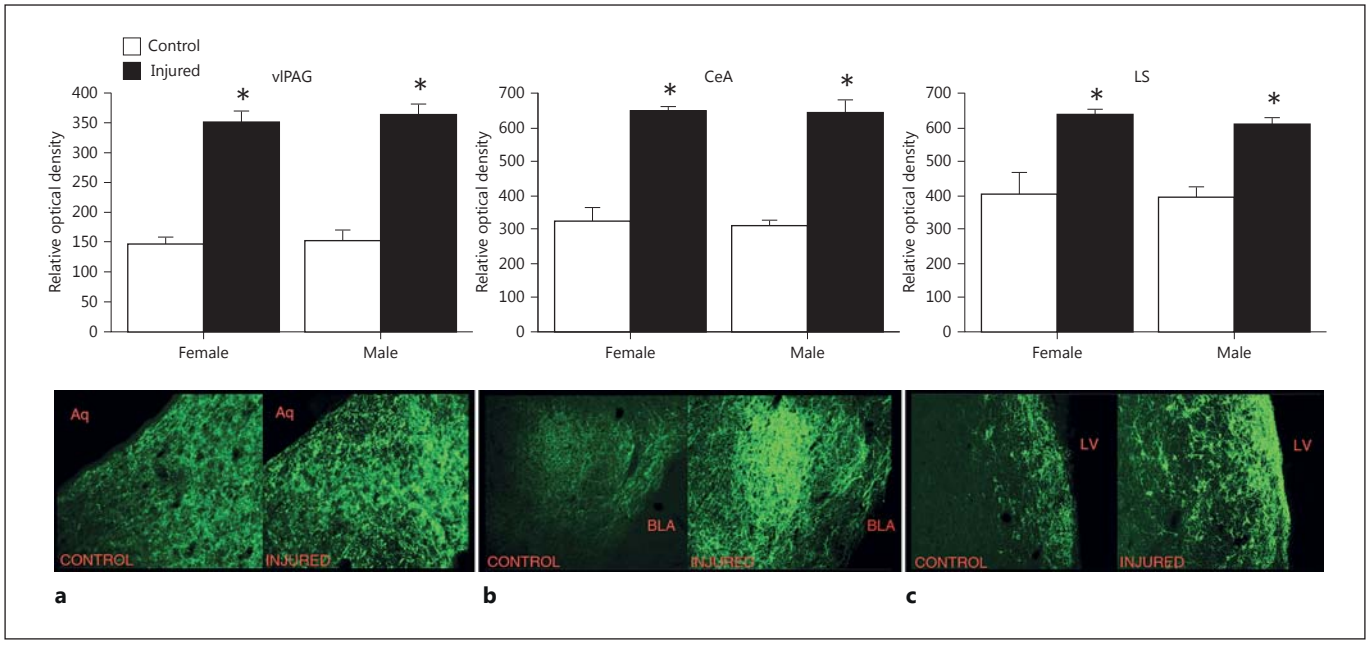

Fig. 4. Neonatal injury increases adult met-enkephalin protein immunoreactivity in pain- and stress-related brain regions. Consistent with proenkephalin mRNA expression, relative optical density of met-enkephalin immunoreactivity was significantly increased by neonatal injury in the mid through caudal vlPAG (**a**) consistent with our previous reports, CeA (**b**), and LS (**c**). Aqua-duct (Aq), basolateral amygdala (BLA), lateral ventricle (LV). Data are shown as 2-way ANOVA (mean ± SEM); n = 7 subjects per group. Significant main effect of treatment was observed in **a–c**. * $p < 0.05$: significant group differences as measured post hoc by Student's t test.

Opioids Are Necessary for Impaired Stress Response

Our anatomical data demonstrated a significant increase in enkephalin expression in neonatally injured animals. Therefore, we next determined whether the increase in stress threshold observed in the FST was opioid-mediated. Rats (n = 8–9/treatment/sex) were given the opioid receptor antagonist, naloxone HCl intraperitoneally 15 min before the FST (fig. 5a). As no significant effect of sex was observed in our previous studies (fig. 2), data are collapsed across sex and analyzed for significant main effects of treatment and drug using a 2-way ANOVA. Systemic naloxone significantly reduced LTI of neonatally injured adult rats in comparison to vehicle control injured rats (drug: $F_{2, 43} = 9.58$; $p = 0.0004$) (fig. 5a). Importantly, LTI of neonatally injured adults was similar to control levels in the presence of 1 or 5 mg/kg naloxone (fig. 5a). Again, we observed no effect of treatment ($F_{1, 43} < 1$; $p = 0.51$) or drug ($F_{2, 43} < 1$; $p = 0.97$) in the duration of immobility (fig. 5b) and no significant changes in fecal boluses were observed (data not shown).

Neonatal Injury Impairs Adult Stress-Induced Analgesia

It is well established that acute stress activates neural systems that inhibit pain. As our data show that neonatal injury decreases adult sensitivity to stress (fig. 2, 5), we next assessed whether adult stress-induced analgesia was altered by early-life pain (fig. 7). PWL was measured using a paw thermal stimulator before and after 30 min of restraint stress. No effect of sex was observed so data are collapsed. Restraint stress significantly increased PWL from baseline in the left paw of controls, as compared with injured adults (repeated-measures ANOVA: $F_{1, 22} = 5.58$; $p = 0.028$; fig. 6a). Specifically, PWL for controls increases by 86% from baseline indicating the induction of stress-induced analgesia (post hoc paired t test: $t_{11} = 8.25$; $p \leq 0.0001$; fig. 6a). By contrast, neonatally injured adult rats did not display stress-induced analgesia (post hoc paired t test: $t_{11} = 1.53$; $p = 0.16$; fig. 6a) (PWL before stress 11.4 vs. 12.9 s after stress; 14% change from baseline). A similar trend was observed in the right paw, such that PWL increased by 88% for controls, but only 44% for injured adults following restraint (data not shown). Data

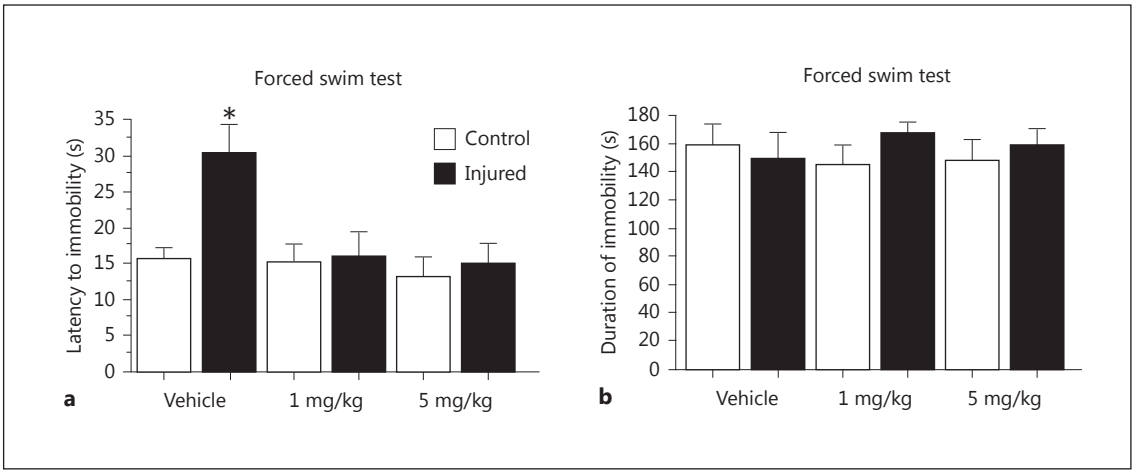

Fig. 5. Naloxone attenuates the injury-induced increase in adult stress threshold. **a** Neonatally injured adults given vehicle had significantly increased LTI as compared with controls. Injured adults given naloxone HCl (1 mg/kg or 5 mg/kg; i.p.) 15 min before FST show significantly reduced LTI such that LTI became similar to controls. **b** Duration of immobility in the FST was not affected by naloxone treatment. Data are shown as 2-way ANOVA (mean ± SEM); n = 8–9 subjects per group. Significant main effects of treatment and drug were observed in **a**. * $p < 0.05$: significant group differences as measured post hoc by Student's t test.

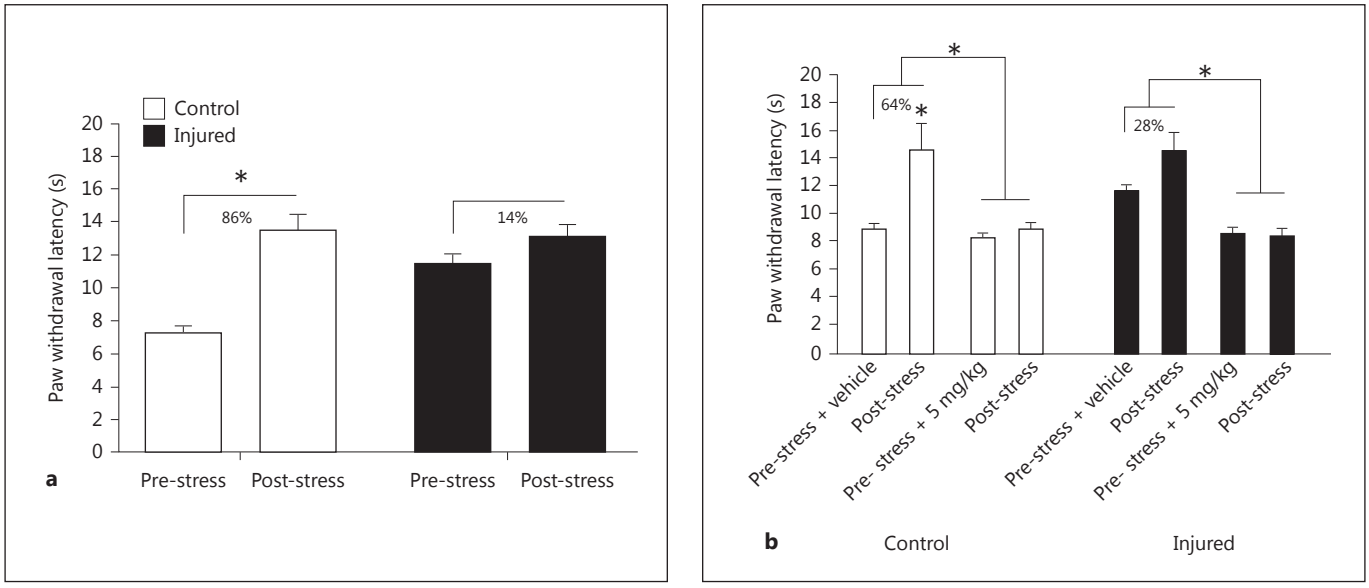

Fig. 6. Neonatal injury impairs adult stress-induced analgesia through an opioidergic mechanism. **a** PWL before and after 30 min of restraint stress. Restraint significantly increased PWL in controls but not neonatally injured adults. **b** Naloxone HCl (5 mg/kg) significantly reduced PWL in neonatally injured adults similarly to controls demonstrating that stress-induced analgesia is opioid-based. As in **a**, neonatally injured adults given vehicle did not exhibit stress-induced analgesia as compared with controls. Data are shown as repeated-measures ANOVA (mean ± SEM) for the left paw as the same trend was observed in the right paw; n = 6 subjects per group. Significant main effect of treatment was observed in **a** and **b**. * $p < 0.05$: significant group differences as measured post hoc by a paired Student's t test.

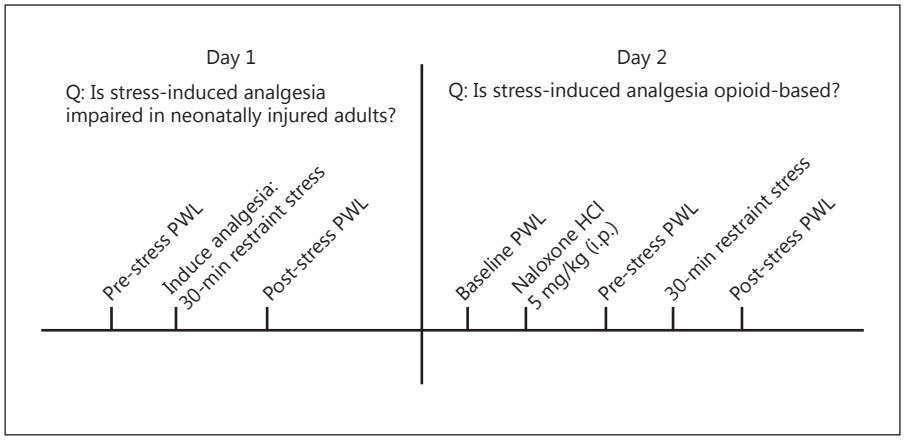

Fig. 7. Protocol for testing the effect of neonatal injury on adult stress-induced analgesia.

are collapsed by neonatal treatment, as no effect of sex was observed for the change in PWL from baseline in either the left (repeated-measures ANOVA: $F_{1, 20} < 1$; $p = 0.84$) or right paws (repeated-measures ANOVA: $F_{1, 20} < 1$; $p = 0.53$).

To determine whether neonatal injury impaired stress-induced analgesia through an opioid-dependent mechanism, naloxone HCl (5 mg/kg; i.p.) was administered 15 min prior to restraint stress. Naloxone completely blocked stress-induced analgesia in the left (repeated-measures ANOVA: $F_{3, 20} = 5.078$; $p = 0.0089$) and right (repeated-measures ANOVA: $F_{3, 20} = 3.41$; $p = 0.038$; data not shown) paw of controls and neonatally injured adults (fig. 6b). Consistent with figure 6a, vehicle-treated controls, but not neonatally injured adults, displayed stress-induced analgesia.

Discussion

The present studies were conducted to determine whether a single inflammatory insult administered on the day of birth impacted adult responses to stress- and anxiety-provoking stimuli through an opioidergic mechanism. Our results show that neonatally injured adults had significantly decreased anxiety-like behaviors and decreased sensitivity to stress as compared with controls. Administration of the opioid antagonist naloxone HCl attenuated stress thresholds in both the FST and stress-induced analgesia test, suggesting the opioid system is necessary for the observed deficits in stress responsiveness. Neonatally injured adults showed significantly increased proenkephalin mRNA and met-enkephalin protein expression in the vlPAG, CeA, LS but not in the NAcc or PVN relative to controls. These data are the first mechanistic demonstration that early-life inflammatory pain changes adult responses to stress through alterations in the endogenous opioid system. Moreover, these data contribute to the growing number of animal studies addressing issues surrounding early-life pain, analgesia and anesthesia and their long-term consequences [19, 42–46].

A Single Neonatal Injury Decreases Behavioral Sensitivity to Aversive Stimuli

Neonatal injury impacted adult anxiety-like behavior in the OF such that adults spent significantly more time in exposed areas than controls, independent of locomotor behavior. These data suggest that injury on P0 significantly decreases adult anxiety-like behaviors or sensitivity to anxiogenic stimuli. In the FST neonatally injured adults had a significantly higher threshold for stress, taking approximately 10–15 s longer (97%) than controls to become immobile. Administration of the opioid antagonist naloxone attenuated the stress threshold of neonatally injured adults such that it was similar to controls, suggesting that our observed decrease in sensitivity to stress-provoking stimuli occurs through an opioid-dependent mechanism. In parallel, neonatally injured adults excreted less fecal boluses in the FST. As opioids are known to regulate behaviors in the FST and decrease colonic motility, these data are consistent with increased expression of endogenous opioids [47, 48].

We hypothesize that the observed changes in anxiety- and stress-related behaviors result from injury-induced augmentation of the enkephalinergic system. In support, viral overexpression of preproenkephalin or direct administration of enkephalin potentiates the anxiolytic effects of

benzodiazepines, increases time in the open arms and blocks swim stress-induced anxiety in the elevated plus maze [49, 50]. Conversely, knockout of the enkephalin gene in mice increases anxiety-like behavior in the OF, suggesting that enkephalin has anxiolytic properties [25, 49, 50].

Importantly, our data are consistent with clinical studies showing that former NICU patients display significantly reduced sensitivity to stress, suggesting early-life trauma reduces sensitivity to aversive stimuli [8, 10, 11, 15]. Reports also show that former preterm infants experience impairments of self-concept relative to their environment, altered ability to externalize, reduced ability to adapt and cognitive inflexibility [15, 17]. Although we cannot specifically delineate many of these behaviors in rodents, increased exploration into an open area exposed to predation or reduced behavioral sensitivity to acute or severe stressors can be likened to insensitivity to salient cues in the environment, inattention and impaired ability to adapt.

Neonatal Injury Mitigates Stress-Induced Analgesia

Consistent with our previous studies, we found that neonatal injury resulted in a significant increase in basal pain sensitivity [22, 23]. Further, we now report that injury on the day of birth attenuates restraint stress-induced analgesia by greater than 100% in adults in comparison to controls. This impairment in stress-induced analgesia was observed in both the neonatally injured and uninjured paws of adults relative to controls. Systemic naloxone HCl prevented stress-induced analgesia [51], suggesting that changes in the endogenous opioid system are responsible for dysregulating normal functioning of the stress system. These findings are consistent with our previous studies showing opioid-dependent increases in basal pain threshold [22, 23], and threshold for forced swim stress.

Previous studies have reported that early-life stress in mice decreases adult stress-induced analgesia [52]. Furthermore, our observed impairment is consistent with clinical reports of attenuated stress-induced analgesia in adolescents and teens that experienced burns early in infancy [9]. Consistent with our observed increase in endogenous opioid tone, high levels of enkephalin are known to dampen the perception of noxious or aversive stimuli, including pain associated with formalin inflammation, anxiety in the elevated plus maze and fecal boluses excreted in response to immobilization stress [50, 53, 54]. In the context of our observed increases in anxiolytic behaviors and opioid-dependent increases in stress and pain thresholds [22, 23], our stress-induced analgesia data are consistent with a general hyposensitivity to noxious or aversive stimuli.

Neonatal Injury Site-Specifically Augments Expression of Enkephalin

The most profound increases in proenkephalin mRNA and met-enkephalin protein occurred in the vlPAG, CeA and LS. Increases in enkephalin expression in these regions provide potential neuroanatomical substrates for impaired responses to stress observed in our neonatally injured adults. Increases in mRNA and protein expression in each region occurred in parallel, and with similar magnitude, suggesting changes in expression are maintained through transcriptional or post-transcriptional epigenetic mechanisms. These finding are in line with other models of early-life perturbation showing concomitant, unidirectional changes in mRNA and protein in adult animals [55, 56]. For example, preproenkephalin mRNA and met-enkephalin protein increase significantly in stress-related brain regions, including the CeA, in response to perinatal stress induced by dam restraint or pup exposure to an infanticidal adult male rat [41, 57]. Overexpression of enkephalin or direct administration of an enkephalin analogue into the CeA dampens anxiety in the elevated plus maze and produces naloxone-reversible analgesia [49, 50, 53]. Conversely, loss of proenkephalin increases anxiety-like behavior, as reflected by a decrease in the number of inner area entries into the OF, decrease in latency to attack an intruder and increase in startle amplitude in response to acoustic perturbations [25, 26]. Collectively, these data suggest enkephalin is critical for reducing responses to anxiety- and stress-provoking stimuli.

The contribution of other endogenous opioids, such as β-endorphin and leu-enkephalin, to the long-term changes in affective behavior we observed cannot be ruled out. Previously, we reported that neonatal injury increases β-endorphin in the vlPAG, but fiber number was low and distribution sparse [23]. Injury also increases leu-enkephalin [23]. However, preproenkephalin is known to yield four times more met-enkephalin protein as compared with leu-enkephalin [58].

Our working hypothesis is that early-life pain experienced during this critical period of development (P0–8 [22]) increases afferent drive to brain regions responsive to noxious input (e.g. vlPAG, CeA and LS). This increase in afferent nociceptive drive results in the activation of supraspinal pain and stress circuits [23, 59, 60]. Subsequent release of endogenous opioid peptides dampens perception, produces analgesia and promotes recovery from the inflammatory insult. As the inflammation associated with intraplantar carrageenan persists for 24–72 h, the release of endogenous opioids is sustained. It is high-

ly probable that the continuous demand for enkephalin programs the methylation or chromatin profile of the enkephalin promoter, such that high production of enkephalin becomes the basal state and persists throughout life.

Our data fit within a broad framework of studies documenting the long-term impact of early-life experience on stress responsiveness [56, 61–65]. To our knowledge, this is the first study to mechanistically establish that early-life pain impairs adult stress through an endogenous opioid-dependent mechanism. Collectively, these data argue that insensitivity to stress and pain [22, 23] we observed are adaptions to the early-life environment. However, decreased ability to evaluate or respond to aversive or noxious stimuli, such as venturing into an area open to predation, excess energy expenditure when faced with the threat of drowning or inability to produce appropriate analgesia, can have severe, even mortal consequences. Thus, these behavioral changes are potentially maladaptive. Admittedly, medical sequelae surrounding prematurity are diverse and complex. However, clinical studies report former preterm infants experience significantly higher behavioral and hormonal thresholds for stress and pain as compared with term-born controls, suggesting reduced sensitivity to noxious or potentially harmful stimuli [8, 11]. Here, our studies delineate specific long-term effects associated with a *single* neonatal injury. These findings should be considered by the clinical community to promote changes in analgesic regimens for NICU patients. Our observations advocate for consistent and appropriate analgesia regimens for NICU patients to reduce the potential for mental health complications associated with premature birth.

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