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Decreased opioid analgesia in weanling rats exposed to endothelin-1 during infancy

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ABSTRACT

Endothelin-1 produces spontaneous nociceptive-associated behaviors that are modulated by the peripheral opioid system. The present study tests the hypothesis that single or repeated exposure to endothelin-1 during infancy decreases opioid analgesia in weanling rats. Morphine analgesia was measured in male and female postnatal day 21 rats following intraplantar endothelin-1 on postnatal day 7, or 11 or both days 7 and 11. In males, exposure to endothelin-1 on postnatal day 11 or both days 7 and 11 produced a statistically significant decrease in morphine analgesia (EC_{50} = 0.902 and 1.326 mg/kg, respectively) compared to control (EC_{50} = 0.486 mg/kg). Similarly in females, exposure to endothelin-1 on postnatal day 11 or both days 7 and 11 produced a statistically significant decrease in morphine analgesia (EC_{50} = 1.367 and 1.226 mg/kg, respectively) compared to control (EC_{50} = 0.468 mg/kg). In addition, females exposed to endothelin-1 on postnatal day 7 exhibited an intermediate decrease in morphine analgesia with an EC_{50} of 0.752 mg/kg. In males, exposure to endothelin-1 decreased mu opioid receptor expression without changing endothelin-A receptor or endothelin-B receptor expression in the hindpaw skin. In contrast, in females, exposure to endothelin-1 increased expression of both endothelin receptors and the mu opioid receptor in hindpaw skin. These findings suggest a sex-difference in the window of vulnerability and the mechanism by which an acute nociceptive event can induce morphine tolerance.

Twenty years ago it was uncommon to administer anesthetics in neonates for painful procedures such as venipuncture, heel stick, invasive procedures or even surgeries [21,24]. Clinical and basic science research has transformed our understanding that not only do neonates feel pain, but also pain experienced at this stage of development can have long-term effects on nociception [2,17,23,26] and analgesia [22,27]. For instance, newborn human infants circumcised without anesthesia exhibit increased pain responses at 4 and 6 month vaccinations as compared to uncircumcised infants [26,29]. Additionally, local anesthetics given at vaccination were more effective in uncircumcised infants compared to circumcised infants [26]. In rodent models, prolonged inflammation during the developmental equivalent of a preterm infant produces nociceptive hypersensitivity and decreased morphine analgesia in adulthood [16,17], suggesting a relationship between analgesic insensitivity and neonatal injury.

Clinically, it is common that full-term infants are exposed to repeated acute procedural pains. Endothelin-1 (ET-1) is endogenously produced at sites of injury and is a potent algogen [13,19]. In adult male rats, ET-1 induced nociception is mediated by binding of ET-1 to endothelin-A (ET_A) receptors, which are located on nociceptors in the skin [13]. Simultaneously, the binding of ET-1 to ET_B receptors, which are located on keratinocytes in the skin, results in the release of β-endorphin. β-Endorphin binds to mu opioid receptors (μORs) located on nociceptors resulting in inhibition of nociceptive signaling [13]. We have previously shown that a single exposure to ET-1 in infant rats produces nociceptive sensitization in males and de-sensitzation in females accompanied by sex-specific change in ET_B receptor expression in the skin [20]. The present study investigates whether single or repeated exposure to ET-1 early in development alters opioid analgesia at weaning. ET-1 was administered on postnatal (P) day 7, a time point that is approximately equivalent to the nervous system development of a full-term human infant [9]. To model repeated injury ET-1 was administered a second time on P11, a time point that is approximately equivalent to a human toddler [9]. Morphine dose–response curves were generated on P21, a time point that is approximately equivalent to the nervous system development of a young human child [9]. In addition, ET-1 induced changes in ET_A, ET_B and μOR expression in the skin was analyzed.

All methods were approved by the Institutional Animal Care and Use Committee at the University of South Carolina School of Medicine. All efforts were made to limit distress and use the minimum number of animals. Male and female Sprague–Dawley rats

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(Charles River, MA) were housed with dam in a 12/12 h light/dark cycle with free access to food and water.

Stock ET-1 (American Peptide, Sunnyvale, CA) was made in sterile endotoxin free saline (1 mg/ml). ET-1 (3.3 nmol in 10 μl) or saline (10 μl) was administered intraplantar in the left hindpaw. Five groups were included in this study. Group 1 ($n = 7$ males and 8 females) were normal naïve rats. Group 2 ($n = 6$ males and 6 females) received saline (S) on both P7 and P11 (S(P7) + S(P11)). Group 3 ($n = 9$ males and 5 females) received saline on P7 and ET-1 on P11 (S(P7) + ET-1(P11)). Group 4 ($n = 8$ males and 7 females) received ET-1 on P7 and saline on P11 (ET-1(P7) + S(P11)). Group 5 ($n = 8$ males and 7 females) received ET-1 on both P7 and P11 (ET-1(P7) + ET-1(P11)).

On P21, opioid analgesia was assessed. Animals were acclimated and baseline mechanical paw withdrawal thresholds were measured prior to the day of the experiment. Rats were placed in ventilated plastic boxes (10 cm × 6 cm × 7 cm) atop a wire-mesh (1 mm × 1 mm) so that mechanical PWTs could be measured from below. A series of 12 von Frey filaments (Stoelting, Wood Dale, IL) were used to evoke a cutaneous flexion withdrawal as previously described [20]. The log stimulus intensity [$\log_{10}$(mg × 10)] of the 12 filaments ranged from 2.44 (40 mg) to 5.18 (15,000 mg). These filaments were applied to the plantar region of the left hindpaw via a modified up–down method to elicit a cutaneous flexion withdrawal [5]. Beginning with the smallest (40 mg) filament, filaments were applied to the mid-plantar region of the left hindpaw for ~2 s. If no response was evoked then the next ascending filament was applied. Application of larger filaments was continued until a paw withdrawal response was evoked. Once a withdrawal response was evoked, the smaller descending filaments were applied until the point at which no withdrawal response was evoked. The filaments were then applied in ascending order a second time until a paw withdrawal response was evoked. The smallest size von Frey filament that evoked at least two withdrawal responses was defined as the paw withdrawal threshold to mechanical stimulation.

Morphine was administered in cumulative doses at 20 min intervals over 2.5 h (morphine doses: 0.1, 0.25, 0.5, 1.0, 1.5, 2, 2.5 and 3 mg/kg). Analgesia was calculated as percent maximum possible effect (%MPE) [30]: %MPE = ([measure value (value following administration of morphine)] − [pretreatment value (baseline value)]) × 100/[cut-off value (15 g filament) − pretreatment value (baseline value)], where 15 is the cut-off value to prevent tissue damage and sensitization. The effective concentration of morphine resulting in 50% of the maximum possible analgesic effect (EC_{50}) was calculated by generating sigmoidal non-linear dose–response curve. Dose–response curves were analyzed for significance using two-way ANOVA followed by post hoc Bonferroni. One-way ANOVA followed by post hoc Bonferroni was used to compare differences between EC_{50}. A value of $P < 0.05$ was considered significant (GraphPad Prism 4.0, GraphPad Software, Inc., San Diego, CA).

Western analysis was used to measure ET_A, ET_B and μOR expression in the plantar hindpaw skin on P21 following intraplantar administration of saline or ET-1 in the left hindpaw on P7 and P11: normal naïve ($n = 3$ males and 3 females), S(P7) + ET-1(P11) ($n = 3$ males and 3 females), ET-1(P7) + ET-1(P11) ($n = 4$ males and 3 females). On P21, rats were asphyxiated with CO_2 and plantar skin from the left hindpaw was quickly removed, frozen on dry ice, and stored at −80 °C until homogenization. As previously described [20], skin was homogenized in 500 μl of lysis buffer (50 mM Tris, 150 nM NaCl, 1% NP-40, 1 mM EDTA, Sigma Protease Inhibitor Cocktail P8340 at 1:100). Samples were centrifuged at 15,700 × g for 30 min at 4 °C, supernatant was removed, and protein quantification was completed (BCA protein assay, Pierce, Thermo Scientific). Thirty-five micrograms of protein underwent polyacrylamide gel electrophoresis followed by transfer to PVDF membrane for western analysis. Membranes were blocked in 5% powdered milk in phosphate buffered saline with 0.05% Tween for 1 h at room temperature. Membranes were incubated overnight at 4 °C with the ET_A, ET_B (1:400, Abcam, Cambridge, MA), or μOR antibody (Neuromics 1:1000). Membranes were washed and placed in HRP labeled secondary donkey anti-rabbit antibody (Jackson Immunoresearch) for 1 h at room temperature. Membranes were washed and bands were visualized using ECL (Thermo Scientific). The same protocol was repeated using a primary antibody against β-actin (1:20,000, Sigma) as a loading control. Films were scanned and ET_A, ET_B and μOR bands were normalized to β-actin expression in same sex naïve skin. All samples were blotted in duplicate. Significance was determined with one-way ANOVA followed by post hoc Bonferroni.

In P21 males, mechanical paw withdrawal thresholds were similar in normal, S(P7) + S(P11), ET-1(P7) + S(P11), S(P7) + ET-1 (P11), and ET-1 (P7) + ET-1 (P11) groups prior to morphine administration (2.3 ± 0.53, 3.3 ± 0.43, 1.8 ± 0.39, 3.8 ± 0.61 and 2.8 ± 0.61 g, respectively). Similar EC_{50} for morphine analgesia was observed in naïve and saline control males (Table 1). Repeated exposure to ET-1 on P7 and P11 produced a right-ward shift in the morphine dose–response curve compared to naïve and saline control animals (Fig. 1A). The right-ward shift represents an approximate 2.7-fold increase in EC_{50} from 0.486 ± 0.148 mg/kg in naïve males to 1.326 ± 0.288 mg/kg following repeated exposure to ET-1 (Table 1). Single exposure to ET-1 on P11 produced an approximate 1.9-fold increase in EC_{50} to 0.902 ± 0.258 mg/kg. In contrast, single exposure to ET-1 on P7 did not alter EC_{50} compared to naïve or saline controls.

In P21 females, mechanical paw withdrawal thresholds were similar in normal, S(P7) + S(P11), ET-1(P7) + S(P11), S(P7) + ET-1 (P11), and ET-1 (P7) + ET-1 (P11) groups prior to morphine administration (2.2 ± 0.62, 2.3 ± 0.74, 2.5 ± 0.45, 3.9 ± 0.33 and 2.6 ± 0.62 g, respectively). Similar EC_{50} for morphine analgesia was observed in naïve and saline control females (Table 1). Single exposure to ET-1 on P7 or P11 or a repeated exposure to ET-1 on P7 and P11 produced a right-ward shift in the morphine dose–response curve compared to naïve and saline control animals (Fig. 1B). Single exposure to ET-1 on P7 or P11 produced an approximate 1.6- and 2.9-fold increase in EC_{50} to 0.752 ± 0.260 and 1.367 ± 0.297 mg/kg, respectively, compared to 0.468 ± 0.158 mg/kg in naïve females. Similarly, repeated exposure to ET-1 on P7 and P11 produced an approximate 2.6-fold increase in EC_{50} to 1.226 ± 0.257 mg/kg (Table 1).

Changes in ET_A, ET_B and μOR expression in the ipsilateral plantar hindpaw were also sex-specific. In P21 males, repeated exposure to ET-1 on P7 and P11 decreased μOR expression, but had no effect on ET_A or ET_B expression compared to naïve males (Fig. 2A and B). In P21 females, single and repeated exposure to ET-1 increased ET_A, ET_B and μOR expression compared to naïve females (Fig. 2A and C).

In the present study, ET-1 exposure on P7 and/or P11 resulted in sex-dependent changes in morphine analgesia on P21. In males, single ET-1 exposure on P7 did not significantly alter EC_{50} compared to saline control males. In contrast, single exposure to ET-1 on P11 or a repeated exposure on P7 and P11 in males significantly increased EC_{50} suggesting that P11 is a critical window during which acute exposure to ET-1 can modulate opioid analgesia. In females, single exposure to ET-1 on P7 significantly increased EC_{50} compared to saline control females with a further increase following repeated ET-1 exposures suggesting that P7 and P11 are critical windows during which acute exposure to ET-1 can modulate opioid analgesia. These findings imply females have an earlier window of vulnerability to ET-1 induced alteration in opioid analgesia when compared to males.

Clinical research demonstrates long-term alterations in nociception and opioid analgesia following neonatal injury, but the direction and precise nature of these changes appears to be dependent upon the type of stimulus, duration of stimulus, and age at