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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 the 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 day 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, 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 mg/kg, 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.

Keywords

endothelin; nociception; morphine; neonatal rat

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 pain experienced at this stage of development can have long-term

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effects on nociception [2, 17, 23, 26] and analgesia [22, 28]. For instance, newborn human infants circumcised without anesthesia exhibit increased pain responses at 4 and 6 month vaccination 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 (μ ORs) 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-sensitization 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 μ OR 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 (Charles River, MA) were housed with dam in a 12/12h 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 l) or saline (10 l) 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 (PWTs) were measured prior to the day of the experiment. Rats were placed in ventilated plastic boxes (10 X 6 X 7 cm) atop a wire-mesh (1 X 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 [$\log_{10}(\text{mg} \times 10)$] 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 hind paw for ~2 seconds. 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 2 withdrawal responses was defined as the PWT to mechanical stimulation.

Morphine was administered in cumulative doses at 20 minute intervals over 2.5 hrs (morphine doses: 0.1, 0.25, 0.5, 1.0, 1.5, 2, 2.5, 3 mg/kg). Analgesia was calculated as percent maximum possible effect (%MPE) [30]: $\%MPE = ([\text{Measure Value (value following administration of morphine)}] - [\text{Pretreatment Value (baseline value)}]) \times 100 / [\text{Cut-off Value (15 gram filament)} - \text{Pretreatment Value (baseline value)}]$, 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 hind paw skin on P21 following intraplantar administration of saline or ET-1 in the left hind paw 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 $-80^\circ C$ until homogenization. As previously described [20], skin was homogenized in 500 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM EDTA, Sigma Protease Inhibitor Cocktail P8340 at 1:100). Samples were centrifuged at 15,700g for 30 min at $4^\circ C$, supernatant was removed, and protein quantification was completed (BCA protein assay, Pierce, Thermo Scientific). Thirty-five μg 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 1% Tween for 1 hr at room temperature. Membranes were incubated overnight at $4^\circ C$ 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 hr 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 μ OR 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 , 2.8 ± 0.61 grams, respectively). Similar EC₅₀ for morphine analgesia was observed in naïve and saline control males (Table 1). Repeated exposure to ET-1 on P7 and P11 produced a rightward shift in the morphine dose-response curve compared to naïve and saline control animals (Fig 1A). The rightward shift represents an approximate 2.7 fold increase in EC₅₀ 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 EC₅₀ to 0.902 ± 0.258 mg/kg. In contrast, single exposure to ET-1 on P7 did not alter EC₅₀ 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 , 2.6 ± 0.62 grams, respectively). Similar EC₅₀ 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 rightward 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 EC₅₀ 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 EC₅₀ to 1.226 ± 0.257 mg/kg (Table 1).

Changes in ET_A, ET_B and μ OR expression in the ipsilateral plantar hindpaw were also sex-specific. In P21 males, repeated exposure to ET-1 on P7 and P11 decreased μ OR expression, but had no effect on ET_A or ET_B expression compared to naïve males (Fig 2A,B). In P21 females, single and repeated exposure to ET-1 increased ET_A, ET_B and μ OR expression compared to naïve females (Fig 2A,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 EC₅₀ 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 EC₅₀ 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 EC₅₀ 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 injury [2, 27]. Recent basic science research indicates sex as an additional contributing factor in the development of long-term changes in nociception and opioid analgesia following neonatal injury [16, 20]. During the neonatal period, males experience a testosterone surge that drives the development of male sexual physiology and behavior in adulthood [1, 3, 6, 8]. Carrageenan induced inflammatory injury on P0 and P8 produces sexually dimorphic changes in nociception during adulthood with females exhibiting greater sensitivity than males [15, 16]. This sexually dimorphic alteration in nociception is not observed when carrageenan is administered on P14, a time point when testosterone levels in males have decreased [16]. It has been suggested that testosterone is neuroprotective in both acute and chronic pain models [4, 12] possibly via limiting inflammation and proinflammatory cytokine activity [18]. In addition, androgens decrease NMDA-mediated responses in several central nervous system regions [4, 25]. In the current study, exposure of males to ET-1 on P7 did not alter opioid analgesia on P21. We would postulate that a testosterone surge in neonatal males may be protective against ET-1 induced changes in opioid analgesia. Conversely, in females, the absence of hormonal influence may be permissive for ET-1 induced changes in opioid analgesia. In males, the change in opioid analgesia induced by ET-1 administered on P11, when hormonal levels are down, further suggest a role for sex hormones in injury-induced opioid tolerance.

The literature suggests adult males have greater opioid analgesia in comparison to adult females [6] implicating organizational effects of sex hormones on opioid analgesia [7]. Neonatal castration produces a right-ward shift in systemic morphine dose response curve [7] and decreases periaqueductal gray mediated morphine analgesia in adulthood [14] resulting in similar opioid analgesia to normal females. Conversely, females exposed to testosterone during the neonatal period have a leftward shift in the morphine dose response curve [7] and increased periaqueductal gray mediated morphine analgesia [14] similar to normal males. When this same gonadectomy was performed in adult males and females it did not change morphine analgesia, implicating the importance of organizational effects of gonadal hormones on sex mediated actions of morphine [7]. Our current findings support an organizational role for sex hormones in opioid analgesia and suggest that the window of vulnerability for this organization is sex specific. This is supported by the observation that exposure to ET-1 on P7 resulted in morphine tolerance in females while exposure to ET-1 on P11 produced tolerance in both males and females.

Furthermore, ET-1 exposure early in development produced sex-dependent changes in endothelin and mu opioid receptor expression in the skin. We have previously shown sex-specific responses to ET-1 when administered on P7 such that males have a greater magnitude and duration of nociception when compared to females [19]. In addition, in response to a single (P11) versus double (P7 and P11) exposure males demonstrate a sensitization to second exposure to ET-1 while females display a de-sensitization upon a second exposure. Thus, ET-1 induced nociception appears to be mediated by age, sex, and the number of exposures to ET-1 [19, 20].

ET-1 induced changes in skin expression of endothelin and mu opioid receptors also appear to be mediated by age, sex, and the number of exposures to ET-1. In females, single exposure to ET-1 on P11 and repeated exposure to ET-1 on P7 and P11 increased expression of ET_A, ET_B and μ OR expression. In contrast, in males, only repeated exposure to ET-1 on P7 and P11 decreased μ OR expression. The significant decrease in μ OR expression observed in the skin following repeated ET-1 exposure in males may contribute to the right-ward shift in the morphine dose-response curve by decreasing peripheral opioid analgesia. In contrast, the absence of a change in endothelin and opioid receptor expression following a single exposure to ET-1 on P11 suggests that the mechanism for opioid tolerance may be mediated by ET-1 induced plasticity at other levels of the endogenous opioid system. Similarly, in females, the increase in ET_A, ET_B and μ OR in the skin suggests that ET-1 induced morphine tolerance may be mediated by changes in the endogenous opioid system at sites other than the skin. There are sexually dimorphic regions of the brain which show differences in cell number, size and morphology [10]. In fact, μ OR binding in hypothalamic medial preoptic area is denser in females compared to males [11]. These sex differences in the central nervous system have been shown to be disrupted following prenatal stress and neonatal gonadectomy [10, 14, 25]. Other studies have demonstrated opioid tolerance following neonatal hormonal manipulation [7,14]. The current study demonstrates that repeated ET-1 induced injury also produces sex-specific opioid tolerance.

Previous studies have demonstrated that neonatal hormones have an organizational effect on opioid analgesia via modulation of central nervous system opioid systems [7,14]. The current findings identify P7 as a critical window during which pain and gonadal hormones may interact not only in organizing the opioid system centrally, but also peripherally in the skin. Understanding the actions of gonadal hormones during the neonatal period is essential to unraveling the complex maturation of the endogenous anti-nociceptive system and the impact of early pain experience on subsequent pain and analgesia.

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Figure 1. ET-1 exposure early in development produces sex-dependent right-ward shifts in the morphine dose-response curve in postnatal day 21 rats

A. Morphine dose-response curves for P21 males exposed to S(P7) + S(P11), S(P7) + ET-1(P11), ET-1(P7) + ET-1(P11), ET-1(P7) + S(P11) or naïve. B. Morphine dose-response curves for P21 females exposed to S(P7) + S(P11), S(P7) + ET-1(P11), ET-1(P7) + ET-1(P11), ET-1(P7) + S(P11) or naïve. Data is average $\pm$ SEM. **, *** $p < 0.01, 0.001$ compared to naïve.

Figure 2. ET-1 exposure early in development produces sex dependent changes in ET_A, ET_B and μOR expression in plantar skin from postnatal day 21 rats

A,B. In male rats, there was a decrease in μOR expression in hindpaw skin following repeated exposure of ET-1 on both P7 and P11 compared to normal naïve. A,C. In female rats, there was an increase in ET_A, ET_B and μOR expression in hindpaw skin following single administration of ET-1 on P11 and repeated exposure to ET-1 on P7 and P11 compared to naïve. * p<0.05 compared to naïve.

Table 1

Single or repeated exposure to ET-1 early in development produces sex-specific increases in the EC₅₀ for morphine analgesia.

	MALE		FEMALE	
Group	EC ₅₀	95% C.I.	Female	95% C.I.
Naïve	0.486	0.342 – 0.630	0.468	0.412 – 0.524
S(P7) + S(P11)	0.444	0.341 – 0.547	0.413	0.220 – 0.606
S(P7) + ET-1(P11)	0.902 ***	0.704 – 1.100	1.367 ***	1.025 – 1.709
ET-1(P7) + S(P11)	0.395	0.282 – 0.508	0.752 *	0.499 – 1.005
ET-1(P7) + ET-1(P11)	1.326 ***	1.091 – 1.561	1.226 ***	0.976 – 1.476

*, ***
p<0.05, 0.001 as compared to S(P7) + S(P11) and compared to Naïve (C.I. = Confidence Interval)