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Sexual dimorphic role of the glucocorticoid receptor in chronic muscle pain produced by early-life stress

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Abstract

Fibromyalgia and other chronic musculoskeletal pain syndromes are associated with stressful early life events, which can produce a persistent dysregulation in the hypothalamic-pituitary adrenal (HPA) stress axis function, associated with elevated plasm levels of corticosterone in adults. To determine the contribution of the HPA axis to persistent muscle hyperalgesia in adult rats that had experienced neonatal limited bedding (NLB), a form of early-life stress, we evaluated the role of glucocorticoid receptors on muscle nociceptors in adult NLB rats. In adult male and female NLB rats, mechanical nociceptive threshold in skeletal muscle was significantly lower than in adult control (neonatal standard bedding) rats. Furthermore, adult males and females that received exogenous corticosterone (via dams' milk) during postnatal days 2–9, displayed a similar lowered mechanical nociceptive threshold. To test the hypothesis that persistent glucocorticoid receptor signaling in the adult contributes to muscle hyperalgesia in NLB rats, nociceptor expression of glucocorticoid receptor (GR) was attenuated by spinal intrathecal administration of an oligodeoxynucleotide (ODN) antisense to GR mRNA. In adult NLB rats, GR antisense markedly attenuated muscle hyperalgesia in males, but not in females. These findings indicate that increased corticosterone levels during a critical developmental period (postnatal days 2–9) produced by NLB stress induces chronic mechanical hyperalgesia in male and female rats that persists in adulthood, and that this chronic muscle hyperalgesia is mediated, at least in part, by persistent stimulation of glucocorticoid receptors on sensory neurons, in the adult male, but not female rat.

Keywords

Early life adverse events, corticosterone, muscle hyperalgesia, neonatal limited bedding, sex differences

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Introduction

Adverse early-life experiences (stress) can produce life-long dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis that contributes to numerous negative health outcomes, including development of chronic musculoskeletal pain syndromes such as fibromyalgia and chronic widespread pain.[1–3] We have previously reported that in male rats, neonatal limited bedding (NLB) stress, a well-established protocol that results in abnormal maternal nurturing behavior[4] and increased basal plasma corticosterone in the neonatal rats,[5] produces muscle hyperalgesia, prolongation of inflammatory mediator-induced hyperalgesia (a form of nociceptor neuroplasticity termed hyperalgesic priming[6]) and nociceptor sensitization in adult male rats.[7] Adverse childhood experiences produce elevated levels of cortisol in adults,[8] and preclinical studies have shown that early-life stress affects HPA axis function in adults, affecting both basal and stress-induced activity.[9] Glucocorticoid

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receptors (GR) are highly expressed in dorsal root ganglia (DRG),[10] with ~60% of DRG neurons expressing GR,[11] GR-immunoreactivity is predominantly localized in peripheral peptidergic calcitonin gene-related peptide-immunoreactive sensory nerve fibers in nociceptors.[11,12] We tested the hypothesis that the muscle hyperalgesia in adult rats, which had been exposed to neonatal stress, could be due to the sustained activation of glucocorticoid receptors[5] producing a persistent effect on GR in primary afferent nociceptors.

Methods

Animals

Primiparous timed-pregnant Sprague Dawley female rats were obtained from Charles River (Hollister, CA). Dams were housed with their litter in standard cages on postnatal days 0–1. On postnatal day 2, litters were assigned to limited bedding (NLB) or standard care (control) conditions, or received corticosterone. Behavioral experiments were performed on 200–300 g adult male and female rats.

Animals used in experiments were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12 h light/dark cycle (lights on 7 am – 7 pm) and environmentally controlled conditions; ambient room temperature (21°C–23°C), with food and water available *ad libitum*. Their care and use in experiments conformed to National Institutes of Health guidelines and measures were taken to minimize pain and discomfort. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Neonatal limited bedding (NLB) stress

We used NLB protocol, a well-established model of early-life stress.[13] Dams and their pups were housed in standard cages on postnatal days 0 and 1, and beginning on postnatal day 2, dams and their pups were placed in cages fitted with a custom stainless steel mesh grid bottom (Ancare, Bellmore, NY), raised ~2.5 cm from the floor of the home cage, to provide space for collection of urine and feces.[14] The nesting/bedding material provided consisted of one sheet of paper towel (~112 × 22 cm), and no environmental enrichment. Litters were left undisturbed during postnatal days 2–9. From postnatal day 10 until weaning, dams and pups were again housed in standard cages with normal bedding (Paperchip® animal bedding, Shepherd Specialty Papers, Watertown, TN), and standard enrichment. On postnatal day 21 pups were weaned and same sex rats housed 3 per cage, in standard housing conditions.

Neonatal corticosterone administration

We used a standard protocol to non-invasively increase plasma levels of corticosterone in neonates by adding corticosterone to the dam's drinking water.[15] Corticosterone (Sigma-Aldrich, St. Louis, MS) was dissolved in ethanol and added to the dams' drinking water (final concentration 200 μg/ml corticosterone, 2.5% ethanol), so that pups received corticosterone via their mothers' milk, during postnatal days 2–9, which parallels the time course of the NLB protocol. This concentration of corticosterone has been shown to approximately double basal plasma corticosterone levels in both dams and pups.[16]

Mechanical nociceptive threshold in skeletal muscle

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a Chatillon digital force transducer (model DFI2, Amtek Inc., Largo, FL).[7] Rats were placed in cylindrical acrylic restrainers designed to minimize restraint stress and allow extension of their hind legs from lateral ports. To acclimatize rats to the testing procedure, they were placed in restrainers and exposed to the testing procedure, daily for 3 days, prior to starting experiments. On the day of the experiment, rats were placed in a restrainer for 30 minutes before experimental manipulations. To determine nociceptive threshold, a 6 mm diameter probe attached to the force transducer was applied to the gastrocnemius muscle to deliver an increasing compression force. The nociceptive threshold was defined as the force, in Newtons, at which the rat withdrew its hind leg. Mechanical nociceptive thresholds was determined by measuring the mean of 3 withdrawal thresholds taken at 5-min intervals; one hind limb of each rat was used. All behavioral testing was done between 10 am and 4 pm (no differences in baseline nociceptive threshold was observed over this time), and was performed blind to treatment condition.

Oligodeoxynucleotide antisense to glucocorticoid receptor mRNA

To investigate whether corticosterone, acting at glucocorticoid receptors (GRs) on sensory neurons, contribute to NLB-induced hyperalgesia, an oligodeoxynucleotide antisense to GR mRNA was administered intrathecally. Oligodeoxynucleotides were synthesized by Life Technologies (Carlsbad, CA). The antisense ODN sequence for GR was 5′ -TGG AGT CCA TTG GCA AAT-3′, and an ODN mismatch of the same sequence as the antisense but with five bases switched

(shown in the bold type face: 5′-TG**A** AGT **T**CA **G**TG **T**CA A**C**T-3′) was used as the control. We have validated intrathecally administered antisense ODN actions on GR expression by Western blotting, demonstrating a decrease in the expression of GR in DRG.[17] Before use, antisense and mismatch ODNs were lyophilized and reconstituted to a concentration of 4 mg/mL in 0.9% NaCl, immediately before intrathecal administration. To administer ODNs (80 mg/20 µl), rats were briefly anaesthetized with 2.5% isoflurane and a 30-gauge hypodermic needle inserted into the subarachnoid space, on the midline, between the L4 and L5 vertebrae. The intrathecal site of injection was confirmed by the elicitation of a tail flick, a reflex evoked by subarachnoid space access and bolus injection.[18]

Statistical analyses

Group data are expressed as mean ± SEM of n independent observations. Statistical analysis of experimental data was conducted using one- or two-way analysis of variance (ANOVA), using Prism 9 (GraphPad Software, San Diego, CA). Where there was a significant main difference between treatment groups, Dunnet's post hoc test was used after the one-way ANOVA (Figure 1), and Šídák's post-hoc test was used after two-way ANOVA (Figure 2). Since some comparisons were between groups of unequal sample sizes, data were analyzed by fitting a mixed model that uses a compound symmetry covariance matrix, and is fit using Restricted Maximum Likelihood (REML) to take unequal sample sizes in to account. The accepted level for significance was $P < 0.05$.

Results

NLB and neonatal corticosterone administration produce muscle hyperalgesia in adult male and female rats

In adult rats that had been exposed to the limited bedding protocol (NLB) during postnatal days 2–9, mechanical nociceptive threshold in the gastrocnemius muscle was significantly lower in both males and females, when compared to male and female rats that had standard bedding during the same postnatal period (Figure 1). Similarly, in adult rats that received

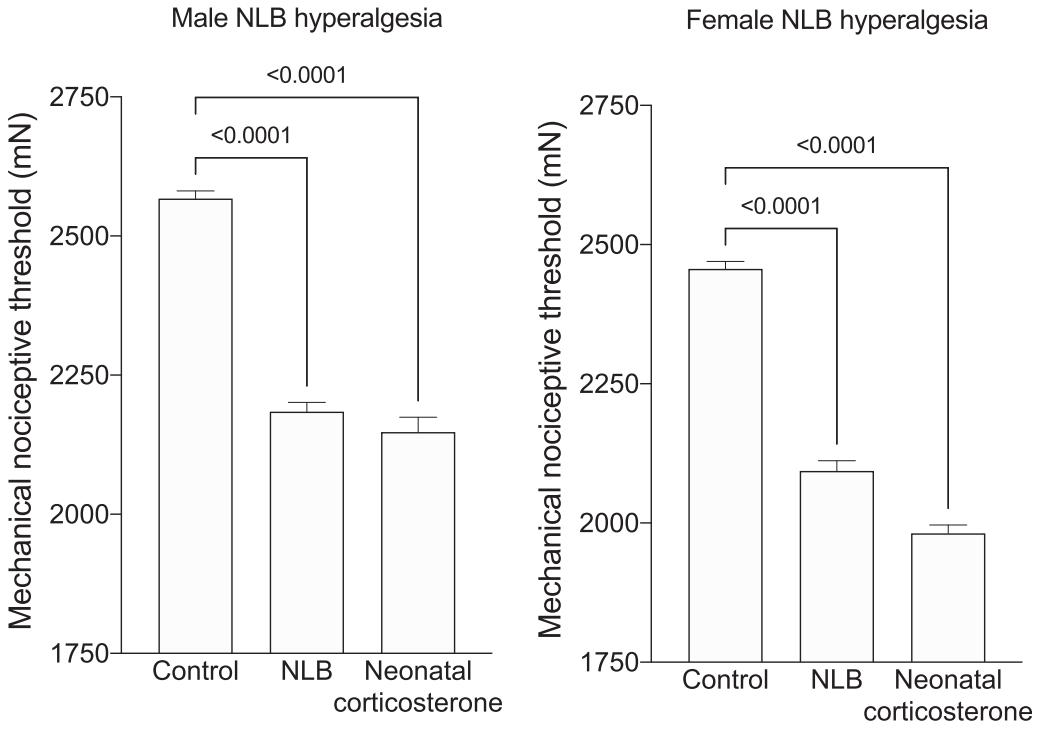

Figure 1. NLB and neonatal corticosterone produce mechanical hyperalgesia in adult male and female rats. Dams and their pups were either housed in standard cages (Control) or in cages with limited bedding (NLB) during postnatal days 2–9 (and then in standard cages). In a separate group, dams' drinking water contained 200 µg/ml corticosterone, during postnatal days 2–9. Both NLB and neonatal corticosterone protocols produced a significant decrease in mechanical nociceptive threshold in adult male and female (8 weeks old) rats; (Males: one-way ANOVA $F(2, 48) = 44.0$, $P < 0.0001$, Dunnett's multiple comparisons test, control vs. NLB and control vs. neonatal corticosterone, both $P < 0.0001$; Females: one-way ANOVA $F(2, 50) = 87.89$, $P < 0.0001$, Dunnett's multiple comparisons test, Control vs. NLB and control vs. neonatal corticosterone, both $P < 0.0001$). Males: control $n = 6$, NLB $n = 23$, neonatal corticosterone $n = 22$; Females: control $n = 6$, NLB $n = 11$, neonatal corticosterone $n = 36$.

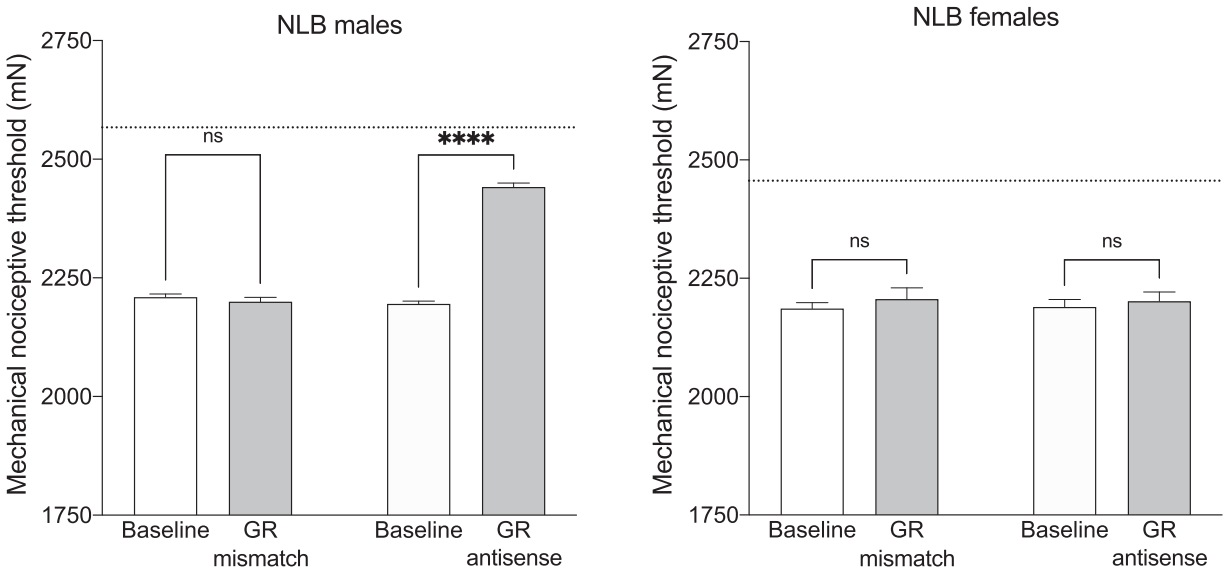

Figure 2 Glucocorticoid receptor (GR) antisense ODN attenuates NLB-induced hyperalgesia in male, but not female, rats. Adult male and female NLB rats received intrathecal injections of antisense or mismatch ODN (40 μg/20 μl, i.t., daily x 3) directed against glucocorticoid receptor mRNA. Adult NLB rats were hyperalgesic (dashed lines indicate muscle mechanical nociceptive threshold in Control, normal bedding rats) prior to receiving ODN. Antisense ODN significantly increased nociceptive threshold in males (Two-way ANOVA, interaction $F(1, 10) = 267.3$, Šídák's multiple comparisons test: mismatch P=ns, antisense $P < 0.0001$), but not in females (Two-way ANOVA, interaction $F(1, 13) = 0.1336$, P=ns, both mismatch and antisense P=ns). Males: both groups $n = 6$; females: antisense $n = 8$, mismatch $n = 7$.

corticosterone (via their mother's milk) during the same postnatal period, days 2–9, muscle mechanical nociceptive threshold was similarly decreased (Figure 1).

Oligodeoxynucleotide antisense to glucocorticoid receptor mRNA attenuated NLB hyperalgesia in adult male, but not adult female rats

In adult rats that had experienced the NLB protocol, mechanical nociceptive threshold in gastrocnemius muscle was significantly lower in both males and females, compared to rats that had standard bedding during the same postnatal period (*cf*. "Control" bedding rats, Figure 1). While administration of ODN antisense for glucocorticoid receptor mRNA significantly reversed NLB-induced hyperalgesia in males, it had no effect in females (Figure 2).

Discussion

Our data support the suggestion that increased corticosterone levels during a critical developmental period (postnatal days 2–9) ,[19,20] produced by NLB stress,[5,13,21,22] or neonatal corticosterone administration,[16] induces a chronic mechanical hyperalgesia in rats of both sexes that persists into adulthood. GRs, present on nociceptors,[11] are thought to play a critical role in the processing of noxious stimuli. Corticosterone and epinephrine, the sympathoadrenal stress mediator, can act at GR and β_2-adrenergic receptors, respectively, on DRG neurons, which synergistically induce hyperexcitability of nociceptive DRG neurons and changes in voltage-gated sodium and potassium currents.[23] In adult males, but not females, NLB-induced chronic muscle hyperalgesia is, at least in part, dependent on GRs on sensory neurons. While the biological basis for this sexually dimorphic contribution of GR on NLB-induced hyperalgesia has yet to be determined, basal levels of corticosterone in adult NLB rats have been reported to be significantly higher in males, but not in females,[24] although not in all studies.[21,25] Furthermore, reactivity of the HPA axis to stress in adult NLB rats is *increased* in male rats, but *decreased* in female.[26] These findings support a role of HPA axis function in the adult male rat in mechanical hyperalgesia. Studies of the effect of neonatal corticosterone administration on basal and stress response corticosterone levels in adults have provided conflicting findings.[14,15,27–30] Our finding that GR ODN treatment reverses NLB hyperalgesia in males, but not in females, may not be due to higher basal and/or greater stress-induced increase in corticosterone in adult NLB males, but rather to high corticosterone levels (due to NLB stress or corticosterone administration) during the critical development period producing epigenetic regulation of the glucocorticoid receptor gene (*Nr3c1*) promotor,[31] and significant increase in *Nr3c1* DNA methylation in nociceptors, changes which can lead to increased visceral hyperalgesia.[32] Of note in this regard,

compared to sex-matched pain-free controls individuals with chronic musculoskeletal pain (fibromyalgia) have altered GR methylation.[33] And, while not specifically evaluated in nociceptors, there are sex differences in *Nr3c1* epigenetics that lead to sex differences in HPA axis function, as well as corticosterone-induced increased axonal sprouting (plasticity) of nociceptive afferents[10] into the superficial layers of the spinal cord dorsal horn[34] which could contribute to enhanced pain.

In summary, early-life stress (NLB) produces a marked, persistent, mechanical hyperalgesia present in adult male and female rats. In male rats this effect is mediated via GRs present on nociceptors. We hypothesize that dysregulated HPA responsiveness in NLB adults and/or epigenetic changes in GR during the neonatal period[9,13,26] exert marked ongoing effects on DRG neurons through GR, in male rats. The lack of a role for GR in NLB-induced hyperalgesia in adult female rats may be due to sex differences in epigenetic regulation of GR, or normal levels of peripheral corticosterone in adult NLB females.[15] Our results support the suggestion that men and women may require different therapeutic approaches to ameliorate chronic musculoskeletal pain, produced by early life adversity.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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