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Neonatal handling (resilience) attenuates water-avoidance stress induced enhancement of chronic mechanical hyperalgesia in the rat

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Abstract

Chronic stress is well known to exacerbate pain. We tested the hypothesis that neonatal handling, which induces resilience to the negative impact of stress by increasing the quality and quantity of maternal care, attenuates the mechanical hyperalgesia produced by water-avoidance stress in the adult rat.

Neonatal male rats underwent the handling protocol on postnatal days 2–9, weaned at 21 days and tested for muscle mechanical nociceptive threshold at postnatal days 50–75.

Decrease in mechanical nociceptive threshold in skeletal muscle in adult rats, produced by exposure to water-avoidance stress, was significantly attenuated by neonatal handling. Neonatal handling also attenuated the mechanical hyperalgesia produced by intramuscular administration of the pronociceptive inflammatory mediator, prostaglandin E_2 in rats exposed as adults to water-avoidance stress.

Neonatal handling, which induces a smaller corticosterone response in adult rats exposed to a stressor as well as changes in central nervous system neurotransmitter systems, attenuates mechanical hyperalgesia produced by water-avoidance stress and enhanced prostaglandin hyperalgesia in adult animals.

Keywords

Neonatal handling; stress; muscle hyperalgesia; resilience; housing density

Introduction

The early postnatal period is a critical developmental time for the establishment of long-term mechanisms that regulate the response to stress [1]. For example, an enhanced

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hypothalamic-pituitary-adrenal (HPA) stress axis response in the adult, both in animal models [2] and clinical studies [3, 4], occurs with exposure to early-life stress, while there is an attenuated HPA axis stress response (stress resilience) in animals exposed to neonatal handling [5, 6]. Early life stress is a major risk factor for greater chronic pain in the adult [7–10]. For example, induction of stress is well known to exacerbate pain severity in patients with chronic pain states, such as fibromyalgia and other widespread pain syndromes [11–13]. Furthermore, we have shown, that exposure to stress neonatally (neonatal limited bedding) decreases mechanical nociceptive threshold in skeletal muscle in the adult rat, and further exacerbates muscle hyperalgesia when the adult rat is exposed to a novel sound stress [14].

It is likely that stress hormone levels are a key determinant in regulating nociceptive threshold. Of note, we have previously shown that induction of chronic stress in the adult rat markedly enhances the duration of mechanical hyperalgesia induced by administration of the pronociceptive inflammatory mediator, prostaglandin E_2 (PGE_2) in skeletal muscle [15]. This enhancement of PGE_2 hyperalgesia produced by stress, which persists unattenuated for at least 2 weeks after the last exposure to the stressor [15], is referred to as "hyperalgesic priming" [16].

In contrast to early life stress enhancing stress responses in the adult, short periods of neonatal handling during the first postnatal week, produces both an increase in the quality and quantity of maternal care [17, 18], as well as lower corticosterone responses to stress exposure and a faster return to basal levels of this stress hormone [5] in the adult rat. This effect of neonatal handling, which has been termed stress resilience [19, 20], has not been evaluated for its impact on pain sensitivity in the adult. Therefore, in the present study, we tested the hypothesis that resilience, induced by neonatal handling would induce, in the adult, resistance to chronic muscle pain (hyperalgesic priming) and inflammatory mechanical hyperalgesia.

Material and Methods

Animals

Primiparous timed-pregnant female Sprague Dawley 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 handled (experimental) or standard care (control) conditions. Subsequent behavioral experiments to measure mechanical nociceptive threshold were performed on 200–350-g (age: 50–75 days) male rats from these litters.

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

Mechanical nociceptive threshold in muscle

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a Chatillon digital force transducer (model DFI2, Amtek Inc, Largo, FL, USA) [21]. Rats were placed in cylindrical acrylic restrainers designed to provide adequate comfort and ventilation, allow extension of the hind leg from the cylinder, and minimize restraint stress. All rats were acclimatized to the testing procedure, and testing was performed in parallel across groups. Rats were adapted to the restrainer for 1 h prior to starting each study and for 30 min prior to experimental manipulations. To measure nociceptive threshold, a 6-mm-diameter probe attached to the force transducer applied to the gastrocnemius muscle, to deliver a compression force that increased with time. The nociceptive threshold was defined as the force, in Newtons, at which the rat withdrew its hind leg from the stimulus. Baseline nociceptive withdrawal threshold was defined as the mean of 2 readings taken at a 5-minute inter-trial interval. Nociceptive thresholds were again measured after injection of PGE_2 (Sigma, St. Louis, MO; 1 μg in 20 μL 0.9% saline) into the belly of the gastrocnemius muscle. Each hind limb is treated as an independent measure and each experiment performed on a separate group of rats. All behavioral testing was done between 10 am and 4 pm.

Neonatal handling

We used a well-established neonatal handling model that produces resilience to stress in the adult rat [22–24]. The neonatal handling protocol used was similar to that previously described [23], and involves removing the pups from the home cage for 15 min daily and placing them in a separate temperature-controlled container. Litters were handled daily on postnatal days 2–9, and on postnatal day 21 pups were weaned and females culled. Weaned male rats were housed either 2 per cage or 3 per cage, dictated by the number of male pups in a litter.

Water Avoidance Stress

In the present study, we used the water-avoidance model for visceral hypersensitivity in the rat, which manifests irritable bowel syndrome-like features [25], and which we have shown to produce mechanical hyperalgesia in skeletal muscle [15]. For 1 hour per day, for 10 consecutive days, rats were placed on an acrylic platform (8 × 8 cm, 10 cm high) in the center of a clear plastic tank (45-cm length × 25-cm width × 25-cm height) filled with room temperature tap water to a depth of 9 cm. One, two and three days after the 10-day stress period, rats were tested for mechanical hyperalgesia in the gastrocnemius muscle.

Statistics

Group data are expressed as percentage change from baseline nociceptive threshold (mean ± SEM of n distinct observations). Statistical comparisons were made by one- and two-way repeated measures analysis of variance (ANOVA) followed, where appropriate, with Sidak's or Tukey's multiple comparisons test (GraphPad Prism). Statistical significance was set at $p<0.05$.

Results

In non-handled control rats, the water-avoidance stress protocol induced a statistically significant decrease in mechanical nociceptive threshold in the gastrocnemius muscle (one-way repeated measures ANOVA, $F_{3,69}=100.7$; $p<0.0001$, Figure 1), and nociceptive threshold measured 1, 2 and 3 days after the last of 10 daily stress exposures were each significantly different from pre-exposure baseline thresholds (Dunnett's multiple comparison test $p<0.05$ at each time point). In neonatal handled rats, the water-avoidance stress protocol induced a statistically significant decrease in mechanical nociceptive threshold in the gastrocnemius muscle (one-way repeated measures ANOVA, $F_{3,111}=7.12$; $p=0.0019$, Figure 1). Furthermore, nociceptive threshold measured 1 day after the last of 10 daily stress exposures was significantly different from pre-exposure baseline threshold (Dunnett's multiple comparison test $p<0.05$ baseline versus 1 day), but there was no significant difference between baseline and day 2, and baseline and day 3 (p=N.S). A two-way repeated measures ANOVA demonstrated a significant time × treatment (neonatal handling) interaction ($F_{3,180}=27.50$; $p<0.0001$), indicating that the effect on nociceptive threshold varied over time; there was also a significant main effect of treatment group ($F_{1,60}=80.43$; $p<0.0001$).

PGE_2 hyperalgesia was also significantly enhanced by water-avoidance stress ($P<0.001$, two-way repeated measures ANOVA, Figure 2). Two-way ANOVA demonstrated a significant time × treatment (water-avoidance stress) interaction ($F_{12,268}=34.34$; $p<0.0001$), and there was also a significant main effect of treatment ($F_{2,67}=56.73$; $p<0.0001$). There was a significant attenuation of PGE_2 hyperalgesia in neonatally handled rats compared to non-handled rats (Tukey's multiple comparison test, $p<0.001$).

During data analysis, there appeared to be a difference in nociceptive threshold in neonatally handled rats housed 2 per cage and those housed 3 per cage (differences in housing density were necessitated by litter size) within experimental groups. Since housing density is an independent determinant of level of stress [26], we separately analyzed data by density of home cage housing. Neonatally handled rats that were housed in groups of 3 did not develop hyperalgesia when exposed to the water-avoidance stress protocol (1-way repeated measures ANOVA, $F_{3,54}=114$, $p=0.338$), while those housed in groups of 2 developed hyperalgesia (1-way repeated measures ANOVA, $F_{3,57}=12.46$, $p=0.0002$, Figure 3), with a significant difference between baseline nociceptive threshold and threshold measured at days 1, 2 and 3 (Dunnett's multiple comparison test, $p<0.05$, Figure 3). There was no significant difference in PGE_2 hyperalgesia between rats housed 2 and 3 per cage (Tukey's multiple comparison test, P=N.S., data not shown).

Discussion

In the present experiments we tested the hypothesis that a protocol that induces resilience to stress, neonatal handling [22–24], attenuates the induction of hyperalgesia produced by exposure of the adult to stress. Rats that underwent neonatal handling, to induce a state of resiliency to stress [22, 23], exhibited a marked (~75%) attenuation of mechanical hyperalgesia induced by exposure to water-avoidance stress as adults. Since water-

avoidance stress in adult rats induces a state of chronic stress [15, 25], the attenuation of lowered mechanical threshold (i.e., mechanical hyperalgesia) in skeletal muscle in rats that had been neonatally handled to induce resilience, suggests that this decrease in threshold is a stress-mediated phenomenon. It has previously been shown that, as adults, rats that had undergone neonatal handling [22–24], have both a lower basal corticosterone level [6] and an attenuated endocrine response to stress [5]. This relationship between stress axis activation and nociceptive threshold is consistent with earlier studies by us [9, 14, 27–29] and others [30–32], demonstrating that chronic stress lowers mechanical nociceptive threshold and enhances inflammatory mediator-induced mechanical hyperalgesia.

There is substantial evidence that events in early life produce long-lasting effects on neuroendocrine stress axis response to stressors in the adult [7–9]. We have previously shown that early-life stress in rats (neonatal limited bedding) both decreases nociceptive threshold and enhances inflammatory mediator-induced hyperalgesia in adults [9], and also enhances hyperalgesia produced by exposure to stress in the adult [14]. We now show that neonatal handling, a protocol that attenuates the stress response in the adult rat [22–24], decreases both stress-induced mechanical hyperalgesia and enhanced inflammatory mediator-induced mechanical hyperalgesia in skeletal muscle, produced by stress exposure in the adult. Since early-life events can markedly affect stress response in adults (e.g., increased incidence of post-traumatic stress [7, 33]), an understanding of what early life factors lead to post-traumatic stress disorders or provide stress resilience in the adult are now being explored [34]. In contrast to neonatal stress, neonatal handling provides a resiliency in the response to stressors in the adult. This has been attributed to the neonatal handling protocol producing both an increase in maternal care as well as providing exposure to a novel environment, both of which are believed to facilitate stress regulation (for a recent review, see McEwen and colleagues [35]). While the mechanisms underlying the stress resiliency produced by neonatal handling are yet to be fully elucidated, neonatal handling permanently changes several transmitter systems within the brain. For example stress-induced increase in glucocorticoid receptor expression in the rat prefrontal cortex in the adult is prevented in neonatally handled rats [36], and neonatal handling also affects several neurotransmitter systems within the CNS, including serotonin [37], GABA [38], acetylcholine [39] and monoamine [40] systems, as well as increased hippocampal NMDA [41] and mu-opioid receptors [42]. Many of these systems are involved in both stress and pain processing and such changes in these systems produced in neonatally handled rats may underlie the effects on stress-induced hyperalgesia we observed in neonatally handled adult rats.

While not part of the initial experimental design, we observed that housing density was an important factor in the impact of neonatal handling on the stress-induced mechanical hyperalgesia in skeletal muscle produced by stress exposure in adults. Specifically, we observed that while rats housed 2 per cage showed a partial (~50%) attenuation of the hyperalgesia, those housed 3 per cage showed a *complete* prevention of the stress-induced mechanical hyperalgesia. Stress-induced enhancement of PGE_2 hyperalgesia, (i.e. hyperalgesic priming [16, 43]) was also significantly (~20–40%) attenuated in the neonatally-handled rats, but unlike stress-induced mechanical hyperalgesia, the magnitude

of the attenuation of priming, prolonged PGE_2 hyperalgesia following stress, was not dependent on housing density. Of note, we have previously shown that both mechanical hyperalgesia induced by an inflammatory mediator as well as hyperalgesic priming are mediated by different mechanisms [44, 45].

While the current study does not distinguish whether housing density potentiates the effect of neonatal handling, from housing density attenuating the effect of water avoidance stress, the differential effect of rats housed 2 versus 3 per cage on stress-induced hyperalgesia may be related to the effect of housing density on stress level. It has previously been shown that rats housed 2 per cage spent only about one fifth the time in the open arm of the elevated plus maze compared to rats housed 3 per cage (and a similar amount of time to solitary housed rats), indicating that there is a significantly greater chronic anxiety level in rats housed 2 per cage [46]. Of note, anxiety is closely correlated with stress hormone levels [47], and housing density is known to affect both the rate of catecholamine synthesis as well as magnitude of release from the adrenal medulla [26]. Other measures of stress level, resting mean arterial pressure and heart rate, were both significantly less for rats housed 4 per cage than for those housed 1 or 2 per cage, and the cardiovascular responses to a mild stressor (regular cage change) was also significantly less in rats housed 4 per cage [48]. Furthermore, while singly housed rats are generally considered to be in the most stressful housing condition [49], when held in a rodent restrainer for tail vein injection, the increase in heart rate for rats housed 2 per cage was greater than those housed singly [48]. Thus, it should be generally considered that 3 per cage housing of rats is the least stressful. Importantly, while it is well-recognized that rat housing conditions are an important factor that should be described in scientific papers [50], housing density, which is rarely reported, may be an important confounding variable in studies evaluating some aspects of pain in preclinical models, especially where stress impacts pain.

Conclusions

Neonatal handling, which induces resilience to stress, attenuates muscle hyperalgesia in adult rats exposed to water-avoidance stress.

Acknowledgments

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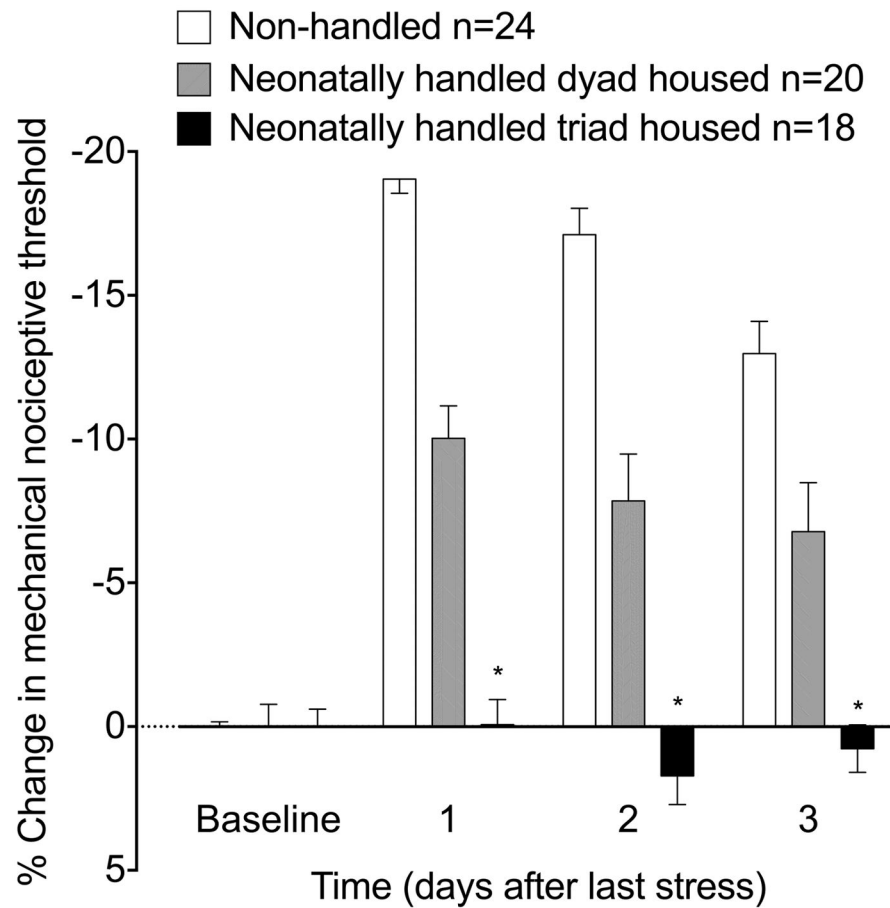**Figure 1. Neonatal handling attenuates stress-induced muscle hyperalgesia in adult rats**
Water-avoidance stress produces a significant decrease in nociceptive threshold in the gastrocnemius muscle (3 d after the last exposure to the stressor). Water-avoidance stress induces reduction in nociceptive threshold that is markedly attenuated in neonatally handled rats. *p<0.05.
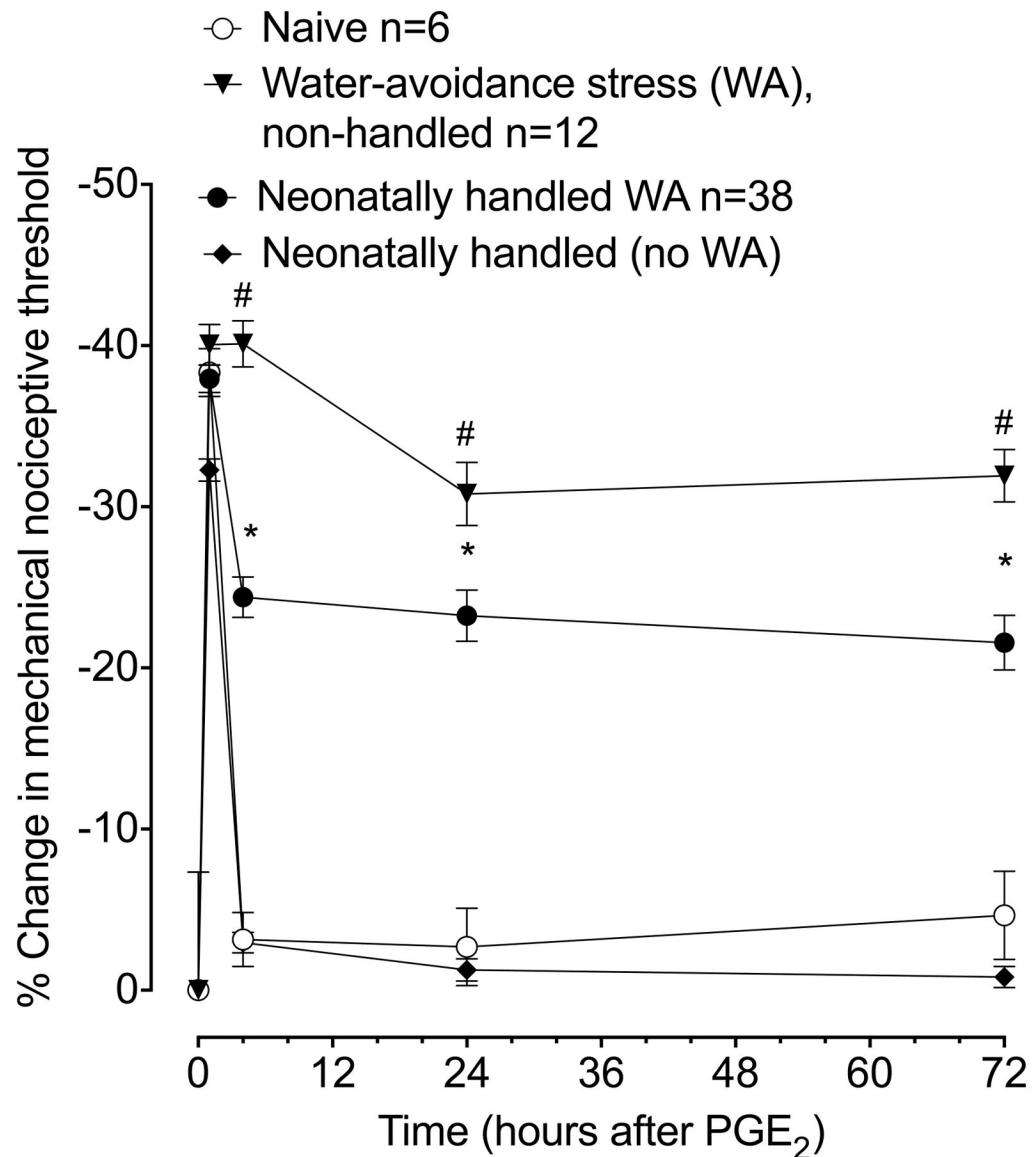
Figure 2. Enhancement of prostaglandin E$_2$ (PGE$_2$) hyperalgesia by water-avoidance stress is attenuated in neonatally handled rats

Mechanical hyperalgesia in the gastrocnemius muscle was measured 1, 4, 24 and 72 h after intramuscular administration of PGE$_2$, in naive non-stressed, neonatally handled non-stressed, water-avoidance stressed rats, and water-avoidance stressed rats that were neonatal handled. In naive non-stressed rats and in neonatally handled non-stressed rats, PGE$_2$-induced a decrease in mechanical nociception present 1 hour post-injection, that returns to near baseline at the 4-h time point. In water-avoidance stressed rats, the decrease in nociceptive threshold induced by PGE$_2$, administered 1 day after the last stress, remained largely undiminished 72 h post-PGE$_2$ administration, and was significantly different from a non-stressed control group of rats (#P<0.0001, 2-way repeated measures ANOVA). In neonatally handled rats, the decrease in nociceptive threshold produced by exposure to

water-avoidance stress is significantly attenuated compared to the non-handled stress group, at the 4-, 12- and 72-h time points (*P<0.0001, 2-way repeated measures ANOVA).
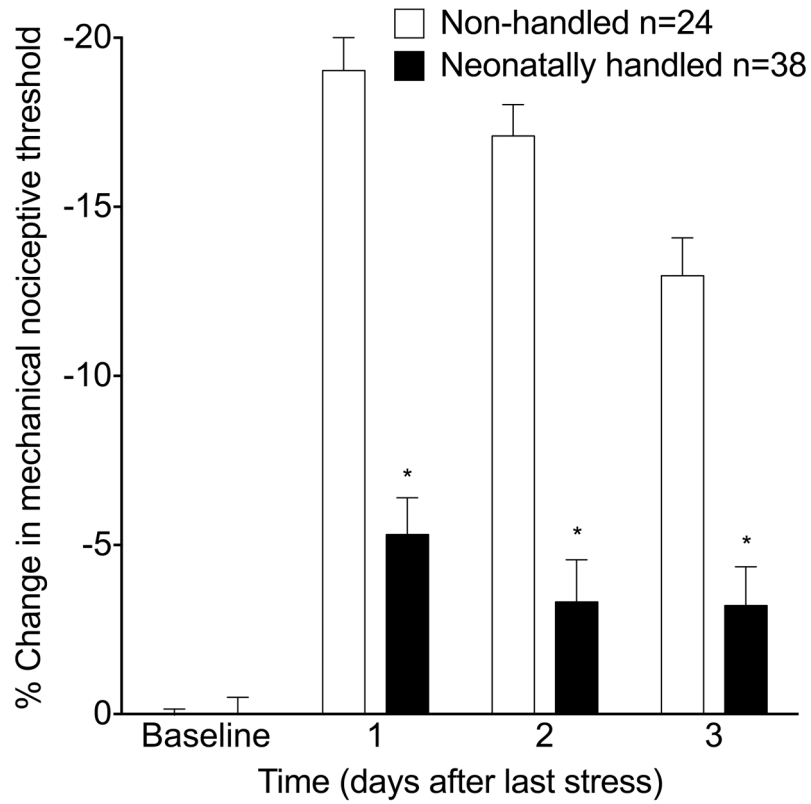
Figure 3. Housing density is a factor in attenuation of water-avoidance stress-induced muscle hyperalgesia by neonatal handling

Water-avoidance stress-induces muscle hyperalgesia that is completely eliminated in 3 rats per cage housed rats, while 2 rats per cage housed neonatally handled rats show a significant attenuation of muscle hyperalgesia ($F_{2,59} = 110.1$, $P<0.001$, 2-way repeated measures ANOVA). * $p<0.05$.