Title
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Introduction

Pain associated with chemotherapy-induced peripheral neuropathy (CIPN), a serious adverse effect of most classes of chemotherapeutic agents, may persist or worsen even months or years after completion of chemotherapy [10,73]. As more effective treatments increase long-term survival in cancer patients, a growing population are living with *chronic* pain, markedly impacting quality of life [82]. We, and others, have observed in oncology patients that stress is a risk factor for CIPN [6,34,59], and that paclitaxel-induced CIPN is worse in patients with greater levels of perceived stress [47]. While both the diagnosis [69] and treatment of cancer are very stressful [9,61], underlying mechanisms for the impact of stress on CIPN remain poorly understood. We have previously shown, in a preclinical model of alcohol-induced painful peripheral neuropathy, a contribution of neuroendocrine stress axes to the severity [17,20]. Thus, differences in stress level and stress responsiveness may help explain why CIPN occurs only in some oncology patients [38,77], and individual differences in severity and duration.

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Authors' contributions: LFF: designed and performed experiments; DA: performed experiments; PG: performed experiments and wrote the manuscript; JDL: designed research, wrote the manuscript. All authors read and approved the final version of the manuscript.
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In addition to the stress associated with the diagnosis and treatment of cancer, we have recently found that a major early life stress, being born prematurely [13], is an important clinical risk factor for CIPN [60]. Importantly, in this regard, evidence from human and animal studies demonstrate that early life is a critical period for setting neuroendocrine stress responsiveness in the adult [23]. Disruption of rodent maternal behavior, by limiting available bedding material to make a nest for her pups (neonatal limited bedding, NLB), produces, in the adult, an increased stress axis responsiveness [26,35,64], which we have observed produces nociceptor hyperexcitability [30] and mechanical hyperalgesia [3,30] in the rat. Conversely, early life tactile enrichment (neonatal handling, NH), enhances the ability of rats to display an adaptive neuroendocrine response to adversity, trauma, threat or other stressors [24,76], a stress-resilient phenotype that we and others have shown to be a protective factor against chronic pain, in the adult rat [5,25]. CIPN occurs in approximately two-thirds of patients receiving paclitaxel [78], a widely used first-line chemotherapy for the treatment of ovarian, breast, lung, cervical, pancreatic and other solid tumors [63]. In the present study, we use our established preclinical model of paclitaxel-induced painful CIPN [2,16,19] to evaluate the relationship between paclitaxel-induced neuropathic pain (hyperalgesia) and stress, including the timing of stress relative to administration of chemotherapy and contribution of neuroendocrine stress hormones acting at their cognate receptors on nociceptors.

Experimental Procedures

Animals

Experiments were performed on male and female Sprague Dawley rats (Charles River Laboratories, Hollister, CA) aged 2 days to 8 weeks. Dams were housed with their litters, and at the end of the third post-natal week, litters were separated by sex and housed 3 per cage, under a 12-hour light/dark cycle, in a temperature- and humidity-controlled room in the University of California, San Francisco Laboratory Animal Resource Center. Food and water were available *ad libitum*. Nociceptive testing was performed between 10:00 am and 5:00 pm. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of California, San Francisco, and adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Mechanical nociceptive threshold

Mechanical nociceptive threshold was quantified using an Ugo Basile Analgesymeter® (Randall-Selitto paw-withdrawal test; Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of a rat's hind paw [74,86]. Nociceptive threshold was defined as the force in grams at which the rat withdrew its paw. Baseline paw-pressure threshold was defined as the mean of the 3 readings taken before a test agent was injected. In each experiment, only one paw per rat was used. Each experiment was performed on a different group of rats. Data are presented as the mean change from baseline nociceptive threshold.

Sound stress

The unpredictable sound stress protocol consists of exposing rats to sound on days 1, 3 and 4. Groups of 3 cage mate rats were placed in a wire mesh box, $31 \times 38 \times 24$ cm, 25 cm from a speaker that emitted pure tones (lasting 5 or 10 s at random times each minute) at 5, 11, 15 and 19 kHz, with amplitudes varying over time independently for each frequency from 20–110 dB sound pressure level; total time in the chamber each day was 30 min [83]. Sham stress animals were placed in the sound chamber for 30 minutes, but without exposure to the sound stimulus. Following sound stress or sham control, rats were returned to their home cages in the animal care facility. Rats were exposed to sound stress either prior to, or after paclitaxel treatment.

Adrenalectomy

Rats underwent surgical excision of both adrenal glands under 3% isoflurane anesthesia. After shaving the abdomen, rats were placed on a thermal blanket and their skin swabbed with povidone iodine solution. To provide peri-operative analgesia, the animals were preoperatively given meloxicam (5 mg/kg, s.c.), as well as bupivacaine infiltrated into the skin in the area to be incised. Two flank wall incisions were made, and each adrenal gland visualized and excised; 5–0 silk suture was used to close abdominal wall and skin incisions, separately. Following surgery, rats' drinking water containing 0.45% saline and 25 μg/ml corticosterone, reduced to 17 μg/ml on day 5 and to 12 μg/ml on day 7 [1], which produces a low physiological (~8 mg/dl peak) plasma level of corticosterone , and simulates the phasic (circadian) corticosterone rhythm, as well as normalizing basal levels of adrenocorticotropic hormone (ACTH) and catecholamines [50], and prevents the decreased weight gain observed in adrenalectomized rats without corticosterone replacement [1].

Neonatal limited bedding (NLB)

Using the well-established NLB model of early life stress [26], beginning on postnatal day 2, dams and their pups were placed in cages fitted with stainless steel wire mesh cage bottoms, raised ~2.5 cm from the home cage floor, to allow collection of urine and feces. The nesting/bedding material provided consisted of one sheet of paper towel (~25 × 33 cm); dams and their litters were left undisturbed during postnatal days 2–9; from postnatal day 10 until weaning, dams and their litters were housed in standard cages, with standard bedding. On postnatal day 21 pups were weaned and same sex rats housed 3 per cage.

Neonatal handling (NH)

We used a well-established neonatal handling (NH) model that produces resilience to stress in the adult rat [52,54,55], previously used by us [4,5]. This protocol involves removing dams from their litters, and gently handling, touching and stroking pups for 15 mins, after which dams and their litters are returned to their home cage. This was conducted daily on pups from postnatal days 2–9. On postnatal day 21 pups were weaned and same sex rats housed 3 per cage.

Drugs

Paclitaxel (Sigma-Aldrich, St. Louis, MO) was dissolved in absolute ethanol and polyethoxylated castor oil (Cremophor EL; 1:1; Sigma-Aldrich) [11,12] and diluted in saline, to a concentration of 1 mg/ml, just before injection. Paclitaxel was administered 1 mg/kg, intraperitoneally, every other day for a total of 4 doses. This produces a preclinical model of chemotherapy-induced painful peripheral neuropathy [2,16]. Meloxicam (Metacam®, Boehringer Ingelheim, Ridgefield, CT) was administered (5 mg/kg s.c.) immediately prior to adrenalectomy surgery. Bupivacaine (0.25%, Hospira Inc., Lake Forrest, IL), infiltrated (~5–8 mg/kg, intradermally, along where the incision was to be performed) prior to adrenalectomy.

β_2-adrenergic receptor antisense:

To investigate whether catecholamines, acting at β_2-adrenergic receptors on sensory neurons, play a role in paclitaxel-induced hyperalgesia, an oligodeoxynucleotide (ODN)-antisense to β_2-adrenergic receptor mRNA was administered intrathecally [17]. The antisense ODN sequence, 5′-AAA GGC AGA AGG ATG TGC-3′ (Invitrogen Life Technologies, Carlsbad, CA) is directed against a unique region of the rat β_2-adrenergic receptor mRNA sequence (GeneBank accession number NM_012492). The mismatch ODN sequence 5′-A**T**A GC**C TG**A **T**GG AA**G** TCC-3′ was designed by mismatching six bases (denoted by bold typeface) of the antisense sequence.

Glucocorticoid receptor antisense:

To investigate if glucocorticoids are involved in paclitaxel-induced hyperalgesia, ODN antisense against glucocorticoid receptor mRNA [17] was used. Intrathecal administration of the ODN sequence 5′-TGG AGT CCA TTG GCA AAT-3′ decreases the expression of glucocorticoid receptors in rat peripheral nerves [43]. As a control, an ODN mismatch of the same sequence, with five bases switched (shown in the bold typeface: 5′-TG**A** AGT **T**CA **G**TG **T**CA A**C**T-3′) was used.

Antisense and mismatch ODNs were lyophilized and reconstituted to a concentration of 2 μg/μl in 0.9% NaCl, immediately prior to administration. To administer ODNs, 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. ODN (80 μg/20 μl) was slowly injected intrathecally; correct injection site was confirmed by observation of a tail flick, a reflex that is evoked by subarachnoid space access and bolus injection [58]. This is a robust and reproducible method for intrathecal ODN administration [87], as a direct communication between the subarachnoid space and dorsal root ganglion (DRG) in rats [39] facilitates rapid entry of ODN into cell bodies in DRG [51]. Animals regained consciousness approximately 1 min after the ODN injection. Of note, in experiments in which rats received both ODNs, antisense ODN for β_2-adrenergic receptor was administered 6 h after ODN for glucocorticoid receptor.

Data analysis

In all experiments, the dependent variable was change in paw-withdrawal threshold, expressed as percentage change from baseline. To compare the hyperalgesia induced by paclitaxel in the different experimental groups, a two-way ANOVA followed by Bonferroni *post-hoc* test, as described in the figure legends. The experiments were performed blind to treatment group. Prism 8.0 (GraphPad Software, Inc, San Diego, CA) was used for the graphics and to perform statistical analyses; A P value < 0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean.

Results

CIPN time-course

We first compared the magnitude and time-course for paclitaxel-induced mechanical hyperalgesia in male and female rats. Administration of paclitaxel (1 mg/kg) every other day for a total of 4 injections produced a decrease in the mechanical nociceptive threshold, mechanical hyperalgesia, over the 4-day course of administration, and peaking by day 7 in both males and females. Nociceptive thresholds in both male and female rats remained significantly lower than pre-paclitaxel baseline even after cessation of paclitaxel administration, and persisted for the duration of the testing period (28 days; Figure 1). There was significantly greater magnitude of paclitaxel-induced hyperalgesia in percentage change from baseline (but not in absolute threshold) in female rats at day 21 and day 28 time points.

Effect of sound stress

We next determined whether exposure to unpredictable sound stress, which enhances inflammatory pain but does not affect nociceptive threshold *per se* [42], affects paclitaxel-induced hyperalgesia. We observed a significant enhancement of paclitaxel-induced hyperalgesia in both male and female rats (for change in baseline, but only in females for threshold) exposed to sound stress prior to starting paclitaxel (Figure 2). In contrast, when males and females were exposed to the same stress *after* paclitaxel treatment, there was no enhancement in the magnitude of paclitaxel-induced hyperalgesia.

Effects of adrenalectomy

Since unpredictable sound stress produces a sustained activation of both neuroendocrine stress axes [44,83], we hypothesized that adrenalectomy would attenuate or prevent paclitaxel-induced hyperalgesia. To test this hypothesis, we administered paclitaxel to rats that had undergone surgical adrenalectomy. Paclitaxel did not produce hyperalgesia in adrenalectomized male or female rats (Figure 3).

Effect of knock down of glucocorticoid and β_2-adrenergic receptors

Since adrenalectomy prevented paclitaxel-induced hyperalgesia, we next determined the contribution of corticosterone and epinephrine, the principal adrenal stress axis mediators of the hypothalamic-pituitary adrenal (HPA) and sympathoadrenal neuroendocrine stress axes, respectively. Both hormones are released from the adrenal gland, and can act at their cognate receptors, glucocorticoid and β_2-adrenergic, on nociceptors [18,67]. To selectively knock

down receptor expression, rats received antisense ODN for glucocorticoid or β_2-adrenergic receptor mRNAs, or their combination, 80 μg/day intrathecally for 10 days; control rats received mismatch ODN (80 μg/day for 10 days). β_2-adrenergic receptor antisense ODN treatment produced a small, albeit significant, decrease in paclitaxel-induced hyperalgesia, in males, while it markedly attenuated hyperalgesia in females. In contrast, antisense ODN to glucocorticoid receptor mRNA produced a marked attenuation of paclitaxel-induced hyperalgesia in males, but did not significantly affect females. The combination of the β_2-adrenergic and glucocorticoid antisense ODNs markedly attenuated paclitaxel-induced hyperalgesia in both males and females (Figure 4).

Effect of neonatal handling and neonatal limited bedding protocols

Since early life stress has been suggested to negatively impact CIPN in oncology patients [60], we evaluated the effect of early life interventions that produce a stress-resilient (NH protocol) or a stress-sensitive (NLB protocol) phenotype in the adult. In rats that had undergone the NH protocol, we observed a significant attenuation of paclitaxel-induced hyperalgesia in adult males, and a small, but significant, attenuation in adult females (Figure 5). In rats that had undergone NLB protocol, there was no change in the magnitude of paclitaxel-induced hyperalgesia in adult male or females.

Discussion

Stressful life events are risk factors for neuropathic pain, including CIPN [6,34,47,59], and stress is prevalent in cancer patients [46,91], where both the diagnosis [69] and treatment [9,61] are extremely stressful. Our primary aim was to use a preclinical model to determine the impact of stress on paclitaxel-induced painful CIPN. Paclitaxel-induced percent change from baseline hyperalgesia, which was significantly greater in females at later time points, was enhanced in male and female rats that had been previously exposed to unpredictable sound stress, which itself does not affect nociceptive threshold [42]; while absolute thresholds for males, were lower at every time point following stress, this did not reach statistical significance. Importantly, when male or female rats were exposed to sound stress *after* cessation of paclitaxel treatment, hyperalgesia was not enhanced. These findings support the suggestion that stress affects the initiation rather than the maintenance of CIPN pain.

To evaluate the mechanism mediating the effect of stress on CIPN, we investigated the role of the two neuroendocrine stress axes, first by administering paclitaxel to adrenalectomized rats, a surgical lesion that eliminates the source of the final mediators for both the sympathoadrenal and HPA stress axes. Seven days after adrenalectomy, paclitaxel-induced hyperalgesia was markedly attenuated, in both male and female rats. These findings are consistent with our previous work demonstrating the dependence of stress levels of sympathoadrenal and HPA stress axis mediators in a model of alcohol-induced neuropathic pain [17].

To determine if the action of key neuroendocrine stress axis mediators, corticosterone and catecholamines (epinephrine and norepinephrine), at their cognate receptors on nociceptors [8,32,81] affect paclitaxel-induced hyperalgesia, we administered antisense ODN

intrathecally to downregulate glucocorticoid and β_2-adrenergic receptors in sensory neurons. Knock down of β_2-adrenergic receptors markedly attenuated paclitaxel-induced hyperalgesia in females, but produced only a small, albeit significant, attenuation in males. In contrast, ODN knockdown of glucocorticoid receptors had no effect on paclitaxel-induced hyperalgesia in females, but produced marked attenuation in males. A combination of both ODNs prevented development of paclitaxel-induced hyperalgesia in both sexes; hyperalgesia was not present at any time point during the 28-day evaluation period, long after the last administration of antisense. The mechanisms underlying the sexual dimorphism of the effects of glucocorticoid and β_2-adrenergic receptors, currently unknown, may be related to sexual dimorphism in: i) sensitivity of β_2-adrenergic receptor-mediated hyperalgesia, males show a much greater hyperalgesia at lower doses of epinephrine [41], so residual receptor expressed after antisense knock down may be sufficient to maintain a paclitaxel-induced hyperalgesia if mediated by increased epinephrine levels; ii) second messenger systems, e.g., we have shown that stimulation of β_2-adrenergic receptors activates the second messenger protein kinase C-epsilon in cultured dorsal root ganglion neurons derived from male but not from female rats [32]; iii) expression of glucocorticoid receptors, which, while not evaluated in nociceptors, is well-established for neurons in the central nervous system [27,72]; or, iv) the HPA [28] and sympathoadrenal [53,89] stress axes. Of note, sex differences have been reported for side effects of cancer chemotherapy, including in CIPN [40,66]. And in a recent preclinical study in rats [94], paclitaxel induced greater mechanical hyperalgesia in females, consistent with our observation. However, while Kim and colleagues [33] observed lower nociceptive threshold for females at every time point they evaluated from 2 to 28 days after paclitaxel, they reported no sex dimorphism, possibly due to the large variance in their data.

The mechanism(s) by which paclitaxel-induced hyperalgesia is dependent on nociceptor glucocorticoid receptors in males but not in females, and why nociceptor β_2-adrenergic receptors play a much greater role in paclitaxel-induced hyperalgesia in females is currently unknown. It has been reported that the greater increase of proinflammatory cytokines and their receptors in DRG of female rats is dependent on female sex steroids, 17β-estradiol and progesterone [94]. Thus, experiments exploring the role of sex hormones and signaling by their receptors, on sensory neurons, would be an important first step toward understanding the sexual dimorphism in the role of neuroendocrine stress hormones.

With regard to sexual dimorphism in stress responses, it has been shown that chronic sleep deprivation, which tonically increases corticosterone levels [56] and adrenergic receptor density [45], exacerbates paclitaxel-induced hyperalgesia [48]. And, there is a sexually dimorphic contribution of stress axes to hyperalgesia, for example in the magnitude of paclitaxel-induced hyperalgesia in Lewis and Fischer F344 rats, which have HPA axis hypo- and hyper-responsivity, respectively [49], as well as marked differences in glucocorticoid receptor density [80].

Human and rodent studies have demonstrated that early life is a critical period for development of adult neuroendocrine responsiveness to stressful stimuli [23]. In clinical studies, it has been observed that adults born prematurely have altered HPA reactivity [22,84] and pain thresholds [92], and have an increased risk for developing CIPN [60]. In preclinical studies, disruption of rodent maternal behavior, by limiting available bedding

material for her neonatal pups' nesting (NLB protocol), produces adults with increased stress axis responsiveness [26,35,64], nociceptor hyperexcitability [30] and mechanical hyperalgesia [3,30]. Conversely, tactile enrichment in early life (NH protocol), induces stress resilience, with an increased capacity of the rodent's adaptive neuroendocrine response to adversity, trauma, threat or other stressors [24,76], and a protective effect against chronic pain in adult rats [5,25]. Adult NH rats have lower basal levels of corticosterone [24] and sympathetic nerve activity [95], as well as lowered adrenal tyrosine hydroxylase activity [71], the rate-limiting step for catecholamine synthesis. The marked attenuation of paclitaxel-induced hyperalgesia in NH males, but minimal in NH females, is similar to our previous observation of sexual dimorphism in the effects of NH on persistent muscle hyperalgesia produced by chronic stress; NH fully prevented stress-induced muscle hyperalgesia in males, but produced only a small reduction of hyperalgesia in female rats [4]. This sexual dimorphism may be related to the finding that NH promotes increased maternal care, thought to be responsible for the development of resilience, and greater maternal behavior is directed to male than to female pups [57,62], which may explain why NH attenuates stress-induced increases in corticosterone levels in male but not female rats [68].

In complementary studies, we evaluated the NLB protocol that produces, in the adult, increased nociceptor hyperexcitability and mechanical hyperalgesia [3,30]. The NLB stress protocol did not, however, affect the magnitude of paclitaxel-induced hyperalgesia in males or females. We had hypothesized that the NLB protocol would exacerbate paclitaxel-induced hyperalgesia, since we have reported that being born prematurely is a clinical risk factor associated with CIPN [60]. It is unlikely that a ceiling effect obscured detection of enhancement of severity of CIPN by NLB treatment, since we observed enhanced CIPN severity in sound stress-exposed rats. Since NLB treatment does not, *per se*, enhance levels of stress mediators, rather, it results in a greater stress mediator reactivity following exposure to stressors in the adult, future studies will evaluated whether early life NLB stress produces a greater enhancement of CIPN in sound stress-exposed rats.

Further studies to elucidate pathophysiological mechanisms by which neuroendocrine stress axes affect CIPN processes, might include examining the role of P-glycoprotein (multidrug resistant protein 1). We have previously shown that inhibiting multidrug resistant proteins, including P-glycoprotein with probenecid, attenuates hyperalgesic priming (a long-lasting latent hyper-responsiveness of nociceptors to inflammatory mediators [75]) produced by carrageenan [21]. A role for P-glycoprotein is supported by several factors, including, that the activity of P-glycoprotein is sexually dimorphic [85,90] and that P-glycoprotein activity is inhibited by chronic stress [14], with both corticosterone [65,79] and a β_2-adrenergic agonist [93] attenuating P-glycoprotein activity. Importantly, following administration, paclitaxel is concentrated in DRGs, where it remains for at least 24 h after a single injection, and the magnitude of paclitaxel-induced hyperalgesia is correlated with paclitaxel levels in DRGs [36]. P-glycoprotein, present in rat DRGs [15], acts as a membrane drug transporter to extrude paclitaxel to the systemic circulation. Of note, the severity of paclitaxel CIPN is dependent on P-glycoprotein polymorphisms [7,88] that have different paclitaxel transport activities [29,31]. Therefore, inhibition of P-glycoprotein function by neuroendocrine stress axis mediators may attenuate the extrusion of paclitaxel from DRGs, thereby increasing the

severity of CIPN. This mechanism also helps explain why stress enhances CIPN when it is given before, but not after, paclitaxel.

In summary, pre-exposure to stress enhances CIPN hyperalgesia in a preclinical model of paclitaxel-induced painful CIPN. In addition, there is sexual dimorphism in the dependence of paclitaxel-induced hyperalgesia on the two main neuroendocrine stress axis mediators, corticosterone and epinephrine, acting at their cognate receptors (β_2-adrenergic and glucocorticoid, respectively), on nociceptors.

To date, no drugs have been developed to specifically prevent or treat chemotherapy-induced painful peripheral neuropathy, and available analgesics that aim to reduce pain symptoms have limited efficacy. Therefore, it remains a pressing need to understand the processes that contribute to the development of painful CIPN, so that effective, mechanism-based, therapeutic interventions can be developed. This has important clinical implications for therapeutic approaches to mitigate a major, dose-limiting side-effect of cancer chemotherapy. While there is evidence for stress-reducing mindfulness-based interventions reducing the magnitude of neuropathic pain intensity [37], our current study suggests that antagonism of glucocorticoid and β_2-adrenergic receptors on nociceptors might be efficacious, albeit sexually dimorphic, as a preventative treatment for taxane-induced painful CIPN.

Acknowledgments

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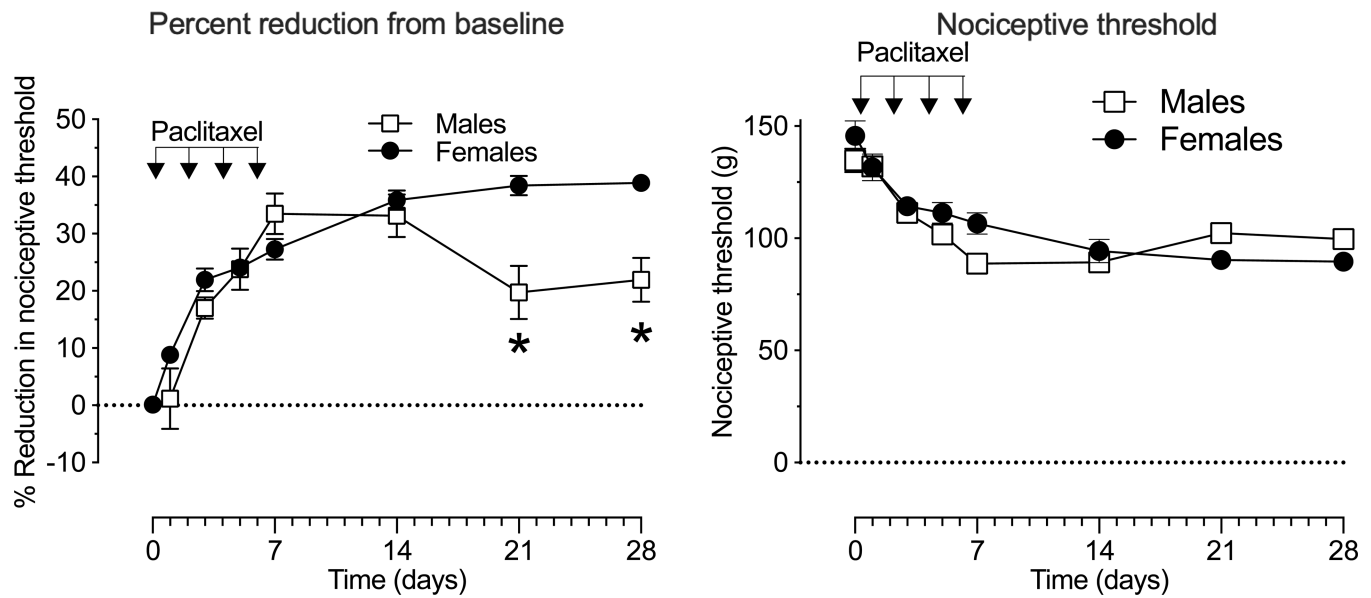
Figure 1. Effect of paclitaxel on mechanical nociceptive threshold in female and male rats.
Female and male rats received paclitaxel (1 mg/kg, i.p.) administered every other day for a total of 4 injections, on days 0, 2, 4 and 6.

Percentage reduction in nociceptive baseline (left panel): The magnitude of hyperalgesia, measured after the last paclitaxel administration (i.e. days 7 – 28), showed a significant interaction effect, but sex differences approached significance differences (P=0.0522). However, post-hoc analysis revealed significantly lower hyperalgesia in males on days 21 and 28 (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 20.52, P<0.0001; Time, $F_{(3, 30)}$ = 3.228, P=0.0363; Sex, $F_{(1, 10)}$ = 4.851, P=0.0522. Bonferroni's post-hoc test, Day 21: P=0.045; Day 28: P=0.0226. n=6 per group).

Nociceptive threshold (right panel): While the magnitude of hyperalgesia, measured after the last paclitaxel administration (i.e. days 7 – 28), showed a significant interaction effect, there was no significance difference for sex (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 16.60, P<0.0001; Time, $F_{(3, 30)}$ = 2.125, P=0.1180; Sex, $F_{(1, 10)}$ = 0.002, P=0.9619).
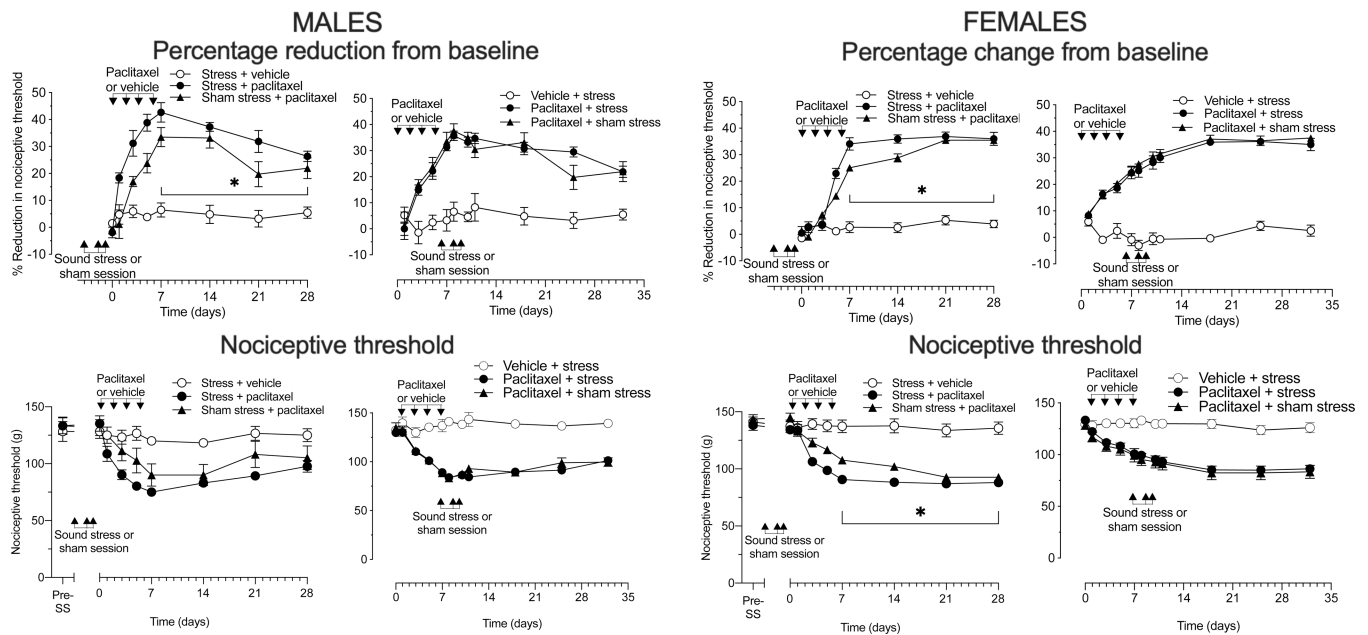
Figure 2. Effect of sound stress on paclitaxel-induced hyperalgesia.

Rats were exposed to unpredictable sound stress before (left panels) or after (right panels) paclitaxel treatment.

Males (left 4 panels)

Stress before paclitaxel (left 2 panels): Sound stress exposure occurred on days −4, −2 and −1. Twenty-four hours after the last exposure to sound stress, paclitaxel (1 mg/kg, i.p.) was administered on days 0, 2, 4 and 6. Mechanical nociceptive threshold was measured before the first sound stress, and again on days 0 (30 min after first dose of paclitaxel), 1, 3, 5, 7, 14, 21 and 28. *Percentage reduction from baseline:* The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 28) was significantly greater in the rats exposed to sound stress (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)} = 4.710$, P=0.0082; Time, $F_{(3, 30)} = 6.52$, P=0.0026; Stress, $F_{(1, 10)} = 83.56$, P<0.0001), sound stress + paclitaxel vs. sham sound stress + paclitaxel *P=0.0459, Bonferroni's multi-comparison test, (n=6 per group). *Nociceptive threshold:* While in paclitaxel-treated rats at every time point from day 7 to 28 in sound stress-exposed rats had a lower threshold than non-stressed rats, this difference was not significant (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)} = 1.58$, P=0.2156; Time, $F_{(3, 30)} = 15.9$, P<0.0001; Stress, $F_{(1, 10)} = 1.43$, P=0.2598).

Stress after paclitaxel (second from left 2 panels): Paclitaxel (1 mg/kg, i.p.) was administered on days 0, 2, 4 and 6, and, sound stress sessions started 24 h after the last paclitaxel injection, on days 7, 9 and 10. Mechanical nociceptive threshold was evaluated on days 0 (30 min after first dose of paclitaxel), 1, 3, 5, 7, 14, 21 and 28. *Percentage reduction from baseline:* There was no significant difference between paclitaxel + sound stress and paclitaxel + sham sound stress groups of rats (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)} = 3.650$, P=0.0235; Time, $F_{(3, 30)} = 14.87$, P<0.0001; Stress, $F_{(1, 10)} = 0.5977$, P=0.4573, n=6 per group). *Nociceptive threshold:* There was no significant difference between paclitaxel + sound stress and paclitaxel + sham sound stress (2-way

repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 1.75, P=0.1782; Time, $F_{(3, 30)}$ = 7.46, P=0.0007; Stress, $F_{(1, 10)}$ = 0.84, P=0.3813).

Females (right 4 panels):

Stress before paclitaxel (second from right 2 panels): Sound stress exposure occurred on days −4, −2 and −1. Twenty-four hours after the last exposure to sound stress, paclitaxel (1 mg/kg, i.p.) was administered on days 0, 2, 4 and 6. Mechanical nociceptive threshold was measured before the sound stress, and again on days 0 (30 min after first dose of paclitaxel), 1, 3, 5, 7, 14, 21 and 28. *Percentage reduction from baseline:* The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 28) was significantly greater in the sound stress exposed rats (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 03764, P=0.7706; Time, $F_{(2.3, 22.6)}$ = 2.83, P=0.1095; Stress, $F_{(1, 10)}$ = 180.5, P<0.0001, n=6 per group). *Nociceptive threshold:* The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 28) was significantly greater in the sound stress exposed rats (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 7.243, P=0.0009; Time, $F_{(2.9, 22.9)}$ = 15.44, P<0.0001; Stress, $F_{(1, 10)}$ = 25.04, P=0.0005).

Stress after paclitaxel (right two panels): Paclitaxel (1 mg/kg, i.p.) was administered on days 0, 2, 4 and 6, and, sound stress sessions started 24 h after the last paclitaxel injection, on days 7, 9 and 10. Mechanical nociceptive threshold was evaluated on days 0 (30 min after first dose of paclitaxel), 1, 3, 5, 7, 14, 21 and 28. *Percentage reduction from baseline:* There was no significant difference between paclitaxel + sound stress and paclitaxel + sham sound stress groups (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 3.650, P=0.0235; Time, $F_{(3, 30)}$ = 14.87, P<0.0001; Stress, $F_{(1, 10)}$ = 0.5977, P=0.4573, n=6 per group). *Nociceptive threshold:* The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 28) was not significantly different in the sound stress exposed rats (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)}$ = 0.0923, P=0.9637; Time, $F_{(2.9, 22.9)}$ = 15.31, P<0.0001; Stress, $F_{(1, 10)}$ = 0.1573, P=0.7000).
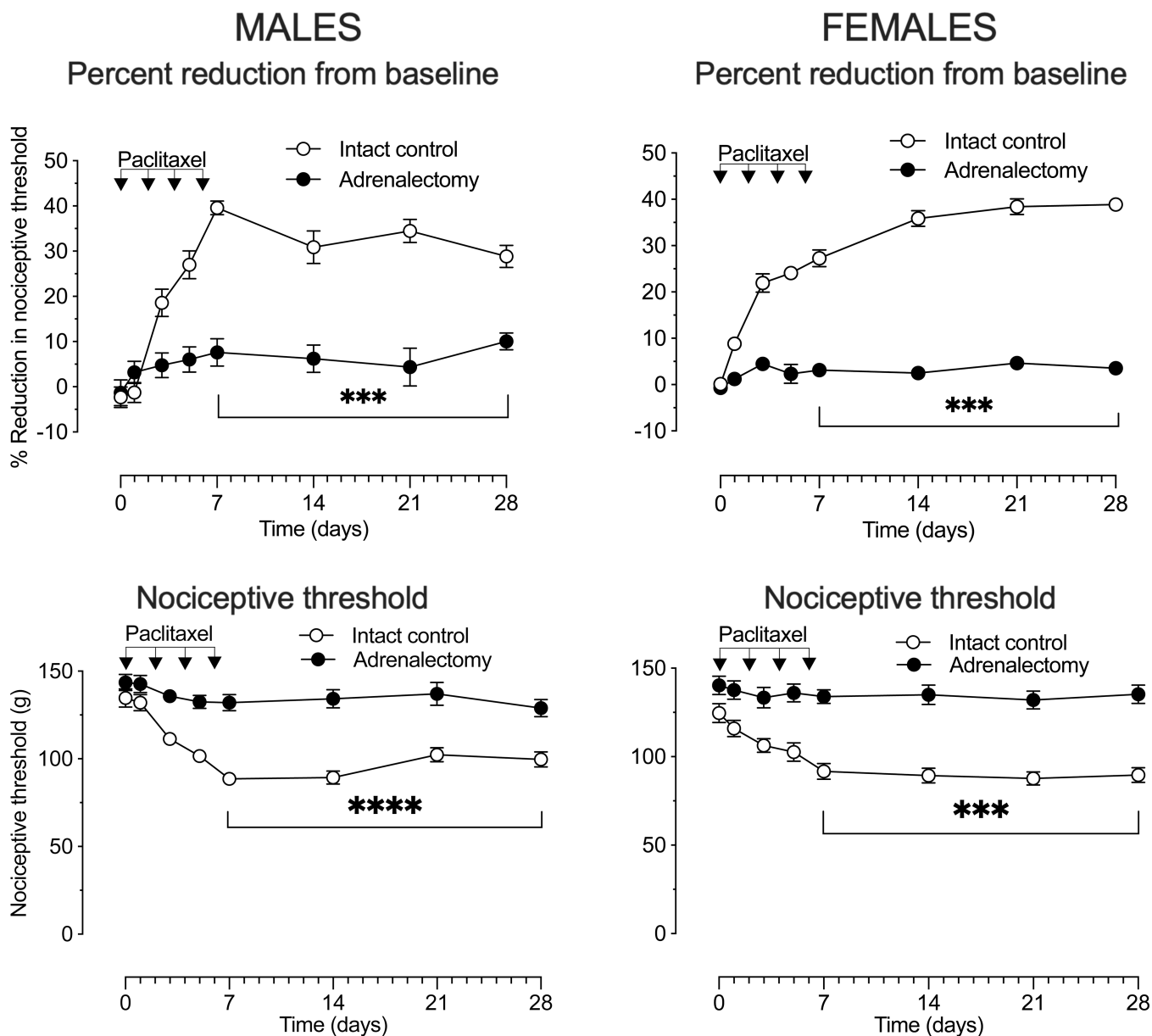
Figure 3. Effect of adrenalectomy on paclitaxel-induced hyperalgesia.
One week after bilateral adrenalectomy (with basal level corticosterone replacement), rats received paclitaxel (1 mg/kg, i.p.) on days 0, 2, 4 and 6. Mechanical nociceptive threshold was evaluated before surgery, and again on days 0 (30 min after first paclitaxel), 1, 3, 5, 7, 14, 21 and 28. *Males*: The magnitude of hyperalgesia measured after paclitaxel administration (i.e., on days 7 – 28) was markedly attenuated in adrenalectomized rats (2-way repeated measures ANOVA: Interaction, $F_{(3, 48)} = 3.373$, P=0.0259; Time, $F_{(3, 48)} = 1.974$, P=0.1304; Stress, $F_{(1, 16)} = 44.59$, P<0.0001). (n=6 per group). *Females*: The magnitude of hyperalgesia measured after paclitaxel administration (i.e., on days 7 – 28) was also markedly attenuated in adrenalectomized rats (2-way repeated measures ANOVA: Interaction, $F_{(3, 30)} = 14.47$, P<0.0001; Time, $F_{(3, 30} = 18.98$, P<0.0001; Stress, $F_{(1, 10)} = 381.0$, P<0.0001. n=6 per group).
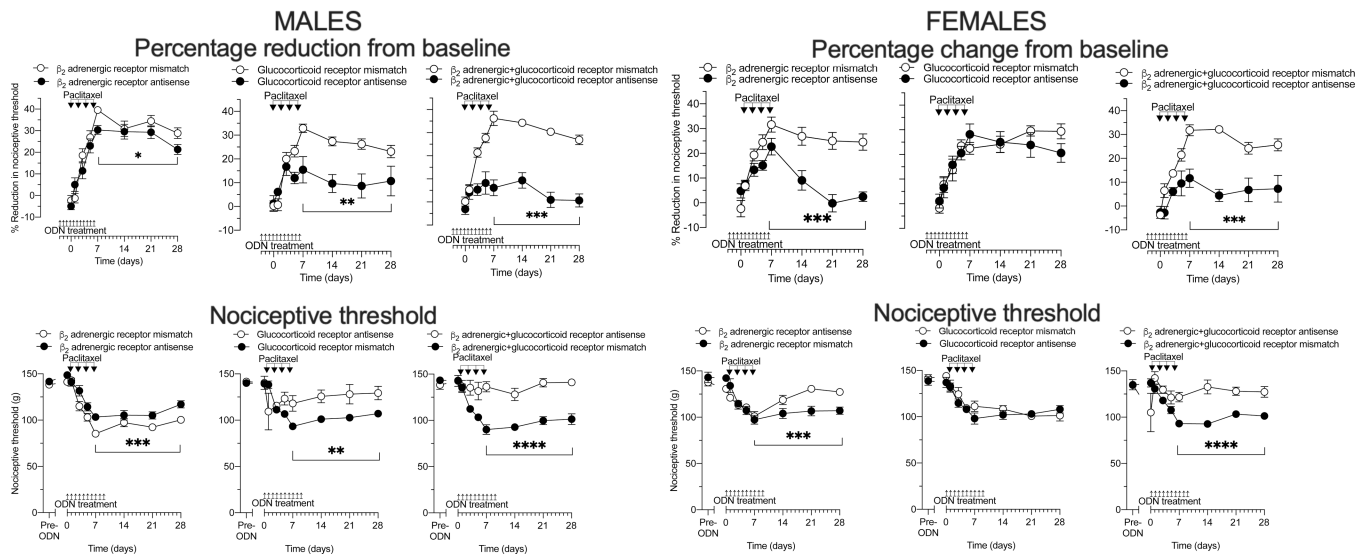
Figure 4. Role of β₂-adrenergic and glucocorticoid receptors in paclitaxel-induced mechanical hyperalgesia in male and female rats.

Male and female rats were treated intrathecally with injections of ODN antisense or mismatch for the β₂-adrenergic receptor (left panels) or glucocorticoid receptor (middle panels) mRNA, or their combination (right panels), for 10 consecutive days (80 μg/day). Beginning on the 4th day of ODN treatment (day 0), paclitaxel (1 mg/kg, i.p.) was administered on days 0, 2, 4 and 6. Mechanical nociceptive threshold was evaluated before ODN treatment was started, and again on days 0 (30 min after first paclitaxel), 1, 3, 5, 7, 14, 21 and 28.

Males: The magnitude of hyperalgesia measured after administration of the last dose of paclitaxel (i.e. days 7 – 28) was significantly attenuated in the β₂-adrenergic receptor antisense ODN treated group (Interaction, $F_{(3, 30)} = 1.022$, P=0.3970; Time, $F_{(3, 30)} = 5.952$, P=0.0026; Treatment, $F_{(1, 10)} = 5.773$, *P=0.0371), glucocorticoid receptor antisense ODN treated group (Interaction, $F_{(3, 30)} = 0.5439$, P=0.6560; Time, $F_{(3, 30)} = 3.602$, P=0.0247; Treatment, $F_{(1, 10)} = 11.68$, **P=0.0066) and in the group treated with the combination of the β₂-adrenergic receptor and glucocorticoid receptor ODN (Interaction, $F_{(3, 30)} = 0.5107$, P=0.6780; Time, $F_{(3, 30)} = 5.261$, **P=0.0049; Treatment, $F_{(1, 10)} = 128.6$, ***P<0.0001), when antisense and mismatch groups are compared over time, two-way repeated measures ANOVA). (n = 6 per group). The glucocorticoid + β₂-adrenergic receptor ODN treated group was not significantly different from glucocorticoid receptor ODN treated group (Interaction, $F_{(3, 30)} = 1.057$, P=0.3818; Time, $F_{(3, 30)} = 1.792$, P=0.1699; Treatment, $F_{(1, 10)} = 1.952$, P=0.1926). *Females*: The magnitude of hyperalgesia measured after the last dose of paclitaxel (i.e. days 7 – 28) was significantly attenuated in the β₂-adrenergic receptor antisense ODN treated group (Interaction, $F_{(3, 30)} = 2.899$, P=0.0512; Time, $F_{(3, 30)} = 10.36$, P<0.0001; Treatment, $F_{(1, 10)} = 38.41$, ***P=0.0001), but was not attenuated in the glucocorticoid receptor antisense ODN treated group (Interaction, $F_{(3, 30)} = 3.003$, P=0.0459; Time, $F_{(3, 30)} = 2.389$, P=0.8685; Treatment, $F_{(1, 10)} = 0.2463$, P=0.6304). Paclitaxel-induced hyperalgesia was significantly attenuated in the group receiving the combination of β₂-adrenergic receptor and glucocorticoid receptor antisense ODN (Interaction, $F_{(3, 30)} = 1.106$, P=0.3622; Time, $F_{(3, 30)} = 1.533$, P=0.2263; Treatment, $F_{(1, 10)}$

= 46.19, ***P<0.0001) groups, when antisense and mismatch groups are compared over time by two-way repeated measures ANOVA). The glucocorticoid + β_2-adrenergic receptor ODN treated group was not significantly different from β_2-adrenergic receptor ODN treated group (Interaction, $F_{(3, 30)}$ = 2.587, P=0.0715; Time, $F_{(3, 30)}$ = 5.804, P=0.0030; Treatment, $F_{(1, 10)}$ = 0.0983, P=0.0.7604. n = 6 per group).
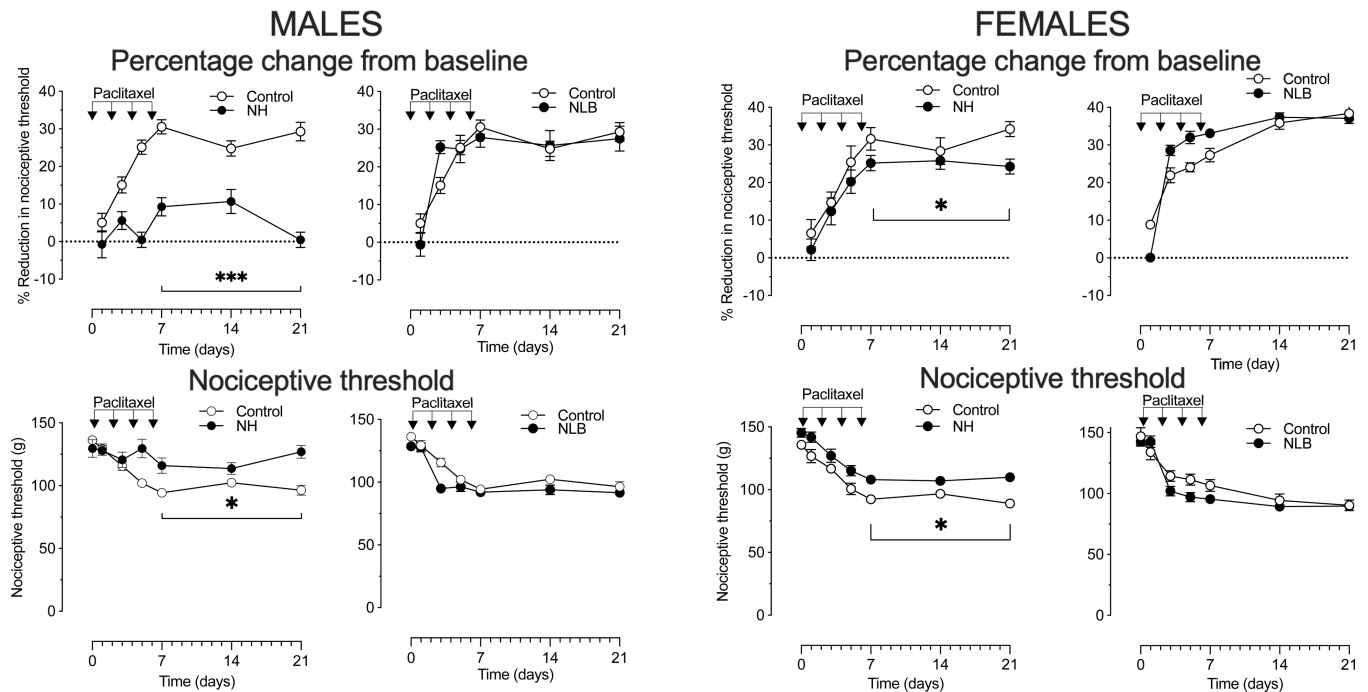
Figure 5. Effect of neonatal handling (NH) and neonatal limited bedding (NLB) on paclitaxel-induced hyperalgesia in male and female rats.

Neonatal rats were exposed to either NH (resilient phenotype) or NLB (stressed phenotype). Approximately 5 weeks later, adult rats received paclitaxel (1 mg/kg, i.p.) administered on days 0, 2, 4 and 6. Mechanical nociceptive thresholds were evaluated before the paclitaxel treatment was started, and again on days 0 (30 min after first paclitaxel), 1, 3, 5, 7, 14, 21 and 28.

Males (left 4 panels)

Percentage reduction from baseline: The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was significantly attenuated in NH males (Interaction, $F_{(2, 20)}$ = 5.636, P=0.0114; Time, $F_{(2, 20)}$ = 2.669, P=0.0939; Neonatal handling, $F_{(1, 10)}$ = 90.92, ***P<0.0001, n=6 per group). In NLB rats, the magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was not significant compared to controls (Interaction, $F_{(2, 20)}$ = 0.5073, P=0.6097; Time, $F_{(2, 20)}$ = 2.547, P=0.1034; Neonatal limited bedding, $F_{(1, 10)}$ = 0.1302, P=0.7258). *Nociceptive threshold:* The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was significantly attenuated in NH males (Interaction, $F_{(2, 20)}$ = 5.515, P=0.0124; Time, $F_{(2, 20)}$ = 2.503, P=0.1071; Neonatal handling, $F_{(1, 10)}$ = 17.52, ***P=0.0019). In NLB rats, the magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was not significant compared to controls (Interaction, $F_{(2, 20)}$ = 0.7462, P=0.4869; Time, $F_{(2, 20)}$ = 2.341, P=0.1220; Neonatal limited bedding, $F_{(1, 10)}$ = 2.562, P=0.1406).

Females (right 4 panels)

Percentage reduction from baseline: The magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was slightly, but significantly less in NH females (Interaction, $F_{(2, 32)}$ = 1.749, P=0.1901; Time, $F_{(2, 32)}$ = 0.5997, P=0.5550; Neonatal

handling, $F_{(1, 16)} = 4.898$, P=0.0418, Control n=6, NH n=12). In NLB females, the magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was not significant from control (Interaction, $F_{(2, 20)} = 6.738$, P=0.0058; Time, $F_{(2, 20)} = 34.14$, P<0.0001; Neonatal limited bedding, $F_{(1, 10)} = 1.275$, P=0.2852, n=6 per group). *Nociceptive threshold:* he magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was slightly, but significantly less in NH females (Interaction, $F_{(2, 32)} = 1.714$, P=0.1963; Time, $F_{(2, 32)} = 0.3806$, P=0.6865; Neonatal handling, $F_{(1, 16)} = 45.52$, P<0.0001, Control n=6, NH n=12). In NLB females, the magnitude of hyperalgesia measured after the last paclitaxel administration (i.e. days 7 – 21) was not significant from control (Interaction, $F_{(2, 20)} = 8.128$, P=0.0026; Time, $F_{(2, 20)} = 39.24$, P<0.0001; Neonatal limited bedding, $F_{(1, 10)} = 1.190$, P=0.3010).