Received: 17 August 2023 | Revised: 25 January 2024 | Accepted: 13 February 2024

DOI: 10.1002/dev.22478

Developmental Psychobiology WILEY

RESEARCH ARTICLE

Repetitive neonatal procedural pain affects stress-induced plasma corticosterone increase in young adult females but not in male rats

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Abstract

Exposure to repetitive painful procedures in the neonatal intensive care unit results in long-lasting effects, especially visible after a "second hit" in adulthood. As the nociceptive system and the hypothalamic–pituitary–adrenal (HPA) axis interact and are vulnerable in early life, repetitive painful procedures in neonates may affect later-life HPA axis reactivity. The first aim of the present study was to investigate the effects of repetitive neonatal procedural pain on plasma corticosterone levels after mild acute stress (MAS) in young adult rats. Second, the study examined if MAS acts as a "second hit" and affects mechanical sensitivity. Fifty-two rats were either needle pricked four times a day, disturbed, or left undisturbed during the first neonatal week. At 8 weeks, the animals were subjected to MAS, and plasma was collected before (t0), after MAS (t20), and at recovery (t60). Corticosterone levels were analyzed using an enzyme-linked immunosorbent assay, and mechanical sensitivity was assessed with von Frey filaments. Results demonstrate that repetitive neonatal procedural pain reduces stress-induced plasma corticosterone increase after MAS only in young adult females and not in males. Furthermore, MAS does not affect mechanical sensitivity in young adult rats. Altogether, the results suggest an age- and sex-dependent effect of repetitive neonatal procedural pain on HPA axis reprogramming.

KEYWORDS
acute stress, corticosterone, HPA axis, neonatal pain

1 | INTRODUCTION

Worldwide, more than 15 million infants are born prematurely (before 37 weeks of gestation) every year, and this number is increasing (Blencowe et al., 2013; Tielsch, 2015). As a result, many newborns require admission to a neonatal intensive care unit (NICU), where they undergo an average of 10–14 daily pain- and stress-full procedures (Carbajal et al., 2008; Cruz et al., 2016). These repetitive procedures experienced in the NICU take place at a time of neurodevelopmental vulnerability, when the nociceptive circuits are still maturing (Beggs et al., 2012; de Kort et al., 2022). This continued stimulation of the developing nociceptive circuits in the NICU leads to long-term effects reaching adulthood (Chau et al., 2019; Grunau, 2013; Matthews, 2002; Walker et al., 2018). For instance, former NICU patients are likely to develop psychiatric disorders, and clinical symptoms of internalization and somatization, all of which are stress sensitive (Grunau et al., 1994;

McLean et al., 2023; Ranger et al., 2014; Vinall et al., 2013). Preclinical studies have shown that the repetitive stimulation of the developing nociceptive circuits has long-term effects on nociception, cognition, and emotional behavior (Butkevich et al., 2022; Chen et al., 2016; Knaepen et al., 2013; Mooney-Leber & Brummelte, 2020; van den Hoogen et al., 2018, 2020). In line with the maturation of the nociceptive circuits, the hypothalamic–pituitary–adrenal (HPA) axis, a system responsible for the stress response, is susceptible to reprogramming by early-life adverse events like those in the NICU (Brummelte et al., 2015; Carbajal et al., 2008; Matthews, 2002; Meesters et al., 2023; Vinall et al., 2013). Because painful procedures in the NICU acutely affect the HPA axis (Carbajal et al., 2008; Grunau et al., 2005; Mooney-Leber & Brummelte, 2017) and may affect its maturation, it is of major interest to study the effects of repetitive neonatal procedural pain on stress responses in later life.

Given the relevance of age in assessing the effects of neonatal pain on the HPA axis reactivity, the present study defined developmental periods based on HPA axis development (Green & McCormick, 2016; McCormick et al., 2016). In females, the prepubertal period extends from postnatal day 22 (P22) to P34 and the postpubertal period from P35 to P59. In males, the prepubertal period is defined between P22 and P40 and the postpubertal period from P41 to P59. From a translational perspective, rodents postpubertal period can be divided between adolescence (from P41 in males, and P35 in females, to P49) and young adulthood (from P50 to P60), which is equivalent to 18–20 years of age in humans (Semple et al., 2013). In males and females, adulthood starts at P60 (Green & McCormick, 2016; McCormick et al., 2016).

Clinical studies have shown that continued neonatal procedural pain shifts the level of the main stress hormone cortisol in former NICU patients (Brummelte et al., 2015; Grunau et al., 2004, 2005; McLean et al., 2023; Mörelius et al., 2016; Olszewska et al., 2022; Stoye et al., 2022). Nevertheless, the long-term effects of repetitive neonatal procedural pain on the HPA axis are still unclear and the directionality of this effect seems to depend on exposure intensity in combination with gender and age (Grunau, 2013; LaPrairie & Murphy, 2010; Matthews, 2002). Several preclinical studies indicate that continued stimulation of the developing nociceptive circuits affects the HPA axis reactivity to acute stress in a sex- and age-dependent manner (Butkevich et al., 2022, 2023; Chen et al., 2016; Davis et al., 2018; Mikhailenko et al., 2021; Mooney-Leber & Brummelte, 2020). Indeed, Mooney-Leber and colleagues concluded that noxious neonatal stimulation causes blunted HPA axis reactivity in adult females only (Mooney-Leber & Brummelte, 2020), and Butkevich and colleagues determined that only adult males presented increased corticosterone levels after acute stress (Butkevich et al., 2022). Using a preclinical rat model, Mikhailenko and colleagues established that neonatal inflammatory-induced noxious stimulation decreases levels of corticosterone 30 min after acute stress in postpubertal adulthood (Mikhailenko et al., 2021). Nevertheless, the effects of repetitive neonatal procedural pain on the stress response of young adult males and females still are to be elucidated.

The first aim of the present study was to investigate the effects of repetitive neonatal procedural pain on plasma corticosterone levels and HPA axis reactivity after mild acute stress (MAS) in young adult female and male rats. To this end, the well-established needle prick rat model of repetitive neonatal procedural pain was used (de Kort et al., 2021; van den Hoogen et al., 2018, 2020), and animals underwent MAS at 8 weeks of age. In the needle prick model, repetitive neonatal procedural pain results in nociceptive behavioral changes only after a second painful event in adulthood ("second hit" model), conforming to a mismatch between early-life programming following neonatal pain and later-life environment (Catuzzi & Beck, 2014; Davis et al., 2018; de Kort et al., 2021; Knaepen et al., 2013; Nederhof & Schmidt, 2012; van den Hoogen et al., 2018, 2020). In light of the known relation between the HPA axis and nociception (Madalena & Lerch, 2017), the second aim of the present study was to investigate whether adult acute stress may act as a "second hit." It thus may affect nociception as evaluated based on von Frey's mechanical sensitivity in animals previously exposed to repetitive neonatal procedural pain.

2 | METHODS

2.1 | Ethics statement

All animal experiments were performed in accordance with the European Directive for Protection of Vertebrate Animal Use for Experimental and Other Scientific Purposes (86/609/EEC) and were approved by the Committee for Experiments on Animals, Maastricht, the Netherlands (DEC 2017-017).

2.2 | Animals

For this study, 52 Sprague–Dawley (SD) male and female rat pups from six time-pregnant SD dams were used (Charles River). Breeding of dams was performed and took place in-house at Maastricht University animal facilities. On the day of birth, referred to as P0, the litters were culled to a maximum of $N = 10$, and pups were randomly assigned using software (random.org) to neonatal condition and adult treatment (Table 1). At P21, pups were weaned and housed in groups of two in same-sex individually ventilated cages, in a temperature (19–24°C) and humidity (55% ± 15%) controlled room with a reversed 12-h/12-h day–night cycle and background music. Ad libitum water and food were available throughout the whole study period.

2.3 | Neonatal procedures

To model repetitive procedural pain exposure in the NICU, a repetitive neonatal needle prick model was implemented as previously described by Knaepen et al. (2013). Newborn pups were noxiously stimulated four times a day via unilateral 2-mm calibrated needle pricks in the

TABLE 1 Group distribution per litter.

Litter	1				2				3		4		5				6			
Neonatal condition	NP		DC		NP		DC		UC		NP		DC		NP		DC		UC	
Young adult treatment	MAS	H	MAS	H	MAS	H	MAS	H	MAS	H	MAS	H	MAS	H	MAS	H	MAS	H	MAS	H
N females	1	0	1	2	2	2	2	2	2	2	0	1	1	0	2	1	1	1	2	2
N males	2	2	2	0	1	0	1	0	2	2	2	1	1	2	0	1	1	0	2	2

Note: For this experiment, 52 female (F) and male (M) Sprague–Dawley rats were born from six time-pregnant dams. Neonatal conditions (NP: needle pricks; DC: disturbed control) and adult treatment (MAS: mild acute stress; H: handling) were randomly assigned at birth using a software. Undisturbed control (UC) pups were randomly assigned to postpubertal treatment prior to weaning.

midplantar surface of the left hind paw from P0 to P7 (needle prick, NP, $N = 18$) (Figure 1). Control animals were shortly handled at the same hourly intervals as the NP animals (disturbed control, DC, $N = 18$) or were left undisturbed (undisturbed control, UC, $N = 16$).

2.4 | Von Frey for mechanical sensitivity in neonates

Paw withdrawal thresholds (PWTs) of the ipsi- and contralateral hind paws were assessed before (BL) and 1, 3, and 5 h after the last noxious stimulation or handling using a dorsal von Frey design (Marsh et al., 1999). Ascending von Frey filaments (bending force 0.407 g, 0.692 g, 1.202 g, 2.041 g, 3.63 g [from P4 onward], and 5.495 g [from P6 onward]; Stoelting) were applied five times to the dorsal surface of the hind paws. The number of positive responses (paw withdrawal or flinching behavior evoked by the filaments) per filament was recorded, and behavioral testing was discontinued when five positive responses were observed. A 50% PWT was calculated using a sigmoidal curve fitting in GraphPad Prism 9.5.1 (GraphPad Software).

2.5 | Von Frey test for mechanical sensitivity in young adults

Mechanical sensitivity was assessed weekly by determining the PWT of the hind paws in response to calibrated von Frey filaments. Briefly, animals were placed in a transparent box resting on an elevated mesh floor. After a 10-min acclimation period, a series of von Frey filaments (bending forces 1.202 g, 2.041 g, 3.63 g, 5.495 g, 8.511 g, 15.136 g, and 28.84 g; Stoelting) were applied to the plantar surface of the hind paw for 5 s using the up-down method. Mechanical sensitivity was assessed weekly from 3 to 7 weeks of age and 24 h after MAS (Figure 1). Male and female rats were tested separately. Researchers were blinded to treatment groups during behavioral testing and throughout the entire (postweaning) experimental protocol (NP = 18; DC = 18; UC = 16).

2.6 | Adult acute stress test and blood sampling

To assess the HPA axis response to acute stress in young adult animals ($N = 28$; NP female = 5; NP male = 5; DC females = 5; DC males = 5; UC females = 4; UC males = 4), they were subjected to acute mild stress (MAS) at 8 weeks of age, and blood samples were collected (Van den Hove et al., 2014). To minimize stress during blood sampling, animals were handled for 4 days before the MAS (Figure 1) (Fluttert et al., 2000). Briefly, the animal was taken from their home cage, and placed in a cloth, and front and hind paws were restrained by the experimenter. The animal was lightly turned on its side and the experimenter ran their finger on the tail three to five times to mimic blood collection. On the day of the MAS, the animal was taken from their home cage and immediately placed in a cloth. The lateral tail vein was identified by turning the animal on their side and a small 1- to 2-cm incision was made with

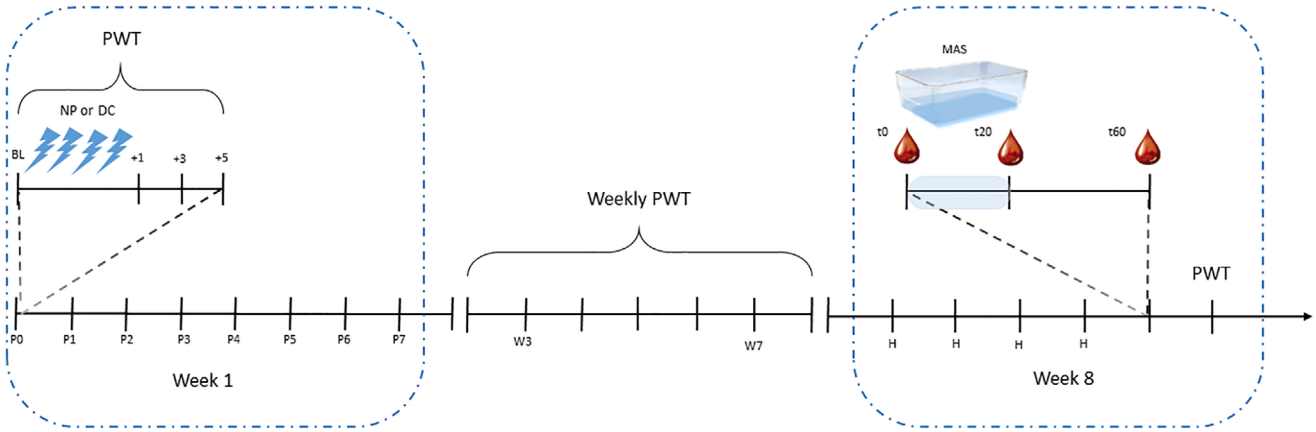

FIGURE 1 Experimental design and timeline. From postnatal day 0 (P0) to P7, animals were either needle pricked (NP, $N = 18$), handled (DC, $N = 18$) four times a day, or were left undisturbed (UC, $N = 16$). Paw withdrawal threshold (PWT) was assessed before (BL) and 1, 3, and 5 h (+1, +3, +5) after the last needle prick or handling using von Frey filaments. At 3 weeks, animals were weaned and PWT was measured weekly from week (W)3 to W7. At 8 weeks, all animals were habituated (H) to tail vein blood sampling for 4 days. On the 5th day, animals were either stressed with an acute mild acute stress (MAS) (NP = 10; DC = 10; UC = 8) or handled (NP = 8 DC = 8; UC = 8). Blood samples were collected before (t0), right after MAS (t20), and at recovery (t60). Finally, PWT was assessed 1 day after MAS.

a razor blade. A total of 100 μL of blood was collected using heparin Microvette (ref #16.443; Sarstedt) (t0). The animal was then placed in a mouse cage (35 × 19 × 14 cm) with 1.5 cm of 21°C water for 20 min. After 20 min in the mouse cage, the animal gently was dried and blood was collected by re-opening the tail vein incision (t20). Then, the animal immediately returned to the home cage. Sixty minutes after the first blood sampling, a last blood sample was taken (t60) to assess recovery of corticosterone levels. All blood samples were taken within 3 min of removal from the home cage. To control for the effect of MAS on nociceptive behavior, a separate set of animals ($N = 24$; NP female = 4; NP male = 4; DC females = 4; DC males = 4; UC females = 4; UC males = 4) were handled, as previously described, at t0, t20, and t60. Researchers were blinded to neonatal conditions and young adult treatment.

2.7 | Plasma corticosterone levels

Immediately after collection, blood samples were centrifuged at maximum speed (18,000 rpm) at 4°C for 10 min. Plasma samples were extracted and stored at −80°C until further processing. Plasma corticosterone levels were analyzed using a standard CORT ELISA kit (catalog # RE52211; Tecan) per the manufacturer's recommendations (Smith et al., 2020). Samples were run in duplicates and the researcher was blinded to neonatal condition. MAS-induced corticosterone increase was calculated by normalizing t20 and t40 values over t0.

2.8 | Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). As planned before the collection of data, differences in mechanical sensitivity during the neonatal period and postweaning were analyzed using a repeated measure analysis of variance (ANOVA) with Holm–Sidak post hoc correction. If a sex effect was not observed, data were pooled by condition to increase power. Changes in corticosterone levels before and after MAS were analyzed using a one-sided repeated measure ANOVA followed by Holm–Sidak post hoc correction, as planned before sample collection. The presence of outliers was tested using the robust regression and outlier removal method ($Q = 1\%$) and if needed, values were excluded from subsequent analysis. All statistical analyses were conducted using GraphPad Prism 9.5.1 (GraphPad Software) and results were considered significant at $p < .05$.

3 | RESULTS

3.1 | Neonatal needle pricks, but not handling, decrease the ipsilateral mechanical PTW

From P0 to P7, 52 animals were subjected daily to four needle pricks (NP) and handling (DC) or were left undisturbed. To verify the model (de Kort et al., 2021; Knaepen et al., 2013; van den Hoogen et al., 2018, 2020), mechanical sensitivity was tested daily with von Frey before and 1, 3, and 5 h after the last needle pricking or handling session. As sex did not significantly affect ipsilateral ($F_{1,32} = 0.2171$, $p > .05$) and contralateral ($F_{1,32} = 0.0781$, $p > .05$) baseline PWT at P0, males and females were pooled to increase power. PWT was shown to significantly increase over time ($F_{31, 1054} = 11.40$, $p < .001$), as expected with the increasing weight and thickened skin texture (Figure 2). Neonatal condition significantly affected PWT ($F_{1, 34} = 32.79$, $p < .001$), and an interaction effect between time and neonatal condition was observed ($F_{31, 1054} = 11.40$, $p < .001$). Post hoc analysis revealed that NP animals had significantly lower PWT at P5 + 1 h; P5 + 3 h; P5 + 5 h; and from P6 + 1 h to P7 + 5 h compared to DC animals.

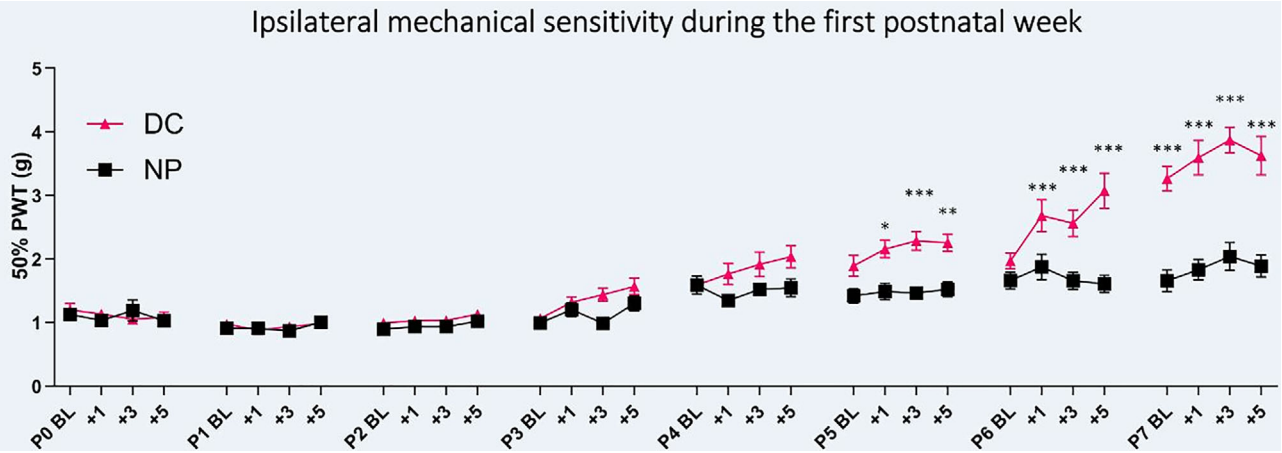

FIGURE 2 Mechanical sensitivity after needle prick or handling during the neonatal week. From postnatal day 5 (P5), repetitive needle prick (NP, $N = 18$) results in decreased ipsilateral paw withdrawal threshold (PWT) compared to handling (DC, $N = 18$) ($F_{1, 34} = 32.79$, $p < .001$). From P0 to P7, PWT significantly increased over time independently of neonatal condition ($F_{31, 1054} = 11.40$, $p < .001$). P0–7, postnatal day 0 to 7; BL, baseline von Frey measurement; +1/+3/+5, von Frey measurement 1/3/5 h after last needle pricks on P0–7. Data plotted as mean ± SEM. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

3.2 | Female but not male rats previously exposed to neonatal needle pricks have reduced corticosterone increase after MAS

At the age of 8 weeks, corresponding to young adulthood, animals were either subjected to MAS or handled. Plasma was collected immediately after the animal was removed from the cage (t0), immediately after ending MAS (t20), and at recovery (t60). Corticosterone levels were assessed at the three time points, and males and females were analyzed separately. Sex significantly affected plasma corticosterone levels at baseline ($F_{1, 19} = 43.17$, $p < .0001$), t20 ($F_{1, 21} = 50.88$, $p < .0001$), and t60 ($F_{1, 20} = 11.86$, $p < .01$). Hence, male and female levels were analyzed separately. In both females and males, time significantly affected corticosterone plasma levels (females $F_{1.590, 17.49} = 29.61$, $p < .01$; males $F_{1.643, 15.60} = 34.10$, $p < .01$). Post hoc comparisons revealed that in females and males, the neonatal condition did not affect t0 levels of corticosterone plasma levels. For all neonatal conditions (NP, DC, and UD) and sex, MAS results in an increase in plasma corticosterone levels at t20 ($p < .05$) (Figure 3d,e), except for NP females, which showed a trend to stress-induced increase in corticosterone levels at t20 ($p = .07$) and t60 ($p = .09$) (Figure 3c). At t20 and t60, no main effect of neonatal condition was observed on plasma corticosterone levels (females $F_{2, 11} = 1.113$, $p > .05$; males $F_{2, 10} = 1.22$, $p > .05$). In young adult males, one result was identified as an outlier at t0 and therefore removed from subsequent analysis.

MAS-induced corticosterone increase was calculated by normalizing t20 and t40 values over t0 (Figure 3a,b). While neonatal condition did not significantly affect corticosterone percentage changes from t0 ($F_{2, 11} = 1.10$, $p > .005$), post hoc analyses revealed that only in females neonatal NP minimizes corticosterone response to MAS at t20 compared to DC (neonatal handling) ($p = .02$) (Figures 3a, S1, and S2). Analysis of corticosterone percentage change in male rats indicated a significant effect of time ($F_{2, 19} = 31.33$, $p < .01$), but no effect of neonatal conditions ($F_{2, 10} = 0.05207$, $p > .05$) (Figures 3b, S3, and S4).

3.3 | MAS in young adult rats previously exposed to neonatal procedural pain does not affect mechanical sensitivity

After weaning at week 3 and up to 7 weeks of age, ipsi- and contralateral mechanical sensitivity was assessed weekly with von Frey. Overall and in line with previous research, time affected PWT ($F_{4, 193} = 18.26$, $p < .001$) (de Kort et al., 2021; van den Hoogen et al., 2016). No effect of neonatal condition was noted on ipsilateral ($F_{2, 49} = 0.9267$, $p > .05$) and contralateral ($F_{2, 49} = 0.1860$, $p > .05$) PWT.

To investigate whether neonatal condition affects mechanical sensitivity after adult MAS, ipsi- and contralateral PWTs were assessed using von Frey filaments a day after MAS. As sex did not significantly affect ipsilateral ($F_{1, 40} = 0.5345$, $p > .05$) nor contralateral ($F_{1, 38} = 0.2739$, $p > .05$) PWT, data from male and female animals were pooled to further increase statistical power. Neonatal condition did not affect ipsilateral ($F_{2, 46} = 1.56$, $p > .05$) nor contralateral ($F_{2, 42} = 2.10$, $p > .05$) PWT (Figure 4). Although young adult treatment statistically affected ipsilateral PWT 24 h after MAS ($F_{1, 46} = 5.29$, $p = .026$), post hoc analyses revealed no significant effect of adult treatment within the neonatal condition (NP + stress vs. NP + handling; DC + stress vs. DC + handling; UC + stress vs. UC + handling) (Figure S5). There was no interaction between neonatal condition and adult treatment ($F_{2, 46} = 0.139$, $p > .05$). On the contralateral PWT, the robust regression and use of an outlier test resulted in the exclusion of four out of 52 values.

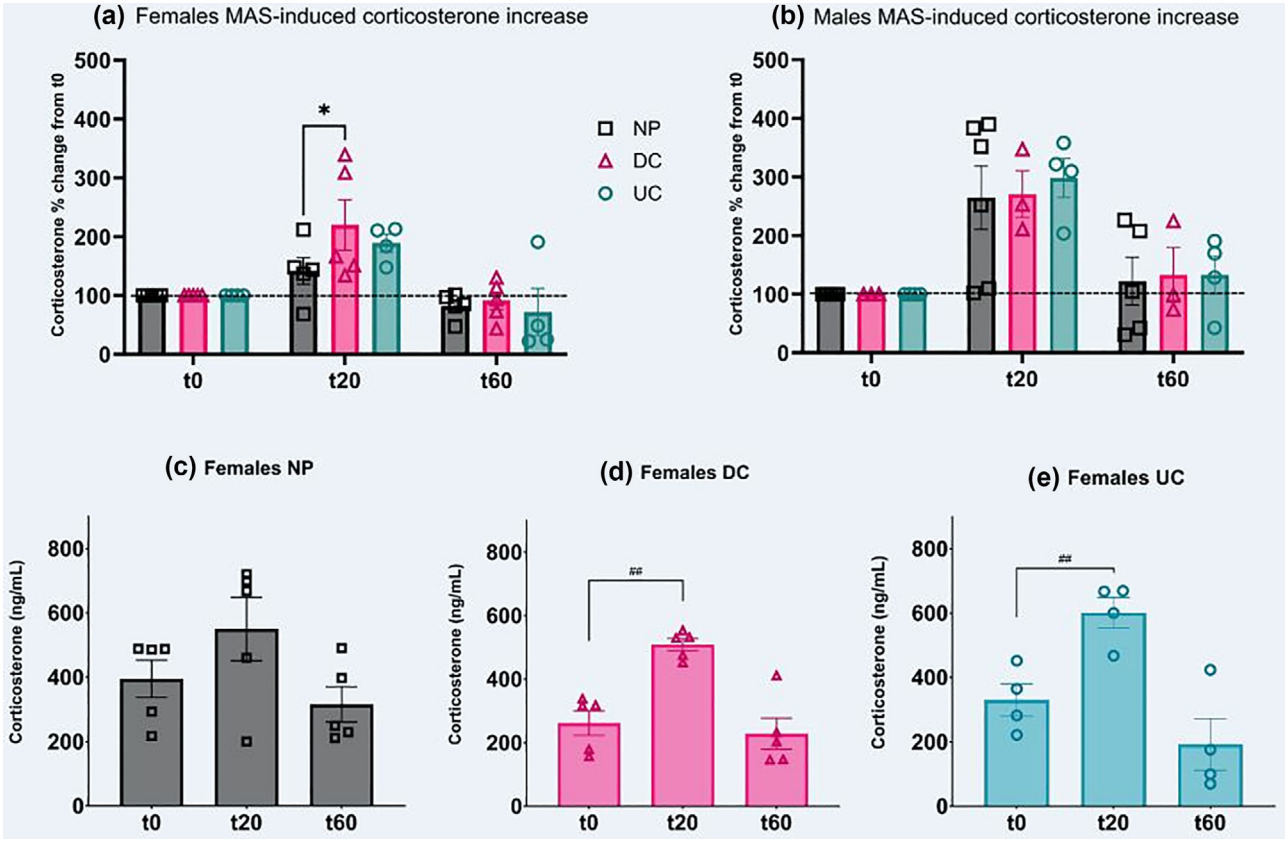

FIGURE 3 Neonatal needle pricks minimize acute stress-induced corticosterone increase in young adult females only. (a) Females previously exposed to neonatal needle prick (NP, $N = 5$) showed a reduced stress-induced corticosterone increase compared to neonatal handled females (DC, $N = 5$) ($p = .02$). No difference is noted between NP and UC females (UC, $N = 4$) ($p > .05$). (b) Neonatal conditions did not affect stress-induced corticosterone increase in males. (c) Only females exposed to neonatal needle pricks did not present an increase in corticosterone plasma levels after mild acute stress (MAS) ($p = .07$). Females from the disturbed control group (d) and the undisturbed control group (e) had a significant increase in plasma corticosterone levels after MAS ($p < .05$). t0, corticosterone levels at removal from home cage; t20 corticosterone levels immediately after MAS exposure; t60, corticosterone levels at recovery. Data plotted as mean ± SEM. Females NP versus females DC #$p < .05$. t0 versus t20 *$p < .01$.

4 | DISCUSSION

First, the study aimed to investigate the effects of repetitive neonatal procedural pain on plasma corticosterone levels and HPA axis reactivity after MAS in post pubertal females and males. Results show that following MAS in young adulthood, both male and female animals developed a stress-induced increase in plasma corticosterone levels. Furthermore, repetitive stimulation of the developing nociceptive circuits (from P0 to P7) results in a reduced stress-induced plasma corticosterone increase in females compared to controls. This effect was not observed in males. These results are somewhat similar to previous work from Mooney-Leber and colleagues, reporting that females exposed to neonatal needle pricks (from P1 to P4) developed a delayed return to baseline plasma corticosterone levels after acute stress in adulthood (Mooney-Leber & Brummelte, 2020). It should, however, be noted that Mooney-Leber and Brummelte did not only measure corticosterone levels but also assessed spatial memory 10 days before restrain stress, which may contribute to their results. The observed lack of effects of repetitive needle pricks on young adult male corticosterone reactivity may stem from the timing and age of stress exposure. Indeed, Chen and colleagues described a reduced stress-induced corticosterone increase in prepubertal but not in postpubertal males exposed to continued stimulation of the developing nociceptive circuits (Chen et al., 2016). Prepubertal males exposed to repetitive neonatal pain possess fewer corticotrophin-releasing hormone (CRH)-positive cells but higher CRH expression in the amygdala compared to controls, an effect not seen in females (Davis et al., 2021; Zuke et al., 2019). Effects of repetitive neonatal pricking on stress-induced corticosterone levels in adulthood need further investigation but, for now, cannot be excluded. Sex difference can also not be excluded, as females were not included (Chen et al., 2016). However, a single injury with 1% carrageen agent at P0 results in attenuated stress-induced corticosterone response in adult males and females (Victoria, Inoue, et al., 2013). Taken together, males exposed to repetitive stimulation of the developing nociceptive circuits display a reduced HPA axis response in the prepubertal period (Chen et al., 2016), while females are more vulnerable in the postpubertal period (Mooney-Leber & Brummelte, 2020). Those results are contrasted by studies concluding the absence

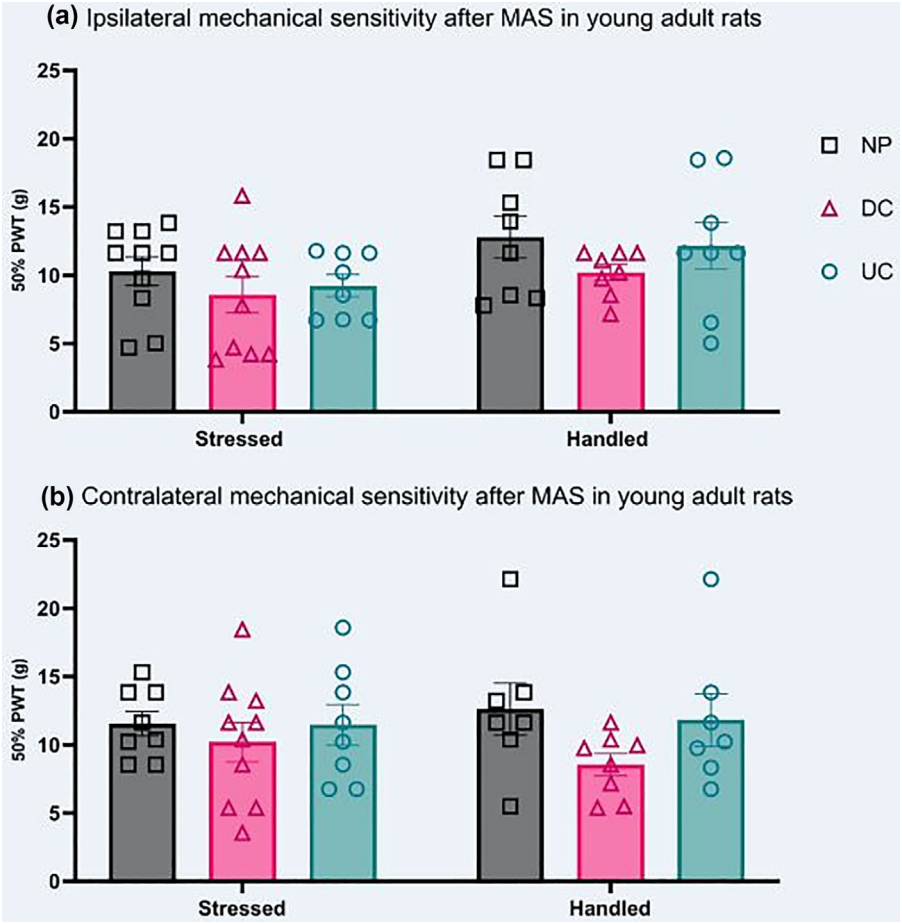

FIGURE 4 Paw withdrawal threshold (PWT) after later-life mild acute stress (MAS) in animals exposed to repetitive neonatal pain. Neonatal condition did not affect ipsilateral (a) or contralateral (b) PWT after MAS ($F_{2,46} = 1.56$, $p > .05$). MAS reduced ipsilateral PWT ($F_{1,46} = 5.29$, $p = .026$), but not contralateral PWT ($F_{1,42} = 0.00410$, $p > .05$). NP, needle prick ($N = 18$); DC, disturbed control ($N = 18$); UC, undisturbed control ($N = 16$). Data plotted as mean ± SEM.

of an effect of repetitive neonatal pain in prepubertal males and females (Davis et al., 2018; Walker et al., 2003).

While corticosterone is the most widely used marker for stress and the activity of the HPA axis in rats, other neurohormones are known to be involved in the modulation of the stress response. For instance, CRH is secreted by the hypothalamus and eventually prompts the release of adrenocorticotropic hormone (ACTH) by the pituitary glands. Furthermore, the HPA axis response depends on negative feedback from glucocorticoids on the glucocorticoid receptors (GR) and the mineralocorticoid receptors (MR), an adaptive response under the influence of sex hormones and epigenetically sensitive to early-life events (De Kloet et al., 1998; Goel et al., 2011; Keller-Wood & Dallman, 1984; Nicolaides et al., 2015). Early-life adversities, including repetitive neonatal procedural pain, promote continued increase of glucocorticoids levels in rodents and human alike (Grunau et al., 2004; McLean et al., 2023; Mooney-Leber et al., 2018; Olszewska et al., 2022; Victoria, Karom, et al., 2013). Rodent females may be more sensitive to the heightened glucocorticoids levels as suggested by their higher ACTH response at P8 following ether inhalation (Hary & Dupouy Isabelle Grégoire, 1986). On the other hand, the attenuated ACTH-stimulated adrenal responsivity observed in P14 males indicates a longer stress hyporesponsive period in males (Yoshimura et al., 2003). Possibly, due to the continued high glucocorticoid levels in the neonatal period, early-life stress enhances GR promotor acetylation and expression in the amygdala of adult females but not males (Louwies & Greenwood-Van Meerveld, 2020; Prusator & Greenwood-Van Meerveld, 2017). A previous study observed that a single painful event in the neonatal week increases GR expression in the paraventricular nucleus, decreases its expression in the hippocampus of adult rats, and results in attenuated stress-induced corticosterone response in adult rats (Victoria, Inoue, et al., 2013). Repetitive neonatal procedural pain was shown to also result in increased hippocampal GR levels in pre- and postpubertal males; females were not included in this study (Chen et al., 2016). The sex-dependent epigenetic regulation of the GR expression likely results in upregulation of GR and inhibition of the HPA axis, eventually leading to lower acute stress-induced corticosterone increase in young adult females. To untangle the effects of repetitive neonatal procedural pain on stress and the HPA axis, as well as gender-specific effects, future

research must focus on the distribution of GR and MR receptors and investigate their regulation by epigenetics, as well as the effect of GR-mediated alterations on the epigenome of males and females (Bartlett et al., 2019).

The needle prick model, while validated as a repetitive procedural pain model (see Section 3.1 and Figure 2), provides information on the cumulative effect of neonatal stress and pain (Mooney-Leber & Brummelte, 2017). The limited stress-induced corticosterone increase in NP females compared to DC suggests that the reprogramming of the HPA axis by repetitive neonatal procedural pain induces stress resilience in young adult females, contrary to the milder neonatal stress by handling alone (DC). This outcome adds to previous studies demonstrating that early-life moderate stressors, such as repetitive neonatal procedural pain, lead to adult resilience in rodents (e.g., reduced state anxiety, fear conditioning, and stress response) (Davis et al., 2018; de Kort et al., 2021; Palermo et al., 2020; Pfau & Russo, 2015; Victoria et al., 2015; Zuke et al., 2019). This effect of repetitive neonatal procedural pain on cortisol levels was also shown in school-age children with former NICU history (Brummelte et al., 2015; Lowe et al., 2023), although other clinical studies highlight cortisol level increase in young children (Grunau et al., 2004; Olszewska et al., 2022; Ranger et al., 2014). Unfortunately, clinical studies did not include possible gender differences in their analysis. Altogether, our results and previous clinical and preclinical studies suggest an age- and sex-dependent effect of repetitive neonatal procedural pain on the HPA axis (Brummelte et al., 2015; Chen et al., 2016; Green & McCormick, 2016; Grunau et al., 2004; Lowe et al., 2023; McLean et al., 2023; Mooney-Leber & Brummelte, 2020; Olszewska et al., 2022). Given that many studies did not include longitudinal and gendered effects, conclusions must be taken with precaution and additional longitudinal research including gender-related aspects is required.

The second aim of this study investigated whether repetitive neonatal procedural pain leads to a nociceptive maladaptive response to acute stress ("second hit") in the young adult. In line with previous studies, neonatal condition does not affect the healthy development of mechanical sensitivity from weeks 3 to 7 (de Kort et al., 2021; van den Hoogen et al., 2016). Our results demonstrate that NP animals did not develop stress-induced mechanical hypersensitivity after MAS "second hit" in the postpubertal period. Post hoc analysis did not reveal an effect of MAS across neonatal conditions, although an effect of MAS was noted on ipsilateral but not contralateral mechanical sensitivity. This result likely reflects a type I error rather than a behavioral effect, as the latter effect was independent of neonatal condition and limited to the ipsilateral paw in the control groups, which received identical treatment on the ipsi- and contralateral sides. The absence of effect of MAS on mechanical sensitivity in young adult animals is in line with clinical reality, where preintervention stress does not induce an increase in presurgery pain (Mamie et al., 2004). Preoperative stress, however, has the potential to affect the intensity and duration of postoperative pain: in another preclinical model, daily immobilization stress of adult rats for 4 consecutive days did not elicit changes in baseline mechanical sensitivity but did result in an increased duration of postoperative pain (Cao et al., 2015).

Although repetitive neonatal procedural pain does not result in a maladaptive nociceptive response following MAS in young adult animals, inadequate early-life programming of the nociceptive response after stress may be age dependent. Indeed, a previous study observed that neonatal stress (via handling or neonatal pain) causes tactile hypersensitivity in postweaning animals after fear conditioning (Davis et al., 2018). In combination with the already reported increased duration of adult postoperative pain in the needle prick animal model (Knaepen et al., 2013; van den Hoogen et al., 2018, 2020), designs must take into account possible synergistic effect of repetitive neonatal procedural pain and preoperative stress on the duration of postoperative pain in the postpubertal and adult rats.

A limitation of the present study might be the fact that the impact of the MAS as a "second hit" is not sufficient to trigger the HPA axis maladaptive response emerging from repetitive neonatal procedural pain. Indeed, the intensity and duration of stress, whether acute or chronic, are known to result in differential effects on the HPA axis response (Madalena & Lerch, 2017; Olango & Finn, 2014). A study by Victoria and colleagues indicates a bidirectional HPA axis response following inflammatory neonatal pain whether exposed to adult acute or chronic stress (Victoria et al., 2015). Hence, future studies should be designed to investigate chronic stress as a "second hit" and its effects on nociception in postpubertal and adult animals previously exposed to repetitive neonatal procedural pain.

ACKNOWLEDGMENTS

The completion of this research project would not have been possible without the scientific discussions with colleagues L. Heijmans (PhD), T. J. de Geus (MSc), I. Rudnick-Jansen (PhD), and M. Mons (PhD). We also would like to thank D. Hermes, W. Gerritsen, S. Claessen, and members of the animals' facility for their technical support and expertise.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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How to cite this article: Baudat, M., Simons, S. H. P., & Joosten, E. A. J. (2024). Repetitive neonatal procedural pain affects stress-induced plasma corticosterone increase in young adult females but not in male rats. *Developmental Psychobiology*, *66*, e22478. https://doi.org/10.1002/dev.22478