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ABSTRACT

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3 Oxytocin (OT), known for its neurohormonal effects around birth, has recently been suggested for
4 being a critical determinant in neurodevelopmental disorders. This hypothalamic neuropeptide
5 exerts a potent analgesic effect through an action on the nociceptive system. This endogenous
6 control of pain has an important adaptive value but might be altered by early life stress (ELS),
7 possibly contributing to its long-term consequences on pain responses and associated
8 comorbidities. We tested this hypothesis using a rat model of neonatal maternal separation (MS)
9 known to induce long term consequences on several brain functions including chronic stress,
10 anxiety, altered social behavior and visceral hypersensitivity. We found that adult rats with a
11 history of MS were hypersensitive to noxious mechanical/thermal hot stimuli and to inflammatory
12 pain. We failed to observe OT receptor-mediated stress-induced analgesia as well as OT anti-
13 hyperalgesia after carrageenan inflammation. These alterations were partially rescued if MS pups
14 were treated by intraperitoneal daily injection during MS with OT or its downstream second
15 messenger allopregnanolone. The involvement of epigenetic changes in these alterations was
16 confirmed since neonatal treatment with the HDAC inhibitor SAHA, not only normalized
17 nociceptive sensitivities, but also restored OT receptor-mediated stress-induced analgesia as well
18 as the endogenous anti-hyperalgesia in inflamed MS rats. There is growing evidence in the
19 literature that ELS might impair the nociceptive system ontogeny and function. This study suggests
20 that these alterations might be restored while stimulating OT receptor signaling or HDAC
21 inhibitors, using molecules that are currently available or part of clinical trials for other
22 pathologies.
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Pharmacological rescue of nociceptive hypersensitivity and oxytocin analgesia impairment in a rat model of neonatal maternal separation

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**Pharmacological rescue of nociceptive hypersensitivity and oxytocin analgesia impairment in a
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RESUME

Oxytocin (OT), known for its neurohormonal effects around birth, has recently been suggested for being a critical determinant in neurodevelopmental disorders. This hypothalamic neuropeptide exerts a potent analgesic effect through an action on the nociceptive system. This endogenous control of pain has an important adaptive value but might be altered by early life stress (ELS), possibly contributing to its long-term consequences on pain responses and associated comorbidities. We tested this hypothesis using a rat model of neonatal maternal separation (NMS) known to induce long term consequences on several brain functions including chronic stress, anxiety, altered social behavior and visceral hypersensitivity. We found that adult rats with a history of NMS were hypersensitive to noxious mechanical/thermal hot stimuli and to inflammatory pain. We failed to observe OT receptor-mediated stress-induced analgesia as well as OT anti-hyperalgesia after carrageenan inflammation. These alterations were partially rescued if NMS pups were treated by intraperitoneal daily injection during NMS with OT or its downstream second messenger allopregnanolone. Involvement of epigenetic changes in these alterations was confirmed since neonatal treatment with the **histone deacetylase (HDAC)** inhibitor SAHA, not only normalized nociceptive sensitivities, but also restored OT receptor-mediated stress-induced analgesia as well as the endogenous anti-hyperalgesia in inflamed NMS rats. There is growing evidence in the literature that ELS might impair the nociceptive system ontogeny and function. This study suggests that these alterations might be restored while stimulating OT receptor signaling or HDAC inhibitors, using molecules that are currently available or part of clinical trials for other pathologies.

INTRODUCTION

Oxytocin (OT) is a nonapeptide synthesized by hypothalamic neurons mainly located in the paraventricular (PVN) and supraoptic (SON) nuclei [1]. OT acts both as a neurohormone when released into the blood via the posterior pituitary or as a neurotransmitter when synaptically released into the spinal cord (SC) and supraspinal regions. Recently, our laboratory identified a small population of 30 parvocellular neurons in the PVN which coordinate the peripheral and spinal release of OT to limit inflammatory pain symptoms [2]. Indeed parvocellular OT neurons of the PVN trigger OT secretion in superficial and autonomic layers of the SC where OT receptors are found [1]. OT displays anti-nociceptive properties by increasing GABAergic inhibition in the SC thus decreasing excitability in superficial and deep SC neurons [1]. In addition to these central-related effects, a peripheral component of OT-mediated analgesia via a direct effect at the dorsal root ganglion (DRG) level has been documented [3].

Activation of the OT analgesic system occurs under different conditions such as inflammatory sensitization [2], neuropathic pain [4], or forced swim stress (FSS) [5]. In the carrageenan model of inflammatory sensitization, spinal OT levels are increased for at least 24 h and OT receptor-mediated local production of the neurosteroid allopregnanolone (AP) leads to a sustained GABAergic activity to maintain spinal antinociceptive control [6]. After a non-noxious FSS, OT is also responsible for significant and transient stress-induced analgesia (SIA) [5]. In this context, a deficit in OT signaling might be related to neurodevelopmental brain pathologies such as autism spectrum disorders [7; 8].

The development of the nociceptive system starts before birth and follows a similar pattern in the rat and in the human fetus [9; 10]. It is well accepted that the developmental stage of the rat just after birth is similar to that of the human fetus at the beginning of the 3rd trimester of gestation.

1 Descending controls of pain mature during the first 3 weeks after birth in the rat [11] and begin to
2 mature during the end of the 3rd semester in humans [12]. At the SC level, spinal inputs and
3 chloride homeostasis undergo re-organization steps after birth to be fully matured [13; 14] making
4 them possibly vulnerable to early adverse events. Neonatal maternal separation (NMS) is a well-
5 established model of early life stress (ELS) that induces long term neuroendocrine and behavioral
6 changes in rodents, with impact on the HPA axis, social and cognitive behaviors, and sensory and
7 nociceptive systems [15; 16]. Long-term consequences of NMS are indeed visceral hypersensitivity
8 [17], epigenetic changes, and associated sensory dysfunctions [18; 19]. Recently, a chronic adult
9 treatment with histone deacetylase (HDAC) inhibitor was shown to be efficient to counteract
10 NMS-induced visceral hypersensitivity [19].

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26 In this study, we used behavioral measures and *in vivo* electrophysiological recordings from SC
27 dorsal horn neurons to characterize the long-term consequences of NMS on the oxytocinergic
28 descending control of pain. Then, we aimed to prevent the development of NMS pain phenotype
29 using pharmacological tools, targeting the OT system or epigenetic factors early during the
30 postnatal period.
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MATERIALS AND METHODS

Animal care and neonatal maternal deprivation procedure

Female Wistar rats with litters were housed in a temperature-controlled room (22°C) under a 12h light-dark cycle (lights on at 07:00 AM), with *ad libitum* access to food and water. All experiments were conducted in conformity with the recommendations of the European Committee Council Direction of September 22, 2010 (2010/63/EU), with an authorization for animal experimentation from the French Department of Agriculture (License 67-116 to PP) and with a procedure agreement evaluated by the regional ethical committee (CREMEAS authorization number APAFIS#7678-20 16112223027528 v2).

At birth, litters were randomly assigned to two groups: Neonatal NMS and non-separated control litters. The litters allocated to the NMS group were removed from the nest cages 3 h per day from postnatal days 2 (P2) to 12 (P12) and placed on a heating pad (37°C) in a separate cage. The litters allocated to the control group remained with their mother in their home cages during the entire NMS interval and received no special handling other than that necessary to change the bedding of their cage. Pups were weaned at P21 and housed four rats per cage before being randomly chosen at 8 weeks or more for behavioral analysis or electrophysiological recordings. **In each experiment, animals were chosen randomly in at least two different litters. Both males and females were used in this study, as no sex specific differences could be seen for the different neurophysiological and behavioral measures.**

Measure of basal mechanical and thermal nociception

Mechanical nociception was measured on adult rats with a calibrated forceps (Bioseb, Vitrolles, France) as previously described [20]. Briefly, the habituated rat was loosely restrained with a towel

1 masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps
2 were placed at each side of the paw and a gradually increasing force was applied. The pressure
3 producing withdrawal of the paw corresponded to the nociceptive threshold value. Thermal
4 nociception was measured by a blind experimenter with the Hargreaves method, as previously
5 described [21]. Briefly, rats were placed in plexiglas boxes (20cm x 25cm x 45cm) for a 15-20
6 minutes habituation period and then hindpaws were exposed to a radiant heat and the latency
7 time required to induce paw withdrawal was recorded and considered as the nociceptive heat
8 threshold. For both tests, measurements were performed three times for each hindpaw and
9 values were averaged.

10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 Forced swim stress

27 At adulthood, rats were exposed to a forced swim stress procedure (FSS) in a Plexiglas® cylinder
28 (diameter: 30 cm; wall height: 60 cm), filled with water (height: 40 cm) at a temperature of 20°C
29 for 10 minutes. After FSS exposure, animals were gently dried. Mechanical thresholds were
30 measured before (baseline) and 15 min after FSS in both NMS and control animals. This protocol
31 has been described to be associated with a plasmatic and spinal release of oxytocin [5]. The
32 oxytocinergic contribution of FSS to SIA was confirmed in a subset of rats receiving an intrathecal
33 (i.t) injection of 10µL of OTR antagonist dOVT ((d(CH₂)₅¹,Tyr(Me)²,Thr⁴,Orn⁸,des-Gly-NH₂⁹)-
34 Vasotocin, Bachem, Germany) at 200µM or 10µL saline 30 minutes before FSS.
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54 Carrageenan Inflammatory pain model

55 To induce a painful inflammatory sensitization in the rat, 150µL of carrageenan (3% in NaCl 0.9%,
56 Sigma Aldrich, Germany) were injected in the plantar surface of the right hindpaw for behavioral
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1 experiments, whereas injections in both hindpaws were performed for electrophysiological.

2 Control animals received either similar injections of NaCl 0.9% or no injection.
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8 In vivo recording of spinal cord neurons and analysis 9

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11 Male and female rats were anaesthetized with isoflurane (2 %; pushed by pressurized air at a flow
12 rate of 0.5 L.min⁻¹) and body temperature was regulated using a thermostatically controlled
13 heating blanket (Harvard Apparatus Ltd, France) maintaining the body temperature at 37°C.
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Animals were set up in a stereotaxic frame (La Précision Cinématographique, Eaubonne, France), with the cervical and sacral vertebrae firmly held. The lumbar SC (L4-L5) was exposed by laminectomy. After removal of the meninges, the SC surface was covered with a thin isolating layer of mineral oil. This opening allowed us to record SC neurons and apply several drugs directly on the SC. At the end of the experiment, animals were killed with an overdose of isoflurane.

Single unit extracellular in vivo recordings were made from wide dynamic range (WDR) neurons, located in lamina V and receiving peripheral sensory messages mediated by A β , A δ and C-type primary afferent fibers. In the lumbar enlargement of the SC, we monitored voltage changes through stainless microelectrodes (FHC, Bowdoin, USA) connected to an extracellular differential amplifier (DAM-80, WPI, Aston, UK). Electrical signals were then digitized (Power 1401 CED, Cambridge, UK), processed with the Spike 2 software (CED Cambridge, UK). Neurons were characterized by their responses to transcutaneous electrical stimulation of the hindpaw receptive field as previously described [22]. Electrical stimulation was applied through two thin cutaneous pin electrodes placed in the center of the receptive field on the hindpaw. Increasing intensities were applied from 1 V to C fiber activation threshold (duration of 1 ms). Trials consisted of a train of 90 electrical stimuli (3 x C-type fiber threshold) applied repetitively (1 Hz) to the excitatory

1 receptive field. This method has been usually used to obtain a stable and reproducible “wind up”
2 [23], a property of C-type nociceptive fibers. The electrically-evoked response of the neuron was
3 captured and displayed as a post-stimulus histogram, for A-type and C-type discharge. Action
4 potentials appearing early after the stimulus artifact were classified as A-type (0-50 ms) whereas
5 the delayed response corresponded to C-type (50-800 ms).
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16 Spinal cord sampling and PCR analysis

17 Lumbar spinal cord samples were collected, reconstituted in a guanidine thiocyanate/ β -
18 mercaptoethanol preparation using ultraturax and stored at -80°C. Total RNA was extracted
19 according to a protocol derived from the original procedure of Chomczynski and Sacchi [24],
20 consisting of two independent total RNA extractions separated by a DNaseI treatment ((TURBO
21 DNase™, Ambion, Life technologies, Saint Aubin, France), as previously described in detail [25].
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800ng RNA were used for reverse transcription with the RT iScript kit (Bio-Rad, Marnes-la-Coquette, France). Quantitative PCR was performed using SYBR Green Supermix (Bio-Rad), on the iQ5 Real Time PCR System (Bio-Rad). Amplifications were carried out in 42 cycles (20s at 95°C, 20s at 60°C and 20s at 72°C). Standardization was made possible using standard curves made from serial dilutions of samples, and amplification data is shown as relative gene expression, calculated as the ratio between cDNA concentration of the gene of interest and that of the housekeeping gene. Preliminary experiments showed that among the different classic housekeeping genes routinely analyzed in the laboratory, and compared with 18S ribosomal RNA, hypoxanthin-guanine phosphoribosyltransferase (HPRT) transcripts remained highly stable among the different samples. Therefore, HPRT was selected as the housekeeping gene to analyze and evaluate potential changes in gene expression in all experiments presented here. To specifically amplify mRNA encoding various rat proteins, we designed specific primer sets (sense and antisense

1 respectively) using Oligo6.0 and M-fold softwares. Primer sequences for all genes of interest can
2 be found in supplementary table 1. Samples were accurately dispensed in duplicates using a
3 robotic workstation (Freedom EVO100, Tecan, Lyon, France), and amplification efficacy given by
4 standard curves was always close to 100% ($\pm 2\%$), whilst amplification specificity was assessed by a
5 melting curve study.
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15 Neonatal rescue procedure

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18 To reverse NMS long term consequences on pain behavior, a pharmacological neonatal treatment
19 was performed in NMS pups. During NMS protocol, all pups in the litter were injected with the
20 same molecule and then tested at adulthood to investigate the long-term efficiency of neonatal
21 treatment. OT (Bachem, Weil am Rhein, Germany), the HDAC inhibitor SAHA (Suberoylanilide
22 hydroxamic acid, Vorinostat, ApexBio Technology, Houston, USA), or vehicle (NaCl, oil or 10%
23 DMSO respectively) were injected intraperitoneally (i.p. 10 μ L) at 1mg/kg and 5mg/kg respectively
24 once daily (o.d) during the NMS procedure (*i.e* from P2 to P12). In another subset of animals,
25 allopregnanolone (AP, Santa Cruz biotechnology, Dallas, USA) was injected subcutaneously at
26 10mg/kg once every two days during the NMS procedure. To avoid unnecessary supplementary
27 handling of the pups, injections were performed at the beginning of the separation process. Pups
28 were then normally weaned at P21, and no special handling was performed until P45, when the
29 efficiency of neonatal treatment was assessed for each neonatal group using behavioral testing.
30 First, mechanical thresholds were measured prior (baseline) and after FSS (SIA measurement).
31 Then one week later, the efficacy of the OT descending control of pain after carrageenan
32 inflammation of the hindpaw was measured with the same protocol as the one previously
33 described (using spinal dOVT injection 24H after inflammation).
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Drugs and Treatments

OT receptor (OTR) agonists and antagonist, as well as V1aR antagonist were prepared as a concentrated solution in water (1mM) and diluted to the needed concentration in NaCl (0.9%) before experiment. The selective OTR antagonist dOVT (d(CH₂)⁵-Tyr(Me)-[Orn⁸]-vasotocine) was used at a final concentration of 1 μM in Saline (NaCl 0.9%). dOVT was injected i.t. (volume 20 μL) or directly on the spinal cord in electrophysiological recordings (volume 500 μL). V1aR antagonist [Phenylacetyl¹,O-Me-D-Tyr²,Arg^{6,8},Lys⁹]-vasopressinamide (AV1AR: Sigma Aldrich, Saint-Louis, France) was injected i.t at a concentration of 1μM (volume 20μL). OT and TGOT ((Thr⁴,Gly⁷)-Oxytocin) (Bachem, Weil am Rhein, Germany) were used as an agonists for OTRs. OT was dissolved in saline and injected i.t or i.p. at a concentration of 10 μM or 1mg/kg, respectively. TGOT was also dissolved in NaCl 0.9% and injected at a concentration of 1 μM (i.t.). Allopregnanolone and SAHA were freshly prepared before the experiment. Allopregnanolone was used at 10mg/kg and dissolved in mineral oil by sonication at 4°C. The non-selective HDAC inhibitor SAHA was dissolved in 10% DMSO and injected i.p. at a concentration of 5mg/kg.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 software (LaJolla, CA, USA). For each experiment, parametric analysis was used only after verifying the normal distribution of the values. Unpaired Student t-test was used to compare baseline nociceptive sensitivity and OTR and oxytocinase (OTase) expression in NMS and CTRL rats. Repeated measures Two-Way ANOVA followed by Sidak post hoc test was used to analyze inflammatory symptoms and to analyze wind-up efficiency. Repeated measures Two-Way ANOVA followed by Tukey's post hoc test was used to evaluate the effect of

1 OT and TGOT spinal application in control and NMS rats. One way ANOVA and Tukey post-hoc
2 tests were used to compare the effects of spinal application of dOVT in control or NMS rats, to
3 evaluate the efficiency of SIA in both groups, to analyze the success of the rescue experiments on
4 baseline nociceptive sensitivities and to evaluate spinal mRNA expression of 3 α -hydroxysteroid
5 dehydrogenase (3 α -HSD), 5 α -reductase type II (5 α -red II), P450scc and translocator protein
6 (TSPO). Mann-Whitney test was used to compare the effects of spinal application of the V1aR
7 antagonist in control and NMS rats. Kruskal Wallis (KW) followed by Dunn's post hoc test was used
8 to compare wind-up frequencies and spinal expression of histone deacetylases (HDAC) transcripts.
9 Differences were considered to be statistically significant for $p < 0.05$.
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RESULTS

Consequences of NMS on the nociceptive sensitivity and on the efficacy of oxytocinergic SIA

We first characterized the long-term effects of NMS on baseline mechanical and thermal nociceptive thresholds (Figure 1A), as well as the intensity of stress-induced mechanical analgesia (SIA) measured after FSS (Figure 1B). Compared to the control group, NMS animals exhibited hypersensitivity to both mechanical and thermal stimulation at adulthood ($P > 100$). NMS rats displayed significantly lower mean pressure thresholds (control: 445.5 ± 6.1 g, $N = 12$; NMS: 268.5 ± 7.1 g, $N = 12$; $p < 0.001$, unpaired Student t-test, $t = 18.83$, $df = 22$) and paw withdrawal latencies to noxious heat (control: 21.1 ± 0.8 s, $N = 12$; NMS: 12.6 ± 0.4 s, $N = 12$; $p < 0.001$, unpaired Student t-test, $t = 9$, $df = 22$).

Figure 1 near here

Previous studies showed that both central and peripheral release of OT contributed to SIA after a FSS [26; 22]. Because NMS is known to alter the normal reactivity to stress and lead to a dysfunction of the HPA axis, we investigated the efficacy of the oxytocinergic component of SIA in NMS animals (Figure 1B). As expected, the FSS paradigm induced a significant analgesia 20 minutes after swim in the control group (Figure 1B). Mean baseline-corrected mechanical thresholds were increased by 239 ± 12.3 g in the control group ($N=8$) but only by 10.7 ± 23.4 g in the NMS group ($N=8$). An injection of dOVT prior to FSS decreased SIA intensity to 56.9 ± 20.1 g in the control group ($N=8$) and had no effect in the NMS group ($N=8$) where it varied of 20.75 ± 20.7 g ($p < 0.001$, One-Way ANOVA, $F = 28.9$). These results suggest a blunted adaptation to stressful conditions in NMS rats associated with possible dysfunction of the oxytocinergic descending control of pain.

Behavioral response to a painful inflammatory sensitization by carrageenan

We next compared the intensity and time course of inflammatory pain symptoms triggered by an intraplantar hindpaw injection of carrageenan to possibly highlight differences in the expression of mechanical/thermal hyperalgesia in control and NMS rats (Figure 2). In both groups, carrageenan injection induced a sustained mechanical and thermal hyperalgesia which peaked 7 h after injection, as previously described [6]. Although basal mechanical nociceptive thresholds (i.e., before carrageenan injection) were significantly lower in NMS rats, the time course of mechanical hyperalgesia was not significantly different between both groups (Figure 2A1-B1). In the control group, mean pressure threshold dropped from 445.8 ± 18.7 g to 30.1 ± 5.1 g, 7 h after injection (N = 6; repeated measures two-way ANOVA, treatment x time, $F_{(12,120)} = 63.81$, $p < 0.001$) and mechanical hyperalgesia lasted for at least 6 days for control. As mentioned above, at baseline, NMS rats exhibited a lower mean pressure threshold of 295.5 ± 4.8 g (N = 6), which further decreased to 70.8 ± 12.6 g, 7 h after carrageenan injection. As illustrated in Figure 2B1, hyperalgesia was detected at a significant level for 6 days (N = 6; repeated measures two-way ANOVA, treatment x time, $F_{(12,120)} = 30.78$, $p < 0.001$). Concerning thermal hot sensitivity, 7 h after carrageenan injection mean latencies decreased to 3.3 ± 0.6 s and 4.2 ± 0.8 s in control and NMS rats (N=6 per group) respectively. Interestingly, thermal hot hyperalgesia persisted for at least 1 days for control rats and up to 4 days in NMS rats (Figure 2B2) (repeated measures two-way ANOVA, treatment x time, $F_{(8,80)} = 13.67$, $p < 0.001$ for CTRL and $F_{(10,100)} = 10.69$, $p < 0.001$ for NMS).

Figure 2 near here

Oxytocinergic contribution to the intensity of carrageenan-induced pain symptoms and to the spinal cord processing of nociceptive messages

In the carrageenan model of inflammatory sensitization, spinal concentrations of OT are elevated during the early phase of inflammation and have been shown to limit pain sensitivity [22]. To investigate whether NMS affects OTR anti-hyperalgesia, i.t application of the selective OTR antagonist dOVT was performed 24 hours after carrageenan injection. As OT may act via the activation of vasopressin V1a receptors (V1ARs) at high concentrations, we also examined a possible contribution of these receptors using i.t application of V1aR selective antagonist.

In the control group and as previously shown [6], a single i.t injection of dOVT (1 μ M, 20 μ L), induced a rebound of mechanical and thermal hyperalgesia. Anti-hyperalgesia mediated by OTR could be seen in the CTRL group as a decrease in the mean baseline-corrected mechanical threshold 4 h (*i.e.* 28h after carrageenan) after dOVT injection by -75.6 ± 8.4 g (N= 10 Figure 3A). This decrease was absent in the saline-treated group (-2 ± 8.4 g, N=10) (One-way ANOVA F = 17.5, $p < 0.001$). For thermal withdrawal latency (Figure 3B), OTR anti-hyperalgesia could also be seen as a significant decrease in the mean values after dOVT compared to saline (N = 10; One-way ANOVA, F = 9.3, $p < 0.001$). This corresponded to a change of -3.9 ± 0.6 s (N=10) and of 1.1 ± 0.8 s (N=10), for the dOVT and saline groups, respectively. Intrathecal injection of the V1aR antagonist was without effect on mechanical (before: 155.25 ± 3.2 g; after i.t. injection: 177.25 ± 18.2 g, N = 4; Mann-Whitney test: U = 4.0; $p = 0.3429$) or thermal nociceptive threshold (before: 10.0 ± 0.9 s; after i.t. injection: 11.0 ± 0.9 s, N=4; Mann-Whitney test: U = 5.0; $p = 0.4571$) in the control group of rats.

Both results confirmed OTR activation to be responsible for a limitation of pain symptoms early after carrageenan injection in control animals. Interestingly, there were no changes in the mean

1 mechanical (dOVT: delta of 5.8 ± 10.8 g, N=8; saline: delta of -27.2 ± 9 g, N=7; One-way ANOVA
2 $F=17.5$ $p<0.001$) and thermal hyperalgesia values (dOVT: delta of -1 ± 0.7 s, N=8; saline: delta of
3 0.1 ± 0.7 s, N=7; One-way ANOVA $F=9.3$ $p<0.001$) in NMS animals. As for control rats, a single i.t.
4 injection of the V1aR antagonist did not change mechanical (before: 164.65 ± 9.9 g; after i.t.
5 injection: 145.75 ± 10.3 g, N = 8, Mann-Whitney test: $U = 20.0$; $p = 0.2241$) nor thermal nociceptive
6 thresholds (before: 6.13 ± 0.4 s; after i.t. injection: 5.5 ± 0.5 s, N=8; Mann-Whitney test: $U = 22.0$;
7 $p = 0.3514$) in NMS rat.

8 Together, this suggests that NMS has long-term consequences on the efficacy of OTR-mediated
9 spinal analgesia, at least in short-term inflammatory painful sensitization.

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24 **Figure 3 near here**

25 To further characterize this NMS-altered OTR-mediated analgesia in NMS adult rats, we recorded
26 WDR neurons in the SC of anesthetized rats and analyzed the spinal processing of nociceptive
27 messages through the expression of windup, a nociceptive-specific short-term plasticity [27]. As
28 illustrated in figure 4, windup recorded 24 h after carrageenan injection was significantly amplified
29 after the spinal application of dOVT ($1\mu\text{M}$). In control animals (N=6, figure 4A), windup protocol
30 led to an average maximum frequency of 23.6 ± 1.5 spikes/s. This value significantly increased to a
31 maximum frequency of 44.1 ± 4.8 spikes/s after dOVT application (N = 6; two-way repeated
32 measures ANOVA, treatment x time, $F_{(14,70)} = 7.811$; $p < 0.0001$). As shown in figure 4B, we did not
33 see any change in windup frequencies in NMS animals (N=6) after dOVT application (before
34 application: 26.8 ± 4.3 spikes/s; after application: 25 ± 4.1 spikes/s; two-way repeated measures
35 ANOVA, treatment x time, $F_{(14,70)} = 1.366$; $p = 0.1936$). Summary of results can be found in figure
36 4C (Kruskal-Wallis test: $KW = 8.9$; $p = 0.03$).

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1 Altogether, electrophysiological and behavioral experiments suggested that the OT descending
2 inhibitory control of pain is inefficient in NMS rats.
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8 **OTR expression and OTR-induced analgesia**

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11 In order to better understand the possible molecular and cellular alterations induced by NMS, we
12 first analyzed the expression of OTR and a possible dysfunction of its downstream signaling,
13 focusing on the SC as it is the first relay for nociceptive messages. There were no difference in the
14 spinal expression of OTR gene transcripts between control (N=7; 3.1 ± 0.9 spinal relative
15 expression) and NMS (N=8; 2.4 ± 0.2 spinal relative expression) rats (unpaired Student's t-test,
16 $p = 0.44$, $t = 0.78$, $df = 13$), as illustrated in figure 5A.
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30 To further confirm that OTR were not only expressed at similar levels in both groups but were
31 equally functional, we measured the analgesic behavioral response to i.t injection of OT (1 μ M
32 20 μ L) or to the selective agonist TGOT (1 μ M 20 μ L) in CTRL and NMS rats (Figure 5B). In CTRL
33 animals OT (N = 8) or TGOT (N = 8) significantly increased mechanical thresholds 20 minutes after
34 the injection (OT: from 467.4 ± 19.4 g to 741.6 ± 67.1 g; TGOT: from 471.8 ± 14.6 g to
35 813.2 ± 26.3 g; Two-way repeated measures ANOVA, treatment x time, $F_{(4,40)} = 14.67$;
36 $p < 0.0001$). Both OT (N = 7) or TGOT (N = 8) significantly increased mechanical thresholds 20
37 minutes after the injection in a reversible manner in NMS animals (OT: from 288.4 ± 5.0 g to
38 533.9 ± 13.1 g; TGOT: from 246.7 ± 11.2 g to 460.1 ± 17.3 g; Two-way repeated measures ANOVA,
39 treatment x time, $F_{(11,38)} = 66.94$; $p < 0.001$). This result confirmed that spinal OTR are functional in
40 NMS rats and capable of producing efficient analgesia. We thus checked whether NMS could lead
41 to an increase in the spinal degradation of OT. As illustrated in Supplementary Figure 1A, we found
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no difference in the spinal expression of oxytocinase (OTase) mRNA between control and NMS rats (N=8; unpaired Student t-test, $t = 0.1317$, $df = 14$; $p = 0.8971$).

Neonatal treatments with OT and allopregnanolone

Many studies provided evidence that OT in the neonatal period could be protective against early life adverse events or lesions [28; 29]. Moreover, NMS is known to reduce maternal behavior towards the pups and a subsequent altered development of OT systems, possibly leading to blunted OT levels [30]. This includes molecular mechanisms affecting chloride homeostasis and the production of neuroprotective neurosteroids such as allopregnanolone [6].

In a first set of experiments, we treated NMS rat pups o.d. from P2 to P12 with OT (1mg/kg in 0.9% NaCl, i.p., Figure 6). As shown in figure 6A, neonatal OT treatment fully restored normal mechanical nociceptive thresholds when measured at P50 (control: 320.7 ± 10.9 g, $N = 12$; NMS: 235.1 ± 6.9 g, $N = 8$; OT-treated NMS: 320.4 ± 6.3 g, $N = 10$; One-way ANOVA, $F = 23.39$, $p < 0.001$). OT neonatal treatment also restored an efficient analgesia after FSS (Figure 6B; delta for Control group: 261.8 ± 18.6 g, $N = 9$; NMS: 17.8 ± 11.6 g, $N = 8$; OT-treated NMS: 168.9 ± 10.4 g, $N = 9$; One-way ANOVA, $F = 30.08$, $p < 0.001$). In contrast to SIA after FSS, neonatal OT treatment failed to restore OT anti-hyperalgesia 24 hours after carrageenan-induced inflammation, measured as before using i.t dOVT injection (Figure 6C). The dOVT injection did not significantly amplified mechanical hyperalgesia (delta for NMS: 5.8 ± 10.9 g, $N = 8$; OT-pretreated NMS: 45.6 ± 19.7 g, $N = 8$; One-Way ANOVA, $F = 14.28$, $p < 0.001$).

Figure 6 near here

Recently, an original long-lasting molecular mode of action of OT in SC neurons has been proposed. It leads to the stimulation of AP synthesis, a neurosteroid well-known for its analgesic

1 and neuroprotective roles [31]. We thus tried to restore OT function in NMS rats by submitting
2 newborn pups to AP during the NMS period. As illustrated in the different panels of figure 6, a
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4 neonatal treatment with s.c AP (10mg/kg) between P2 and P12 was associated with a mean
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6 mechanical threshold at adulthood which was higher (i.e., hyposensitivity) than those measured in
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8 control animals (Figure 6A; Control: 320.7 ± 10.9 g, N = 12; AP-treated NMS: 394.1 ± 21.9 g,
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10 N = 10; One way ANOVA, F = 23.39, p < 0.001). As for neonatal OT treatment, SIA could be
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12 restored by AP treatment (Figure 6B; baseline corrected mean mechanical threshold in AP-treated
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14 NMS: 351 ± 47 g, N = 8, One Way ANOVA, F = 30.08, p < 0.001). As previously reported,
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16 endogenous OT anti-hyperalgesia could not be revealed after dOVT i.t. injection (Figure 6C; AP-
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18 treated NMS: -23 ± 16 g, N = 8; One Way ANOVA, F = 14.28, p < 0.001). Taken together, our results
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20 likely suggested NMS to be associated with a deficit in OT signaling. However, we found no
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22 significant difference in the spinal transcripts levels of 3α -hydroxysteroid dehydrogenase (3α -
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24 HSD), 5α -reductase type II (5α -red II), nor cholesterol side-chain cleavage enzyme
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26 (P450scc), enzymes involved in AP synthesis (Supplementary Figure 1B; N = 8; One way ANOVA,
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28 F = 0.7409, p = 0.6384). Similarly, spinal translocator protein (TSPO) mRNA levels remained
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30 unchanged in NMS rats compared to controls.
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45 **NMS-induced epigenetic alterations and rescue of MS pain phenotype by targeting histone** 46 47 **deacetylases**

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50 The long-term consequences of NMS on pain sensitivity and on the rat ability to cope with
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52 stress/pain potentially rely on epigenetic mechanisms. Indeed, several reports highlight the effects
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54 of NMS on the HPA axis and on maternal behavior [32; 33]. We thus measured the spinal
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56 expression of a set of transcripts coding for several epigenetic factors at different time points
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1 (from P7 to P100) in NMS and control animals (Figure 7A). We found that mRNA levels for some
2 class I (HDAC 1 and 8) and class II HDAC (HDAC 7) were significantly increased in NMS animals at
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4 P24 (HDAC 1 and 7), P45 (HDAC 1, 7 and 8) and until P100 for HDAC 7 (Kruskal-Wallis; HDAC1 :
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6 KW = 60.33 , p < 0.0001 , N = 7 ; HDAC 7 : KW = 61.41 , p < 0.0001 , N = 7 ; HDAC 8 : KW = 49.28 ,
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8 p < 0.0001 , N = 6).

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11 In particular, HDAC 7 mRNA spinal expression was significantly increased in NMS animals
12 compared to controls at P24 (0.49 ± 0.04 relative expression in CTRL and 1.22 ± 0.11 in NMS), as
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14 illustrated in figure 7B, and levels remained significantly higher until adulthood at P100
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16 (0.36 ± 0.03 relative expression in CTRL and 1.01 ± 0.04 in NMS). These results likely suggest that
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18 NMS triggers long lasting changes in gene expression, involving at least HDAC functions.
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29 **Figure 7 near here**

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31 We hence performed a neonatal chronic treatment from P2 to P12 with SAHA (10 mg/kg i.p.), a
32 non-selective HDAC I and II inhibitor. Neonatal treatment of newborn pups with SAHA during NMS
33 was associated with mean mechanical nociceptive thresholds at adulthood (P50) which were
34 similar to the control group (Figure 7C; Control: 320.7 ± 10.9 g, N = 8; SAHA-treated NMS:
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36 303.8 ± 9.4 g, N = 10; One way ANOVA, F = 14.28, p < 0.001). This treatment also partially restored
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38 SIA after FSS (Figure 7D; SAHA-treated: 130 ± 26.7 g, n = 7; One Way ANOVA, F = 34.73, p < 0.001).
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40 Interestingly, SAHA treatment partially restored OT anti-hyperalgesia after carrageenan
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42 inflammation (Figure 7E; Control: 75.6 ± 8.4 g; SAHA-treated: -41.7 ± 12.3 g; One-Way ANOVA,
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44 F = 15.75, p < 0.0001).
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DISCUSSION

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3 In summary, we provide functional evidence indicating that NMS strongly affects adult nociception
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5 and the efficacy of the oxytocinergic descending inhibitory control. NMS is associated with (i)
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7 hypersensitivity to mechanical and thermal hot stimulation at adulthood, (ii) increased
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9 vulnerability to **thermal inflammatory-induced hyperalgesia**, and (iii) dysfunction of the OT
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11 descending control of pain after non-painful FSS or painful inflammatory sensitization. We further
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13 succeeded in rescuing these abnormalities in the nociceptive phenotype by repeated neonatal
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15 injections of OT, AP, or SAHA. Altogether, our data suggest that NMS has a strong impact on
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17 nociceptive integration, which can blunt the efficacy of descending controls of pain such as the
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19 oxytocinergic system which targets the spinal GABAergic inhibitory system. It also suggests that
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21 the OT or epigenetic systems can be good targets to develop early treatments to counteract the
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23 negative effects of ELS, at least in this model.
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Early life events promote nociceptive hypersensitivity and alter OTR-mediated analgesia

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38 Early life stress includes a wide range of adverse events which have long-term negative effects on
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40 brain function. In humans, ELS has been associated with an increased risk of developing chronic
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42 pain states at adulthood [34], including irritable bowel syndrome [35; 36]. In animal studies, the
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44 visceral hypersensitivity following NMS has been widely demonstrated, and correlated with
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46 differential activation of supraspinal regions, including the periaqueductal gray, which is a key area
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48 for descending pain controls. Here, we confirmed that nociceptive hypersensitivity extends to
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50 somatic mechanical and thermal hot stimuli, as previously published by our laboratory [16] and in
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52 other ELS models [37-40]. Our observation of an increased thermal pain symptom after
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54 carrageenan is consistent with studies showing an increased response after formalin injection [41]
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1 or after partial sciatic ligation [42] in NMS animals. Moreover, OT analgesic action on thermal
2 sensitivity also relies on an action on TRPV1 receptors, since OT seems able to directly activate and
3 promote the rapid desensitization of TRPV1 receptors expressed by C-type sensory fibers [43].
4 Although not tested in this study, we cannot exclude such an effect in NMS animals, as an
5 increased duration of thermal hot hyperalgesia could be due to the loss of OT action on TRPV1
6 receptors.
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16 Nociceptive hypersensitivity could be easily explained by a long-lasting up-regulation of voltage-
17 gated channels Nav1.8 and Nav1.9 in DRG neurons as recently published [16].
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21 In this study, impairment of the oxytocinergic pain control has been revealed after recruitment by
22 a non-painful or painful stress. The hypothalamus, where OT producing neurons are located,
23 seems particularly vulnerable to ELS and a dysfunction of the oxytocinergic system may contribute
24 to these alterations as OT plays a key role in the modulation of stress responses, anxiety, social
25 interactions and pain responses. Despite several trials, we have not been able to demonstrate that
26 endogenous OT concentrations are different between groups. Recently, NMS in rodents was
27 shown to be associated with changes in OTR and V1aR binding sites in specific brain regions [44],
28 suggesting a vulnerability of this system to the early environment. In humans, ELS has been
29 associated with low oxytocin concentrations in CSF [45]. Here, we show that OT analgesia is
30 impaired after NMS, both in painful and non-painful conditions. To exert its analgesic effects at the
31 spinal level, OT relays on three main mechanisms: (i) activation of a spinal microcircuit leading to
32 an increased GABAergic inhibitory tone [46], (ii) decrease of the excitability of SC neurons in the
33 superficial layers [47] and (iii) stimulation of allopregnanolone synthesis which further amplifies
34 GABAergic inhibition by allosteric modulation [6]. Here, we showed that spinal OTR are functional
35 in NMS rats and that OTR activation can induce an efficient and transient analgesia, suggesting
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1 that the lack of OT-analgesia could hence result from supraspinal alterations. In agreement with
2 our previously published results [22; 2], we failed to reveal any spinal contribution of the
3 vasopressin V1A receptor in the fine tuning of basal nociceptive threshold or to induce
4 oxytocinergic analgesia after painful or non-painful stress. This control was important since some
5 discrepancies exist in the literature, often pointing out a specie-specific issue and a concentration-
6 dependent action difficult to compare due to different administration procedures ([48]). Exploring
7 hypothalamic OT neurons pharmacological and electrophysiological characteristics might help us
8 to have a deeper understanding of the mechanisms leading to pain-related ELS consequences. A
9 lack of AP synthesis following OTR activation could also explain the lack of OT anti-nociception
10 following carrageenan inflammation in NMS rats, although we found no change in the spinal
11 expression of transcripts coding for proteins responsible for AP synthesis in NMS rats, nor in the
12 spinal expression of OTase mRNA.
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34 **Rescuing NMS long-term alterations of the nociceptive system**

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37 In our working hypothesis, we asked whether the lack of physical interaction between the mother
38 and the pups could be a critical determinant to explain the deleterious plasticity of the nociceptive
39 system and the deficit in oxytocinergic analgesia. Indeed, mother presence has already been
40 shown to be protective against pain. Kangaroo mother care, in particular, is more frequently
41 recommended and has been shown to have immediate analgesic effect on pain responses in the
42 newborn [49; 50] and to be protective against long term alterations usually following preterm
43 birth [51; 52]. As OT has a key role in the modulation of parental behavior and attachment [53;
44 54], we performed a rescue experiment by repeated neonatal OT treatment during NMS, which
45 reversed some consequences of NMS on pain behavior. In the literature, OT has been proposed as
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1 a therapeutic agent for different neurodevelopmental pathologies such as autism spectrum
2 disorders, since it decreases anxiety and optimizes social behavior [7; 8]. In animal models,
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4 postnatal injections of OT have been shown to prevent feeding, social and memory deficit in the
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6 Prader-Willi model [55; 29]. Here, we only studied the beneficial consequences of an early
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8 treatment with OT on pain behaviors but further experiments would be helpful to confirm if this
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10 treatment could also counteract the detrimental effects of NMS on pain-related comorbidities
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12 such as stress and anxiety. These observations might have great clinical relevance in neonatal
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14 intensive care units since it confirms, once again, the crucial role of physical interactions between
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16 the parents and the newborn to optimize newborn development and install proper homeostatic
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18 controls to cope with stress (including pain).
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26 A close reciprocal relationship exists between the oxytocinergic system and neurosteroids.
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28 Allopregnanolone is not only a downstream second messenger of OTR activation [6], it has well-
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30 known anxiolytic, analgesic and neuroprotective properties [31]. In this study, a rescue experiment
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32 with neonatal allopregnanolone treatment appeared successful to restore normal pain sensitivity
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34 and a functional OT analgesia after FSS. Neonatal allopregnanolone level alterations can indeed
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36 lead to changes in behavior at adulthood [56]. Our result is also in good agreement with other
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38 studies using AP treatment to prevent neonatal anxiety after a short-term NMS (24 hours) [57].
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40 The mechanisms by which AP could be protective against NMS consequences on pain controls are
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42 still unknown. However, neonatal AP level alterations could interact with chloride homeostasis
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44 development, since AP has also been shown to change KCC2 expression in the hippocampus [58].
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52 Eventually, it is not surprising that HDAC inhibitor and epigenetic changes are involved in the long-
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54 term consequences of NMS on nociception and OT analgesia. It has long been known that
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56 neonatal stress has long lasting effects through epigenetic mechanisms [32; 59; 33]. **Epigenetic**
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1 changes in chromatin compaction through histone modification such as histone acetylation have
2 been demonstrated after NMS in different brain areas [59; 60; 19]. Here we also demonstrate
3 that the spinal expression of different pain-related epigenetic factors is altered after NMS, in
4 particular HDAC 1, 7 and 8 which are overexpressed in a long-lasting manner compatible with the
5 observed pain phenotypes. In the pain context, histone acetylation can modulate nociceptive
6 response in inflammatory neuropathic and visceral pain models [61]. Spinal class II HDACs are
7 involved in inflammatory hyperalgesia [62], which could explain the beneficial effects of SAHA
8 treatment observed in this study after carrageenan inflammation, and the critical role of spinal
9 HDAC 7 overexpression in NMS animals. In line, it has been suggested that suppressing HDAC 1
10 expression in the SC attenuated neuropathic pain symptoms [63]. The OTR gene promoter is also
11 subjected to increased association of acH3K14 after NMS in rats, in critical structures for pain
12 control [64]. However, we did not see any difference in the spinal expression of OTR in our
13 study, providing further evidence that histone modification may be necessary but not sufficient to
14 exert significant and long lasting changes in specific gene expression and that epigenetic
15 alterations targeting the OTR gene may actually be region-dependant.

16 Corroborating our results, Moloney and colleagues showed that NMS is associated with an
17 alteration in histone acetylation at the spinal level [19] and were the first to show that adult SAHA
18 treatment can reverse NMS-induced visceral hypersensitivity. Here, we show that a preventive
19 neonatal treatment with the same agent is also effective to prevent mechanical and thermal
20 hypersensitivity at adulthood. At this stage, the underlying mechanisms are still to be identified as
21 well as the molecular targets by which SAHA exerts its protective effects against NMS.

22 In conclusion, NMS induced both a basal hypersensitivity to pain and a dysfunction in OT
23 descending control of pain, in painful and non-painful conditions. Some of these alterations can be

1 rescued by neonatal SAHA or OT/AP treatment, but other alternatives need to be explored to be
2 able to recover completely the OT antinociceptive action under inflammatory conditions. By
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4 studying the mechanisms of action of these pharmacological treatments, we might be able to
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6 identify new therapeutic targets to avoid NMS induced reprogramming of pain circuits.
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10 11 12 13 Acknowledgments

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20 research price in 2013.
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25 Conflict of interest statement

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28 The authors have no conflict of interest.
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FIGURES AND LEGENDS

Figure 1. Altered baseline nociceptive sensitivity and stress induced analgesia efficacy in NMS

A. Baseline nociceptive thresholds for mechanical (left histogram) and thermal hot stimulation (right histogram) between control (black bars) and NMS rats (white bars). Statistical code for: Unpaired Student t-test *** $p < 0.001$ $N = 12$ /group. **B.** Stress induced-analgesia (SIA) detected 20 minutes after a forced swim stress procedure aimed at stimulating OT endogenous controls (10 minutes, water at 20°C) for control ($N = 8$ black bar), NMS ($N = 8$ white bar), control pre-injected with dOVT ($N = 8$ red bar) and NMS rats pre-injected with dOVT ($N = 8$ grey bar). The variation of mechanical threshold in grams has been calculated as: $\Delta = \text{mechanical threshold post FSS} - \text{mechanical threshold pre-FSS}$. Statistical code for One Way ANOVA followed by Tukey post hoc test: *** $p < 0.001$.

Figure 2. Increased sensibility to inflammatory pain in NMS rats. Time course of mechanical (top

panels) and thermal hot (bottom panels) nociceptive thresholds for control (A) and NMS rats (B). Carrageenan injection was done at day 0 just after the daily measure. Note that the duration of thermal hyperalgesia was longer for NMS rats. Statistical code for repeated measures Two Way ANOVA treatment X time followed by Sidak post hoc test to compare ipsilateral and contralateral hindpaws for each time point: ** $p < 0.01$, *** $p < 0.001$ $N = 12$ per group.

Figure 3. Lack of efficiency of OT anti-hyperalgesic control on behavioral symptoms of

inflammatory pain in NMS rats. Changes in the mean mechanical (A) and thermal hot (B) thresholds, 24 h after a carrageenan hindpaw injection, in control ($N = 10$) and NMS rats ($N = 8$) submitted to a single injection of the selective OTR antagonist dOVT (1 μ M, 20 μ L) or saline (NaCl 0.9%, 20 μ L), measured as: $\Delta = \text{threshold post dOVT injection} - \text{threshold pre dOVT injection}$.

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Statistical code for One Way ANOVA and Tukey post hoc test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CTRL: control, NMS: maternal separation, s: saline.

Figure 4. Lack of efficiency of OT anti-nociceptive control on spinal cord wide dynamic range neurons. A and B. Number of action potentials measured during windup when recording from wide dynamic range neurons *in vivo*. Results are given for anesthetized control (CTRL, **A**) and NMS rats (**B**), displaying an hindpaw inflammation for 24 h due to carrageenan injection, and submitted to a repetitive stimulation of the corresponding receptive field on the hindpaw (1Hz, intensity: 3x threshold for C fibers). Windup characteristics are provided before (black symbols) and 20 minutes after local (spinal) application of the selective antagonist dOVT (1 μ M, in red). Statistical code for **repeated measures** Two Way ANOVA Treatment X Time and Sidak post hoc test: * $p < 0.05$, *** $p < 0.001$. **C.** Mean frequencies of action potential discharge at wind-up plateau phase for all groups. Statistical code for Kruskal Wallis test and Sidak post hoc test: * $p < 0.05$ N=6 per group.

Figure 5. Function of OTR in NMS rats. A. Expression of transcripts coding for spinal OTR in control and NMS rats. No significant difference between groups has been detected using Unpaired Student t-test, N=7 control and N=8 NMS. **B.** Changes in mechanical nociceptive threshold **in CTRL and NMS rats** after i.t injection of OT (1 μ M, 20 μ L, **green triangles, N=8 for CTRL and N=7 for NMS**), TGOT (1 μ M, 20 μ L, **orange squares, N=8**) or vehicle (NaCl, 20 μ L, **white circles, N=7**). Statistical code for **repeated measures** Two-way ANOVA treatment X Time followed by **Tukey's** post hoc test: *** $p < 0.001$.

Figure 6. Neonatal treatment with OT or AP can partially suppress NMS consequences. Effect of a neonatal repeated treatment with oxytocin (OT, 1mg/kg o.d. i.p. injections between P2 and P12, purple bars) or allopregnanolone (AP, 10mg/kg i.p. injections between P2 and P12 every 2days, red bars), on mechanical nociceptive thresholds (**A**, N=12 control and N=8 NMS), stress induced

1 analgesia (SIA) (B, N=9 control and N=8 NMS), and OTR-dependent inhibitory control 24 h after
2 carrageenan inflammation (C, n=8 per group). Results are shown for control (CTRL) and NMS rats.
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5 Statistical code for One WAY ANOVA followed by Tukey post hoc test: *p<0.05; **p<0.01;
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7 ***p<0.001.
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10 **Figure 7. Epigenetic alterations following NMS and partial suppression of NMS consequences**

11 **after a neonatal treatment with SAHA. A.** Changes in relative gene expression of spinal
12 transcripts coding for HDAC 1-9 in NMS rats compared to controls, as measured with RT-qPCR,
13 from P7 to P100. A significant increase in the expression of HDAC 1, HDAC 7 and HDAC 8 has
14 been detected at different time points. Statistical code for Kruskal-Wallis followed by Dunn's
15 post hoc test : ++p<0.01; +++p<0.001, with "+" symbolizing and increased in expression in NMS
16 rats compared to controls. **B.** Time course of the expression of transcripts coding for spinal
17 HDAC 7 in control and NMS rats, from P7 to P100. Statistical code for Kruskal-Wallis followed
18 by Dunn's post hoc test : **p<0.01; ***p<0.001. **C to E.** Effect of a neonatal repeated treatment
19 with SAHA (5mg/kg, daily i.p. injection between P2 and P12: dark green), a non-selective HDAC
20 inhibitor, on mechanical nociceptive thresholds (C, N=8 control and NMS, N=10 SAHA treated
21 NMS), stress-induced analgesia (D, N=9 CTRL, N=8 NMS and N=7 SAHA treated NMS), OTR-
22 dependent inhibitory control 24 hours after carrageenan inflammation (E, N=10 ctrl, N=8 NMS N=8
23 SAHA treated NMS). Results are shown for control (CTRL) and NMS rats. Statistical code for One
24 WAY ANOVA followed by Tukey post hoc test for panels C to E : *p<0.05; *** p<0.001.
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51 **Supplementary Figure 1. A.** Expression of transcripts coding for spinal OTase in control and NMS
52 rats, as measured by RT-qPCR. No significant difference between conditions has been detected
53 using unpaired Student t-test, N=8 CTRL and NMS. **B.** Expression of transcripts coding
54 for spinal 3 α -HSD, 5 α -reductase II, P450scc and TSPO in control and NMS rats, as measured by RT-
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1 qPCR. No significant difference between conditions has been detected using One Way
2 ANOVA, N=8 CTRL and NMS.
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5 **Supplementary Table 1.** List of primer sequences used to amplify genes of interest by quantitative
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8 RT-PCR.
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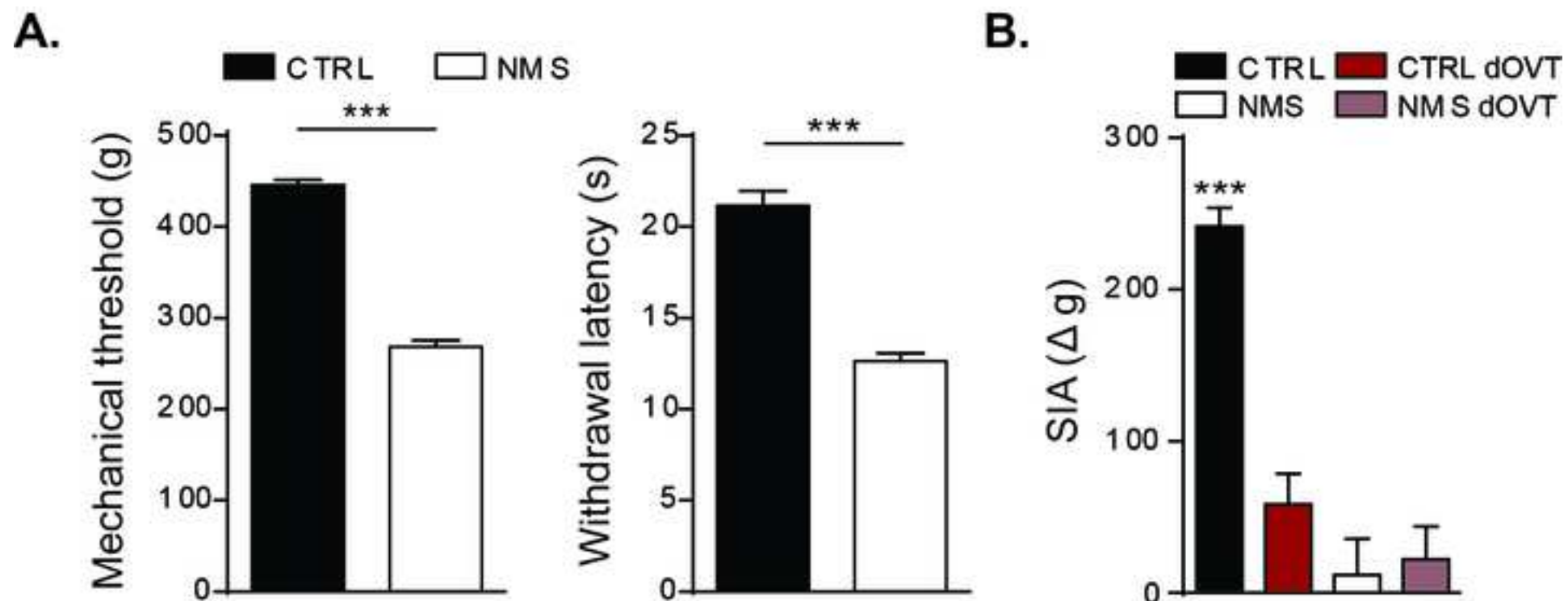
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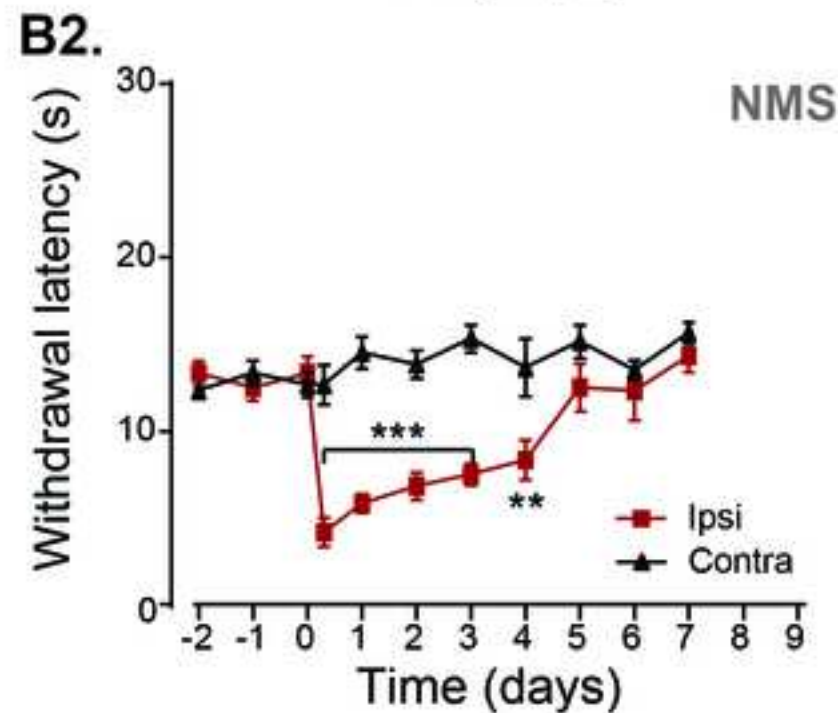
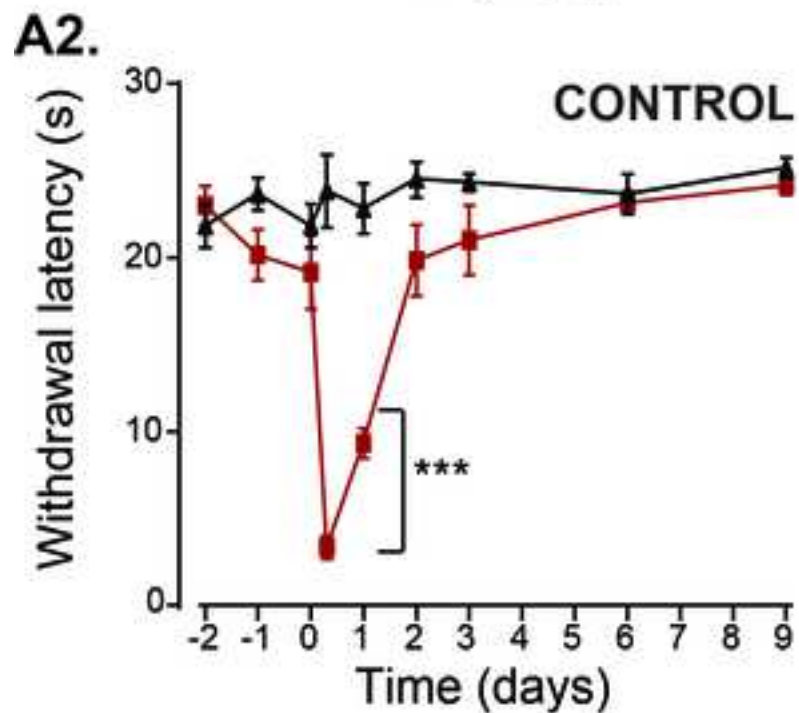
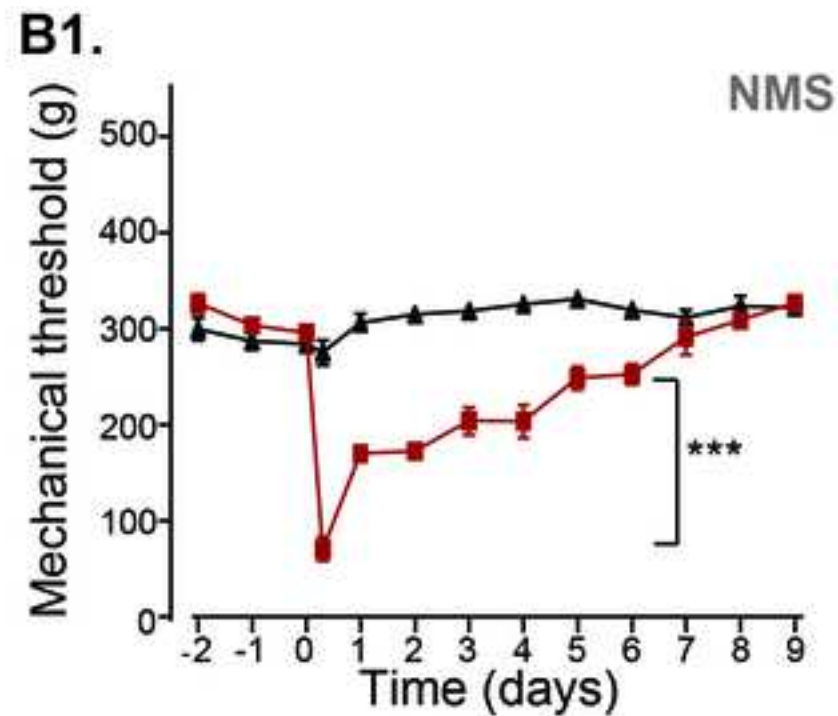
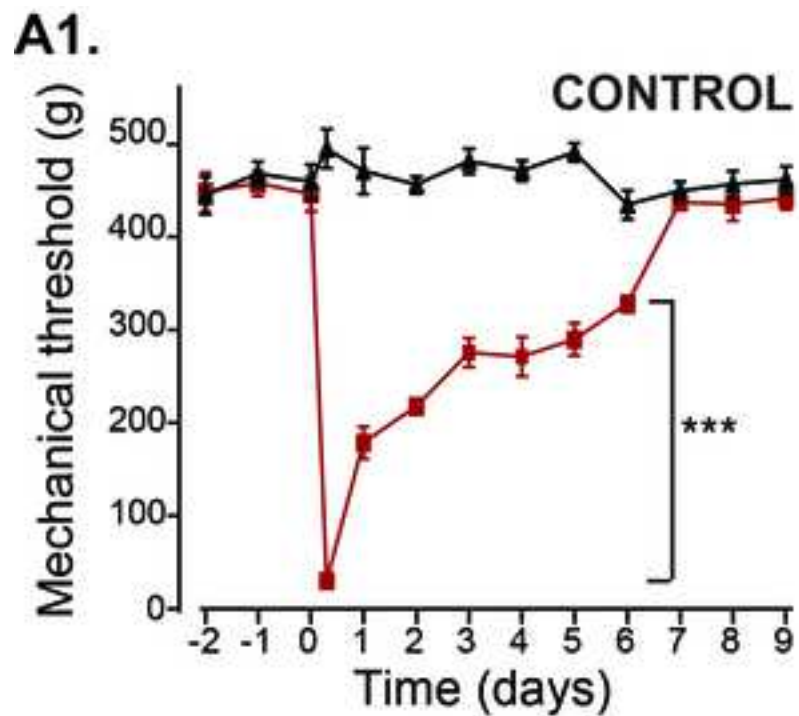
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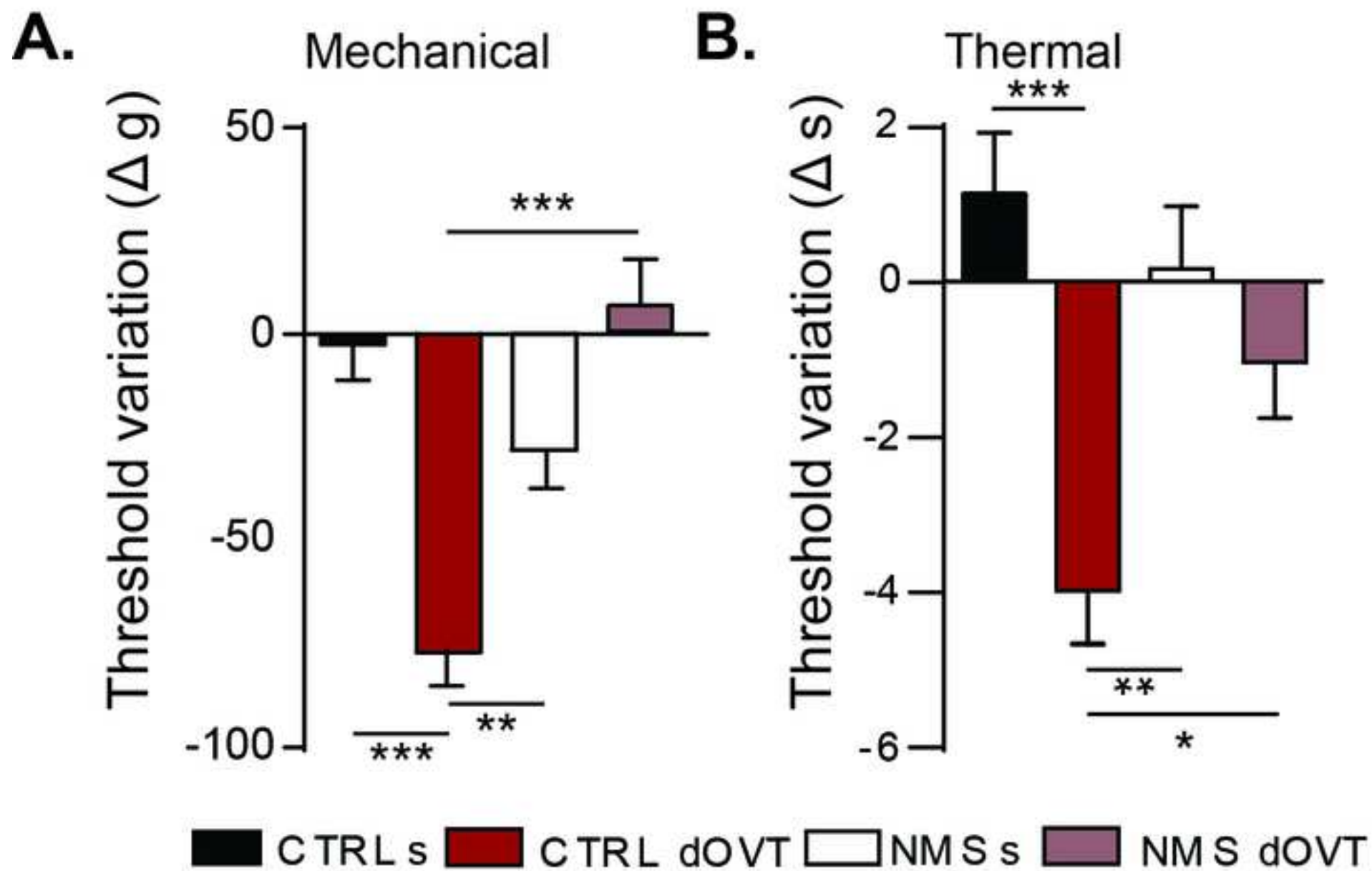
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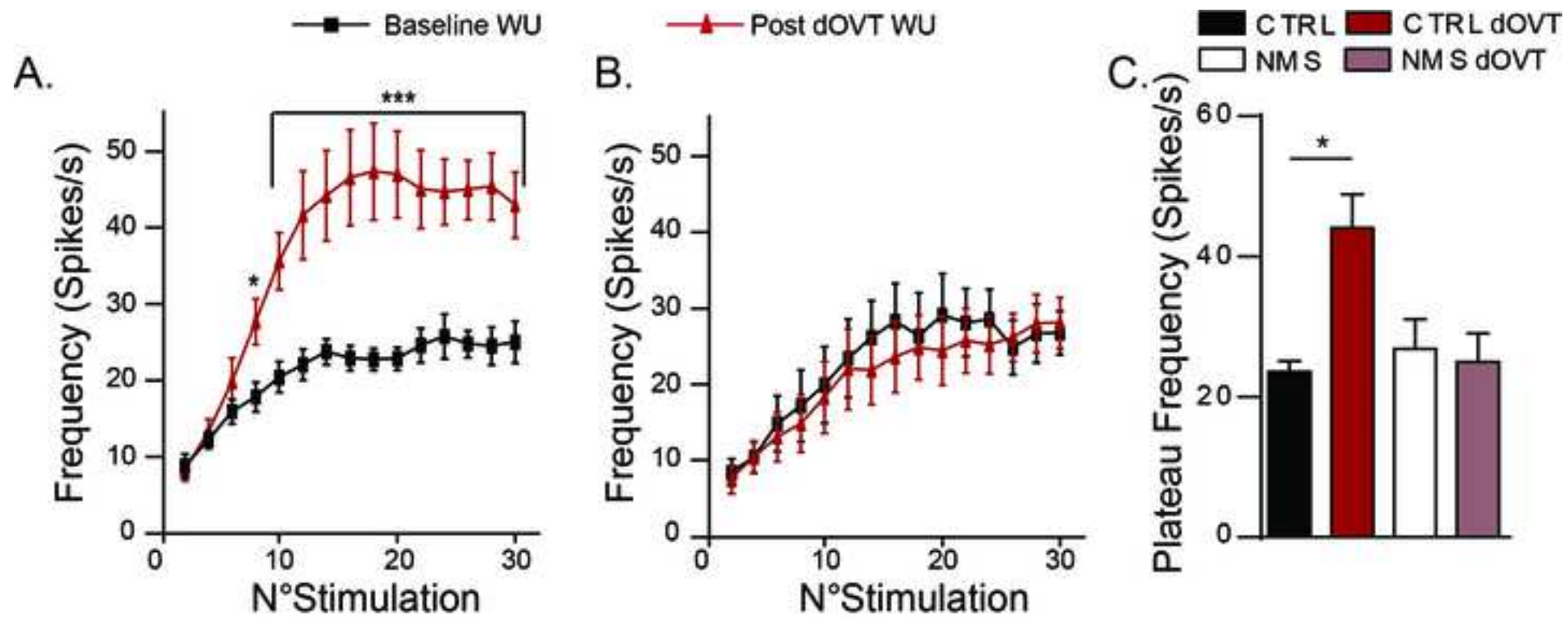
Maternal separation renders adult rats hypersensitive to noxious stimuli and incapable to recruit oxytocin analgesic controls. Rescue is achieved by neonatal treatments with oxytocin, allopregnanolone or HDAC inhibitors.

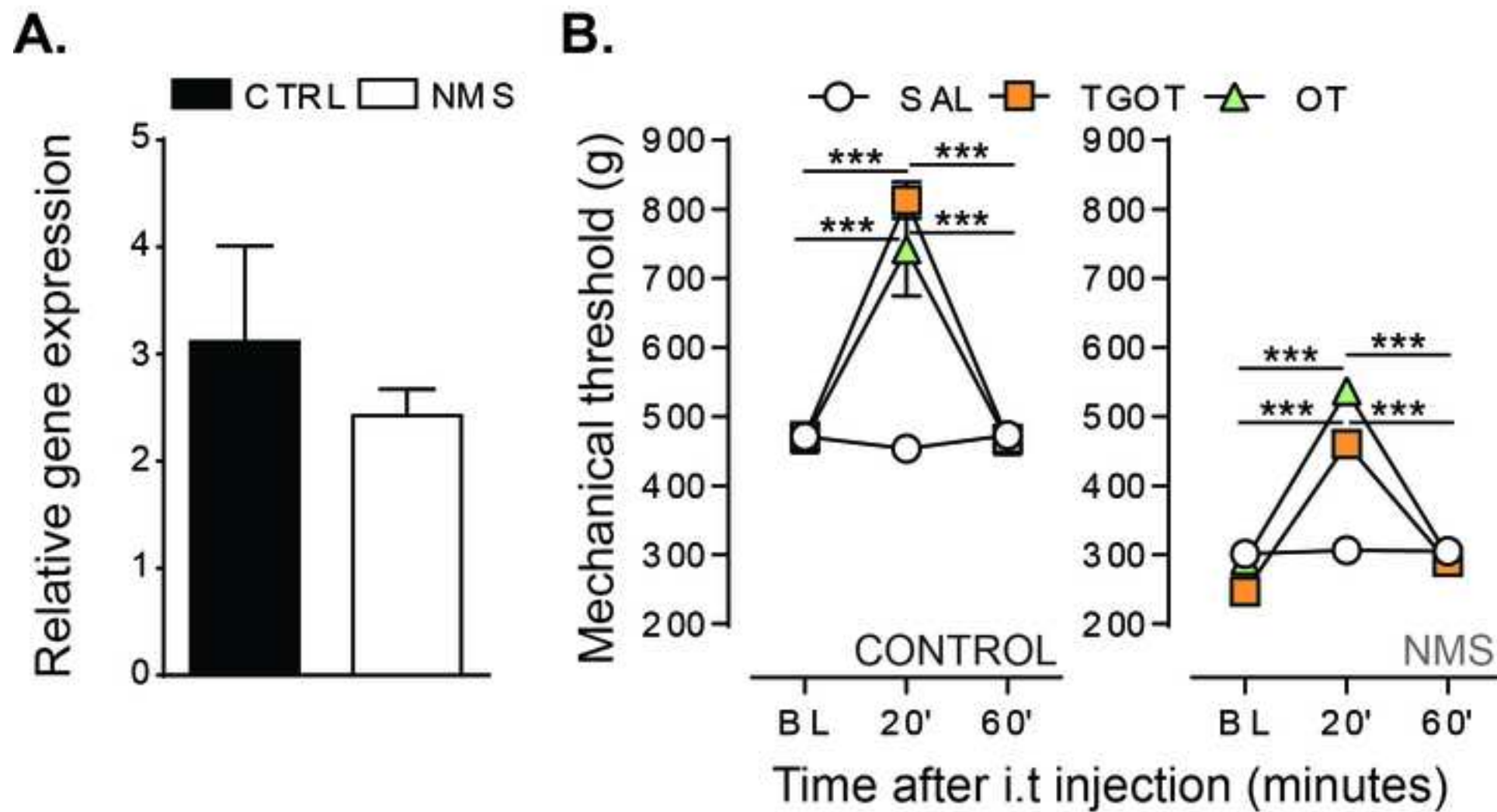
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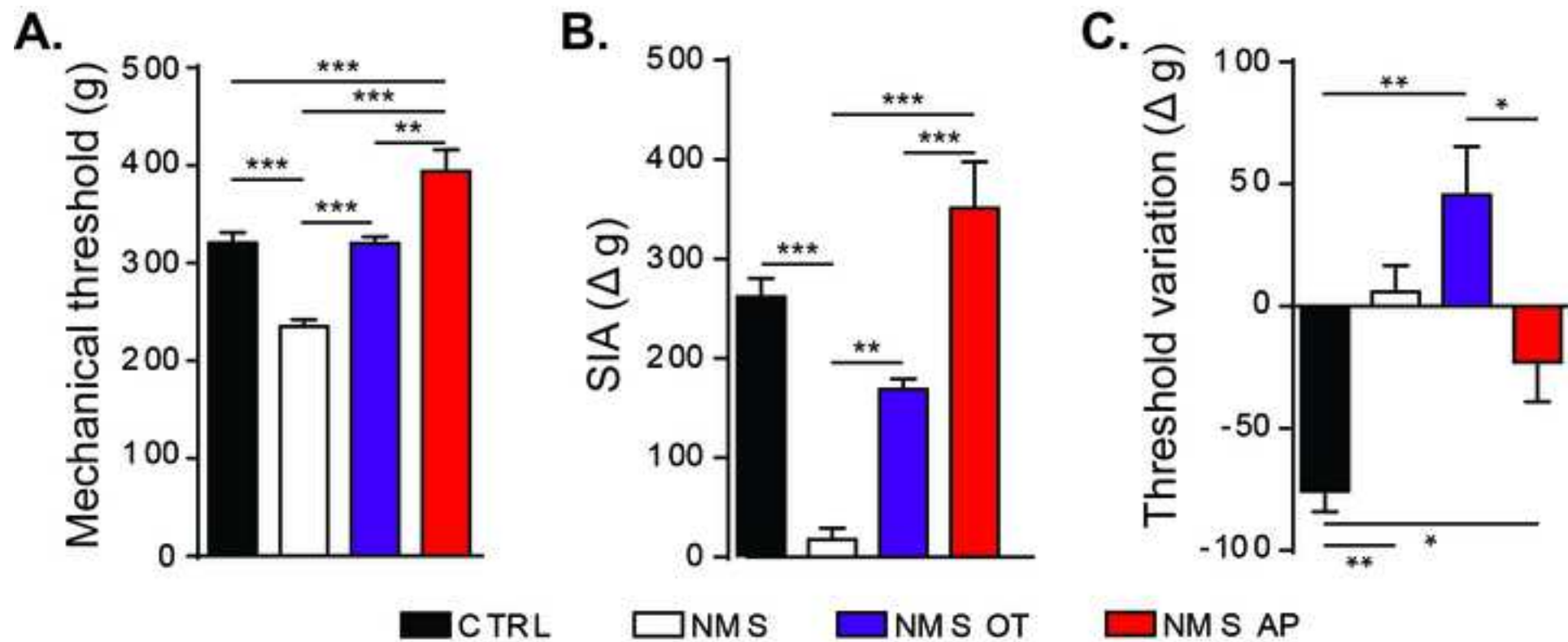


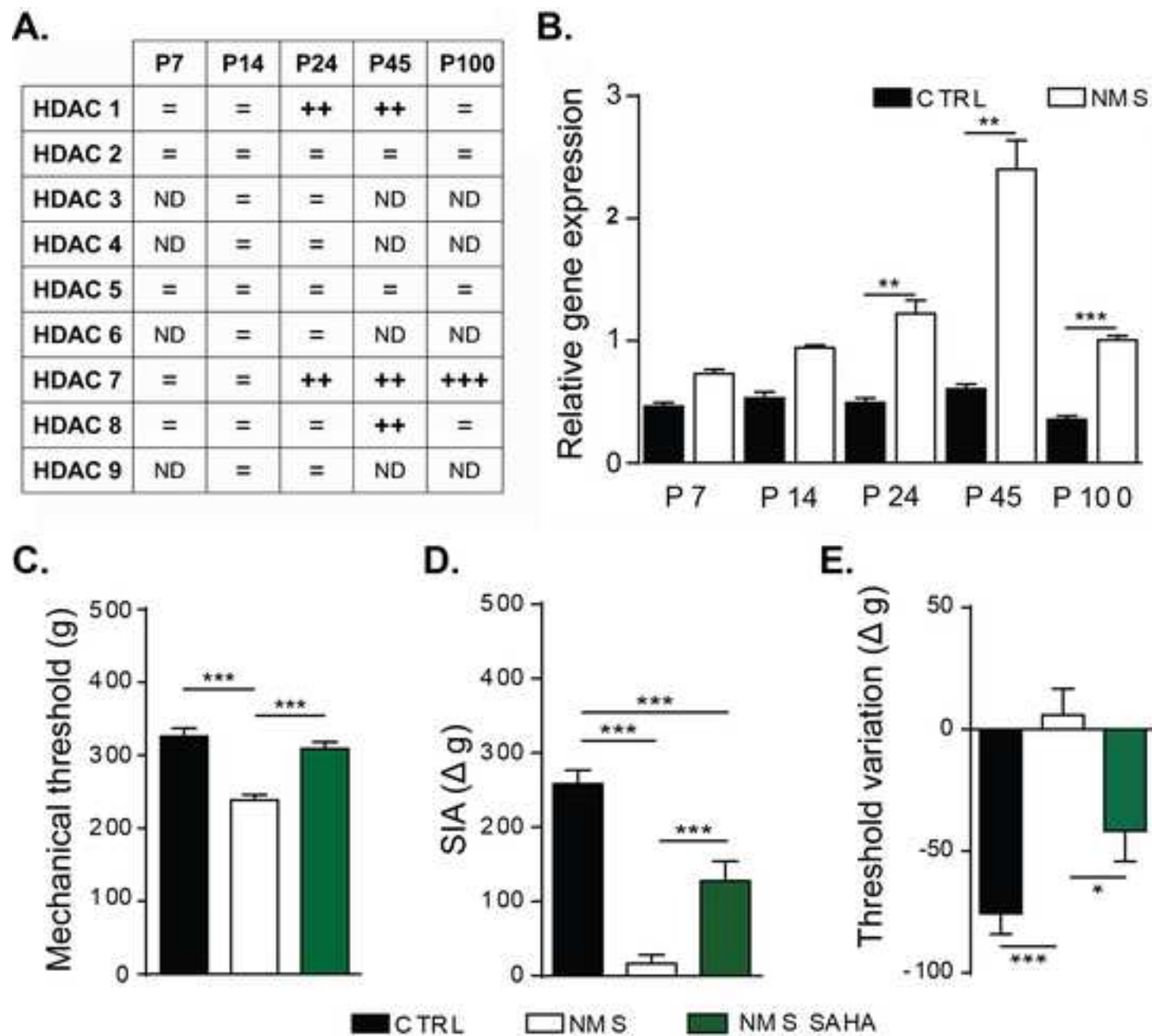


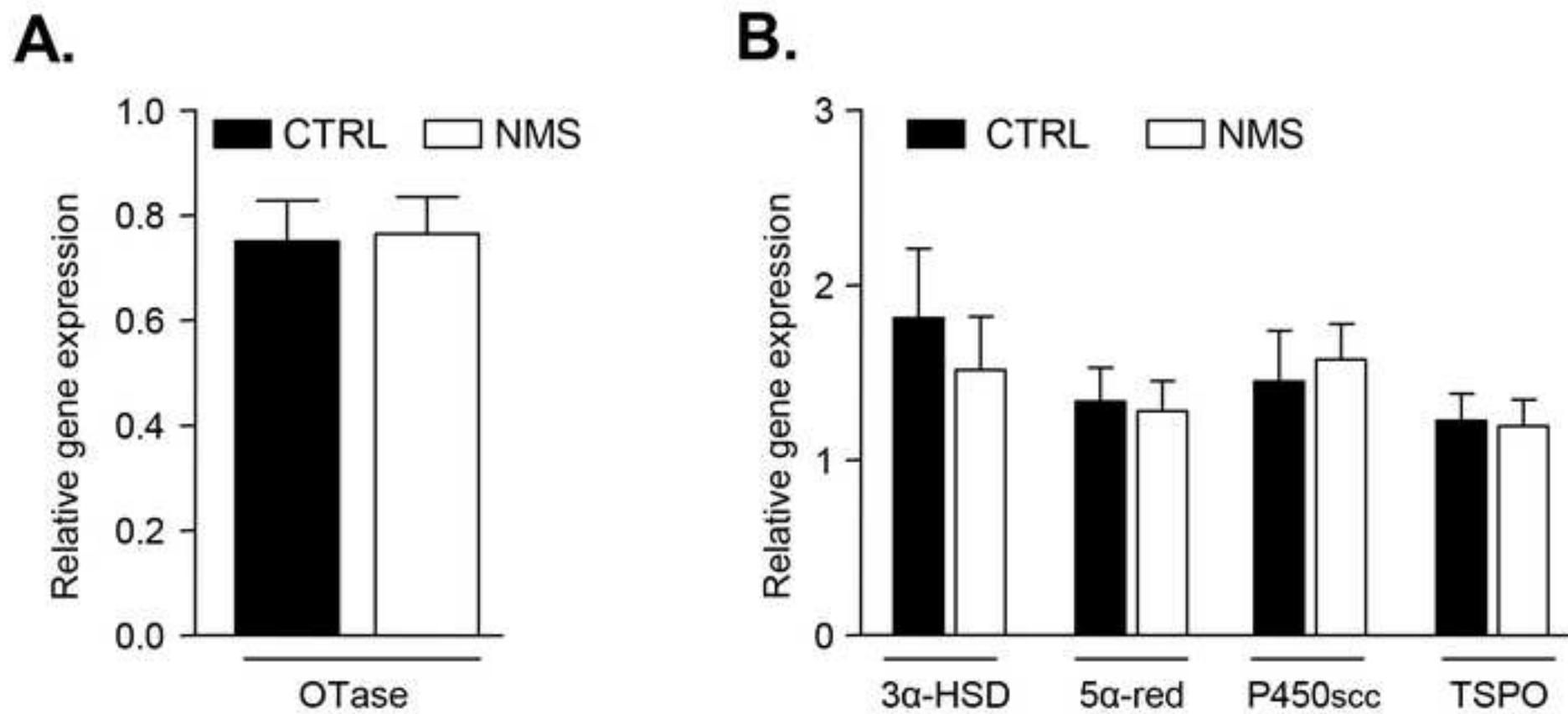












Name	Reference sequence	Amplicon region	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
3α-HSD	NM138547	330 - 423	ACCAGAGCTGGTCCGAACTT	CAAAGCCATTGGGAAATGAA
5α-red II	NM022711	49 - 146	TGCAGATTGTCTGCCATCAG	GTAAGTGGCGGGTTTCCCTA
HDAC 1	NM001025409	247 - 344	CGTTCTATTCGCCAGATAAC	AACTCAAACAAGCCATCAAAT
HDAC 2	NM053447	1415 - 1510	AATTTCCATTCGAGCATCAGA	TCAGCAACATTCCTACGACCT
HDAC 3	NM053448	1238 - 1346	GGGGTCCTGAGGAGAACTAC	CATCCATGCTGCTCTTAAATC
HDAC 4	NM053449	986 - 1089	ATGTACGACGCCAAAGATGA	CTGCTCCGTCTCTCAGCTACT
HDAC 5	NM053450	2084 - 2184	GAGGAGGACTGCATTCAGGTC	GCGAACAACTTTTTGTAAACA
HDAC 6	KY009929	1929 - 2015	AATCGTGGATTGGGATGTTC	CGGTGCAGGGACACGTATAAT
HDAC 7	AF321135	472 - 564	CCCCTGCGTAAACAGTGTCT	CTTCCTGAGCAGGGGATT
HDAC 8	NM001126373	921 - 1023	GACTGTGTCCCTGCACAAGTT	GGGCACATTGACACTGTAGT
HDAC 9	NM001200045	1666 - 1775	CCTGGAGAAGCAGAAGCAATA	CCTCCTCTGCCTCTTCAAGAT
HPRT	NM012583	558 - 647	GGTGAAAAGGACCTCTCGAA	TCAAGGGCATATCCAACAACA
OT	NM012996	80 - 164	CTCTGACCTCCGCCTGCTA	GGAAGACACTTGCGCATATCC
OTR	NM012871	732 - 825	TCGCCGTCTACATTGTACCG	GCTGCCGTCTTGAGTCTCAG
OTase	NM001113403	1934 - 2029	CCAGAGAAAGGGGACTGAGC	TGCCAAAGGTGGCTTGTATC
P450_{scc}	NM017286	679 - 780	GATGTTGGAGGAGATCGTGGA	AAGTCTGGAGGCATGTTGAGC
TSPO	NM012515	152 - 243	ATGGGAGCCTACTTTGTGCGT	GGAGCGAGTGTCAGCGA