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Overexpression of chloride importer NKCC1 contributes to the sensory-affective and sociability phenotype of rats following neonatal maternal separation

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Keywords: neonatal maternal separation; oxytocin; pain; chloride homeostasis; anxiety; sociability

Highlights:

- Maternal separation alters central expression and function of chloride transporters
- Maternal separation induces anxiety-like symptoms and a lack of social preference
- Phenotypic alterations are associated with a pro-inflammatory state
- Neonatal administration of bumetanide or oxytocin normalizes the phenotype

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Declarations of interest: none

Abstract

Background: Early life stress is known to affect the development of the nervous system and its function at a later age. It increases the risk to develop psychiatric disorders as well as chronic pain and its associated affective comorbidities across the lifespan. GABAergic inhibition is important for the regulation of central function and related behaviors, including nociception, anxiety or social interactions, and requires low intracellular chloride levels. Of particular interest, the oxytocinergic (OTergic) system exerts potent anxiolytic, analgesic and pro-social properties and is known to be involved in the regulation of chloride homeostasis and to be impaired following early life stress.

Methods: We used behavioral measures to evaluate anxiety, social interactions and pain responses in a rat model of neonatal maternal separation (NMS). Using quantitative PCR, we investigated whether NMS was associated with alterations in the expression of chloride transporters in the cerebrum and spinal cord. Finally, we evaluated the contribution of OTergic signaling and neuro-inflammatory processes in the observed phenotype.

Results: NMS animals displayed a long-lasting upregulation of chloride importer Na-K-Cl cotransporter type 1 (NKCC1) expression in the cerebrum and spinal cord. Neonatal administration of the NKCC1 inhibitor bumetanide or oxytocin successfully normalized the anxiety-like symptoms and the lack of social preference observed in NMS animals. Phenotypic alterations were associated with a pro-inflammatory state which could contribute to NKCC1 upregulation.

Conclusions: This work suggests that an impaired chloride homeostasis, linked to oxytocin signaling dysfunction and to neuro-inflammatory processes, could contribute to the sensori-affective phenotype following NMS.

1. Introduction

Early life stress induces long-term consequences on brain functions, neuroendocrine reactivity and behaviors (Weinstock, 2001; Talge et al., 2007; Bolton et al., 2017). During the early life, the intense neurodevelopmental plasticity is essential for optimal brain maturation but renders the brain particularly vulnerable to environmental influences. From a developmental point of view, cerebral maturation of the rat at birth is similar to that of the human fetus at the beginning of the third trimester of gestation (Romijn et al., 1991). This period coincides in particular with the maturation of the nociceptive system (Verriotis et al., 2016) and with the maturation of chloride homeostasis, underlying a shift from excitatory to inhibitory GABA function (Rivera et al., 1999; Stil et al., 2009). Two chloride transporters concentrate most of the attention: the cellular membrane expressed chloride ion importer NKCC1 (Na-K-Cl cotransporter type 1) and the chloride ion exporter KCC2 (K-Cl cotransporter type 2), which are responsible for chloride import and export in neural cells, respectively. This maturation process is associated with the release of neurotransmitters involved in the setting of emotions and psychosocial behaviors, such as oxytocin (OT), which are claimed to be inefficient in some neurodevelopmental disorders associated with increased anxiety or autistic spectrum disorders (Young and Barrett, 2015; Gottschalk and Domschke, 2018; Tobon et al., 2018).

In line, many of the neuropsychiatric symptoms associated with neurodevelopmental disorders may result from reduced brain inhibition and an alteration of transmembrane chloride fluxes through GABA_A and glycine receptor channels (Doyon et al., 2016). In particular, functional alterations of NKCC1 and KCC2 transporters seem involved in chronic pain states (Kaila et al., 2014). Downregulation of KCC2 and/or increases in NKCC1 activity, inducing synaptic disinhibition in the dorsal horn of the spinal cord, play a major role in the development and maintenance of pain symptoms (Sung et al., 2000; Coull et al., 2003; Galan and Cervero, 2005; Nomura et al., 2006; Valencia-de Ita et al., 2006; Miletic and Miletic,

2008; Zhang et al., 2008; Hasbargen et al., 2010; Eto et al., 2012; Janssen et al., 2012; Chen et al., 2014; Okada-Ogawa et al., 2015). If the underlying mechanisms of action are still under investigation, evidence suggests a strong link between chloride homeostasis and neuro-inflammatory processes, which actively regulate chloride transporter expression and function (Kaila et al., 2014). In the context of neuropathic pain, it was suggested that activated microglia could release brain-derived neurotrophic factor which could contribute to KCC2 downregulation in spinal neurons and allodynia, even if these results are still controversial (Coull et al., 2005). Furthermore, NKCC1 levels are regulated by inflammatory cytokines and growth factors like tumor necrosis factor- α (TNF- α) (Huang et al., 2014; Weidenfeld and Kuebler, 2017), which can lead to an altered GABAergic activity following inflammatory insults (Price et al., 2005).

Interestingly, disruption of chloride homeostasis due to genetic variation and/or alteration of chloride transporter expression is also seen in several neuropsychiatric disorders, such as schizophrenia (Lewis et al., 2003; Hyde et al., 2011; Sullivan et al., 2015), depressive disorder (Tao et al., 2012), autistic spectrum disorders (Merner et al., 2015) and other affective disorders. In animal models of autistic spectrum disorders, alteration of the excitatory/inhibitory balance is observed (Gibson et al., 2008; Goncalves et al., 2013) and associated with a prolonged depolarizing effect of GABA in juvenile animals (Tyzio et al., 2014). It is also worth mentioning that OT-mediated GABA inhibition during delivery is abolished in these models and contributes to the autistic spectrum disorders-like phenotype (Tyzio et al., 2014). Altogether, these studies strongly suggest that abnormal GABAergic transmission is involved in the pathology of several neuropsychiatric and affective disorders (Luscher et al., 2011; Sibille et al., 2011).

Neonatal maternal separation (NMS) is a well-established animal model of early-life stress in which pups are separated from the dam during the early postnatal period. Long-term

consequences of NMS include alterations of the stress response characterized by hyper-reactivity of the hypothalamic-pituitary-adrenal axis, anxiety-like symptoms (Plotsky and Meaney, 1993), and social deficits (Lukas et al., 2011; Holland et al., 2014). If visceral hypersensitivity has been commonly observed after NMS (Chung et al., 2007; Moloney et al., 2015), our laboratory also recently demonstrated the presence of long-lasting somatic hypersensitivities to thermal hot and mechanical stimuli (Juif et al., 2016) as well as a deficit in OTerpic analgesic controls (Melchior et al., 2018).

While OT appears to be a critical determinant in the long-term consequences of NMS on pain responses and associated comorbidities (Melchior et al., 2018), it has also been involved in chloride homeostasis during development. Early OT signaling enables neuronal maturation through upregulation of KCC2 expression counteracting the function of NKCC1 (Leonzino et al., 2016). As reviewed recently, OT-mediated analgesia mainly relies on an increased GABAergic inhibition in the spinal cord (Poisbeau et al., 2018), and can for example be activated after a forced swim stress (Wotjak et al., 1998; Melchior et al., 2018). This helps to understand that an alteration of chloride homeostasis could impair the beneficial action of OT via chloride mediated GABA_A receptor-channels (Bonin and De Koninck, 2013).

In this study, we used behavioral measures to evaluate the long-term consequences of NMS on the sensory-affective components of pain in adult rats. Complementary to our previous results (Juif et al., 2016; Melchior et al., 2018), we investigated whether impaired chloride transporter expression and OT signaling might contribute to the observed alteration in pain sensitivity and associated affective comorbidities and investigated a possible link with underlying inflammatory reactivity.

2. Methods and materials

2.1. Animals

Pregnant female Wistar rats (Chronobiotron, Strasbourg, France) were used in this study and carefully monitored for delivery. Day of birth was referred to as postnatal day 0 (P0). Mother and pups were housed in a temperature (22°C) and humidity (50 ± 20%) controlled room, under a 12h light-dark cycle (lights on at 7:00 am), with *ad libitum* access to food and tap water. Pups were weaned at P21 and housed in collective cages according to sex. Males and females were used in this study, and results were pooled when no sex-specific difference was observed. All procedures were conducted in accordance with EU regulations and approved by the regional ethical committee (CREMEAS authorization numbers APAFIS#7678-2016112223027528 v2 and APAFIS#10215-201706131614598 v3).

2.2. Neonatal Maternal Separation protocol

Dams and their litter were randomly assigned to the control or NMS group upon parturition. Litters from the NMS group were separated from their home cage for three hours per day, from postnatal day 2 (P2) to postnatal day 12 (P12), and placed in a separate room under a heating lamp to maintain their temperature. Litters from the control group remained undisturbed.

2.3. Behavioral testing

Animals were tested between P45 and P55 and chosen randomly in at least two different litters. Experiments were performed during the light phase, between 9:00a.m. and 4:00p.m. Prior to behavioral testing, animals were habituated to the experimenter with three days of handling.

2.3.1. Evaluation of anxiety-like symptoms with the elevated plus maze

Apparatus consisted in two open arms (45 x 10 cm) and two closed arms (walls 30 cm high) arranged in a plus shape and elevated at a height of 50 cm. Animals were placed in the center of the elevated plus maze with their head facing the open arm opposite the experimenter. Animals were video-tracked for 5 minutes and analyzed with the ANY-maze 5.2 software (Ugo Basile, Gemonio, Italy).

2.3.2. Evaluation of sociability with the three-chamber test

Apparatus consisted in three chambers (80 x 40 x 40 cm) connected by doors (15 x 10 cm). Rats were placed in the center chamber and left to freely explore the apparatus for habituation. After 10 minutes, an empty cylindric wire box was placed in one of the side compartments, and a similar box containing a stranger animal (previously habituated to the box) was placed in the other side compartment. Time spent in both compartments was video recorded during 10 minutes and analyzed using the ANY-maze 5.2 software (Ugo Basile, Gemonio, Italy) to assess sociability.

2.3.3. Evaluation of mechanical nociception with the calibrated forceps

Habituated rats were loosely restrained with a towel masking their eyes. Tips of the calibrated forceps (Bioseb, Vitrolles, France) were placed at each side of the hindpaw, and a gradually increasing force was applied until withdrawal of the paw, following a validated procedure (Luis-Delgado et al., 2006). Pressure eliciting withdrawal of the paw was considered as the mechanical nociceptive threshold value.

2.3.4. Swim stress-induced OTergic analgesia

Rats were exposed to a forced swim stress protocol known to induce recruitment of OTergic descending controls of pain (Wotjak et al., 1998; Melchior et al., 2018). Animals were placed in a PlexiglasTM cylinder (diameter 30 cm) filled with 20°C water. After 10 minutes, rats were gently dried. Mechanical nociceptive thresholds were assessed before the swim and

15 minutes afterwards using calibrated forceps. Stress-induced analgesia was evaluated as the delta between threshold values after and before exposure to swim stress.

2.4. Nerve tissue sampling and RT-qPCR

The lumbar segment of the spinal cord was collected as well as the cerebrum (cerebral hemispheres and diencephalon), reconstituted in a guanidine thiocyanate/ β -mercaptoethanol preparation using ultraturax and stored at -80°C . Total RNA was extracted using a protocol adapted from the original procedure of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) consisting in 2 independent total RNA extractions separated by a DNase I treatment (TURBOTM DNase; Ambion, Life technologies, Saint Aubin, France), as previously described in detail (Lelievre et al., 2002). 800ng RNA were reverse transcribed 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 (20 seconds at 95°C , 20 seconds at 60°C , and 20 seconds at 72°C). Standardization was done using standard curves made from serial dilutions of samples, and amplification data are shown as relative gene expression, calculated as the ratio between cDNA concentration of the gene of interest and that of the housekeeping gene. Here, hypoxanthine-guanine phosphoribosyltransferase (HPRT) was selected as the housekeeping gene since its transcripts remained highly stable among the different samples. Primer sets for all genes of interest were designed using Oligo6.0 and M-fold softwares (primer sequences in Supplementary Table 1). Samples were accurately dispensed in duplicates using a robotic workstation (Freedom EVO100; Tecan, Lyon, France), and amplification efficacy given by standard curves was always close to 100 % (± 2 %), while amplification specificity was assessed by a melting curve study.

2.5. Pharmacological treatments

Neonatal treatments: Drugs were freshly prepared each day and administered to pups from P2 to P12, at the start of the NMS protocol (for bumetanide [BTD] and OT), and for half of the litter at a time in control animals (VU0463271) in order to avoid maternal stress and prolonged withdrawal from the home cage. The NKCC1 inhibitor BTD (Merck, USA) was dissolved in mineral oil and administered subcutaneously (s.c.; 10 μ L / 10g of animal weight) at a final dose of 0.5 mg / kg. OT (Bachem, Switzerland) was diluted in NaCl 0.9 % to reach the dose of 1 mg / kg and injected intraperitoneally (i.p.; 0.1 mL / 10g of animal weight). The KCC2 inhibitor VU0463271 (Tocris Bioscience, UK) was dissolved in 20 % dimethyl sulfoxide (DMSO, Merck, USA) and 80 % mineral oil and injected s.c. at a dose of 2 mg / kg.

Adult treatment: In a subset of experiments, BTD was diluted in 10 % DMSO and injected intrathecally (i.t.; 90 μ g / 20 μ L) in NMS animals. Animals were then subjected to pain assessment 30 minutes after i.t. injection.

2.6. Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, USA). For each experiment, normal distribution of values was verified with the Shapiro-Wilk normality test prior to performing parametric analysis. One-way analysis of variance tests (ANOVA) followed by Tukey's post hoc multiple comparison test was used to compare anxiety-like symptoms, nociceptive thresholds and efficacy of stress-induced analgesia between groups. Two-way (condition x chamber) ANOVA (2wANOVA) followed by Sidak's post hoc test was used to assess social preference in the three-chamber test. 2wANOVA (condition x time) was used to compare transcript expression of several genes at different time points and followed by Sidak or Tukey's post hoc test. Differences were considered to be statistically significant for $p < 0.05$.

3. Results

3.1. NMS-induced alterations in chloride transporter expression in the cerebrum and spinal cord

To highlight possible alterations in chloride homeostasis in the cerebrum and spinal cord of NMS rats, we analyzed the expression of chloride transporters NKCC1 and KCC2, responsible for most of the chloride import and export in neural cells, respectively (**Figure 1**).

Figure 1 near here

As shown in figure 1A, NKCC1 mRNA expression in the cerebrum was significantly higher in NMS animals (2wANOVA, condition x time, $F_{(3,32)} = 11.46$, $p < 0.0001$) from P14 (CTRL: 1.49 ± 0.05 ; NMS: 2.30 ± 0.07) to P100 (CTRL: 0.54 ± 0.05 ; NMS: 0.94 ± 0.07). In the case of KCC2, mRNA expression in the cerebrum was significantly lower in NMS animals (2wANOVA, condition x time, $F_{(3,40)} = 4.83$, $p = 0.0058$) from P24 (CTRL: 1.66 ± 0.03 ; NMS: 1.34 ± 0.04) to P45 (CTRL: 2.12 ± 0.07 ; NMS: 1.65 ± 0.06), but not at P14 (CTRL: 1.30 ± 0.05 ; NMS: 1.13 ± 0.1) nor P100 (CTRL: 2.25 ± 0.10 ; NMS: 2.25 ± 0.04). We also used the spinal cord as a target tissue being at the crossroads of ascending pain information processing, descending modulatory control and behavioral motor responses (including autonomic). As shown in figure 1B, spinal NKCC1 mRNA expression was significantly higher in NMS animals (2wANOVA, condition x time, $F_{(3,24)} = 29.50$, $p < 0.0001$) from P14 (CTRL: 1.30 ± 0.05 ; NMS: 1.92 ± 0.09) to P100 (CTRL: 0.54 ± 0.03 ; NMS: 1.13 ± 0.07). In comparison to NKCC1, we found no significant difference in KCC2 transcript expression at any of the ages studied (Figure 1A; 2wANOVA, condition x time, $F_{(3,24)} = 0.828$, $p = 0.4914$). Accordingly, the ratio in favor of NKCC1 likely suggests an alteration in the maturation of chloride homeostasis and reduced spinal and supraspinal inhibition in NMS animals.

To illustrate the functional consequences of NKCC1 overexpression on the NMS phenotype, we evaluated the effect of a single i.t. injection of BTD (90µg/20µL) on the previously described nociceptive hypersensitivity in NMS animals at adulthood (Juif et al., 2016). Intrathecal BTD induced a significant increase in mechanical nociceptive thresholds 30 minutes after injection, from 283.0 ± 8.18 g to 383.13 ± 16.88 g (Figure 2A; 1wANOVA, $F_{(3,36)} = 37.16$, $p < 0.0001$). Mean mechanical thresholds however still remained significantly lower in BTD-injected NMS animals compared to controls (428.1 ± 31.6 g). In CTRL animals, BTD did not change baseline mechanical thresholds.

Figure 2 near here

We also evaluated if NKCC1 overexpression could be involved in the alteration of the OTergic analgesic controls that we recently described with a protocol of stress-induced analgesia (Melchior et al., 2018). Here, i.t. BTD injection had no significant effect in CTRL animals but fully restored OTergic analgesia, as demonstrated by the significant increase in nociceptive thresholds (1wANOVA, $F_{(3,29)} = 13.71$, $p < 0.0001$) observed 15 minutes after the swim stress (Figure 2B; CTRL: delta 261.78 ± 18.65 g; NMS: 17.88 ± 11.66 g; NMS after BTD i.t.: 257.25 ± 55.56 g). These results strongly suggest that the alteration of chloride homeostasis due to NKCC1 hyperactivity contributes to the NMS nociceptive phenotype as well as failure of OTergic analgesic controls.

3.2. Neonatal treatment with BTD

To confirm the critical role of chloride transporters in complex supraspinal behaviors and in the ontogeny of the NMS phenotype, we treated NMS pups with BTD from P2 to P12 and evaluated their anxiety and social behavior at adulthood (Figure 3).

NMS animals showed at P45 a significant decrease in the time spent in the open arms of an elevated plus maze (Figure 3A; 1wANOVA, $F_{(3,51)} = 11.92$, $p < 0.0001$) compared to controls (CTRL: 89.43 ± 9.54 s; NMS: 36.92 ± 8.76 s), suggesting increased anxiety-like symptoms of NMS rats as described in the literature. Neonatal BTD treatment restored mean values that were similar with those of controls (NMS+BTd: 92.91 ± 6.53 s).

Figure 3 near here

In the three-chamber test, we found that NMS animals did not display any significant social preference and spent as much time in the animal- and object-containing chambers (Figure 3B; time spent with the new animal 255.3 ± 27.92 s, time spent with new objects 256.2 ± 33.55 s, preference ratio 1.27 ± 0.17). This was in sharp contrast with the control group (time spent with the new animal 318.3 ± 14.56 s, time spent with the new object 180.9 ± 10.48 s, preference ratio 1.99 ± 0.20 ; 2wANOVA, condition x chamber, $F_{(3,108)} = 8.936$, $p < 0.0001$). Interestingly, neonatal BTd treatment fully restored social interactions, with animals displaying again a marked preference for the animal-containing chamber compared to the object-containing one, (time spent with the new animal 311.4 ± 27.63 s, time spent with new objects 179.9 ± 23.02 s, preference ratio of 2.2 ± 0.44 s).

In another set of experiments, we evaluated if KCC2 inhibition would be sufficient to induce NMS-like consequences in control animals. We thus performed neonatal injections of the KCC2 inhibitor VU0463271 (s.c.; 2mg/kg) from P2 to P12 in control pups. As shown in figure 3, we found that neonatal blockade of KCC2 resulted in anxiety-like symptoms similar to those observed in NMS animals. Both groups displayed a reduced time spent in the open arm (Figure 3A; CTRL+VU: 46.83 ± 5.39 s). In contrast, VU0463271 neonatal treatment of CTRL animals failed to result in any alterations in social interactions (Figure 3B; time spent with the new animal 366.9 ± 10.82 s, time spent with new objects 144.2 ± 9.91 s, preference ratio 2.7 ± 0.26).

3.3. Neonatal OT treatment rescues NMS-induced alterations of the affective phenotype

As mentioned above, NMS rats exhibited long-lasting nociceptive hypersensitivity and a blunted adaptation to stressful conditions associated with impaired OTergic stress-induced analgesia, which could be restored with neonatal OT treatment (Melchior et al., 2018). After neonatal OT treatment, NMS animals displayed mean nociceptive thresholds similar to control animals, as measured with the calibrated forceps, and a fully functional analgesia after forced swim stress. In an effort to strengthen the crucial role of OT in the neurodevelopment and maintenance of optimal chloride homeostasis, we analyzed the consequence of a neonatal OT treatment on the affective phenotype in NMS animals, evaluating anxiety levels and sociability.

Figure 4 near here

In the elevated plus maze, OT-treated NMS animals displayed a mean time spent in the open arms similar to that of the control group at P45 (Fig. 4A; CTRL: 89.43 ± 9.54 s; NMS+OT: 73.53 ± 6.44 s), in sharp contrast with the anxiety-like profile observed in NMS animals (1wANOVA, $F_{(2,41)} = 9.611$; $p = 0.0004$). Neonatal OT treatment also fully restored social preference values in the three-chamber test (2wANOVA, condition x chamber, $F_{(1,94)} = 14.20$; $p < 0.0001$; time spent with the new animal 355.4 ± 14.33 s, time spent with new objects 140.1 ± 12.16 s, preference ratio 2.9 ± 0.40).

3.4. NMS leads to a pro-inflammatory state

As chloride transporter levels can be regulated by inflammatory factors (Huang et al., 2014; Kaila et al., 2014; Weidenfeld and Kuebler, 2017), we analyzed the expression of several inflammatory mediators in the cerebrum and spinal cord as possible underlying

molecular mechanisms contributing to the altered maturation of chloride transporter in NMS animals (Figures 5 and 6).

In the cerebrum, interleukin-1 α (IL-1 α) levels were not different between groups (Figure 5A, 2wANOVA, condition x time, $F_{(3,40)} = 0.576$; $p = 0.6341$), but we found a transient up-regulation in interleukin-1 β (IL-1 β), detected only at P24 (Figure 5B; 2wANOVA, condition x time, $F_{(3,40)} = 44.32$; $p < 0.0001$), and a durable up-regulation of interleukin-6 (IL-6; Figure 5C; 2wANOVA, condition x time, $F_{(3,40)} = 4.150$; $p = 0.0119$) from P14 to P100 in NMS animals, and of TNF- α from P14 to P45 (Figure 5D; 2wANOVA, condition x time, $F_{(3,40)} = 10.23$; $p < 0.0001$). Cyclooxygenase-2 (Cox-2) levels were also higher in NMS animals from P24 until adulthood (Figure 5E; 2wANOVA, condition x time, $F_{(3,40)} = 28.68$; $p < 0.0001$). As a mirror image with the changes of pro-inflammatory mediators, levels of the anti-inflammatory cytokine interleukin-10 (IL-10) were decreased at P24 and P45 in NMS animals (Figure 5F; 2wANOVA, condition x time, $F_{(3,40)} = 8.364$; $p = 0.0002$).

Figure 5 near here

In the spinal cord, IL-1 α levels were also not different between groups (Figure 6A; 2wANOVA, condition x time, $F_{(3,24)} = 0.581$; $p = 0.6672$), but we found a durable upregulation of IL-1 β (Figure 6B; 2wANOVA, condition x time, $F_{(3,32)} = 12.29$; $p < 0.0001$) and IL-6 (Figure 6C; 2wANOVA, condition x time, $F_{(3,32)} = 25.86$; $p < 0.0001$) transcripts from P14 to P100 in NMS animals. TNF- α mRNA levels were significantly higher in the early postnatal period (Figure 6D; 2wANOVA, condition x time, $F_{(3,24)} = 7.121$; $p = 0.0014$), while spinal Cox-2 levels were significantly higher in NMS animals from P24 until adulthood (Figure 6E; 2wANOVA, condition x time, $F_{(3,32)} = 25.61$; $p < 0.0001$). Levels of IL-10 were decreased throughout all times assessed (Figure 6F; 2wANOVA, condition x time, $F_{(3,32)} = 11.81$; $p < 0.0001$).

Figure 6 near here

These results likely suggest a pro-inflammatory state in the nervous system of NMS animals, possibly explaining the tonic deleterious action of increased NKCC1 activity on anxiety, social and nociceptive behaviors.

4. Discussion

This work provides functional evidence indicating that NMS induces long-term alterations of the sensory-affective state in adult rats. As illustrated by the overexpression of the NKCC1 chloride importer in the spinal and supraspinal levels and by the benefits of its neonatal blockade by BTB, we propose these long-term alterations to result from a dysregulated chloride homeostasis. A reduced inhibitory control in the central nervous system as a result of over-expression of pro-inflammatory mediators could be responsible for the expression of somatic hypersensitivities to pain, increased anxiety and sociability impairments. Based on our results and on the recent literature in the field, we propose neonatal OT deficits to be a critical determinant for these symptoms. A summary of this working hypothesis is shown in figure 7.

Figure 7 near here

In early life, chloride homeostasis is subjected to critical developmental changes, controlling the switch from depolarizing to hyperpolarizing GABAergic currents (Wang et al., 2002; Watanabe and Fukuda, 2015). In the spinal cord, GABA action matures during the first 3 postnatal weeks (Cordero-Erausquin et al., 2005). In supraspinal regions a developmental GABA shift has been observed around P8-10 (Tyzio et al., 2007). Following NMS, this neonatal shift in chloride homeostasis and GABAergic function seems impaired. In particular,

transcripts coding for the chloride importer NKCC1 are durably upregulated suggesting a prolonged immaturity state of neurons and reduced GABAergic inhibition in the cerebrum and spinal cord. The increase in KCC2 levels seems delayed in the cerebrum of NMS animals, with lower levels at P24 and P45 compared to control animals, but not in the spinal cord. This delayed shift has been previously observed in the hippocampus of NMS mice and was linked to a decreased KCC2 expression (Furukawa et al., 2017). Similarly, following a single episode of NMS combined with neonatal anesthesia, rats displayed a long lasting increase in hypothalamic NKCC1 expression and decrease in KCC2 (Ju et al., 2017).

These observations may explain the nociceptive hypersensitivity, anxiety and impaired sociability phenotype of NMS rats. Corroborating our results, gain of function of NKCC1 is also observed in several neuropathic pain models (Hasbargen et al., 2010; Chen et al., 2014) as well as in neuropsychiatric disorders such as schizophrenia (Merner et al., 2016). Similarly, in a model of chemotherapy-induced neuropathic pain, BTB treatment attenuated pain symptoms (Chen et al., 2014). It is also important to note that the impaired behaviors are not suggested to be linked to an altered motor function, which might affect the performance in the elevated-plus maze or three-chamber test. Many studies have demonstrated that NMS animals display similar weight gain as CTRL animals, have similar performance in the rotarod test, and travel similar distances in the open field test, confirming that NMS animals display normal motor function and were able to perform the behavioral tasks (Paternain et al., 2012; Toda et al., 2014; Tata et al., 2015).

In our study, inhibiting NKCC1 function with BTB during the neonatal period successfully normalized the affective phenotype in NMS animals. This is consistent with previous studies in which the depressive and anxiety-like behavior of early-life stressed animals were rescued by neonatal BTB treatment, together with the expression of chloride transporters in the hypothalamus (Ju et al., 2017) or in the hippocampus (Hu et al., 2017).

Clinical trials have also highlighted the therapeutic potential of BTB to improve social behaviors in children with autistic spectrum disorders (Lemonnier et al., 2012). Altogether, disruption of chloride homeostasis appears as a key player in the apparition of several sensori-affective disorders commonly associated with early-life stress.

Based on the well-demonstrated analgesic properties of OT and our recent results demonstrating that neonatal OT is a key determinant of mechanical and thermal hot nociception (Melchior et al., 2018), we show here that neonatal OT also rescues anxiety and social deficits resulting from NMS. It is well known that OT has anxiolytic properties (Gottschalk and Domschke, 2018) and may be useful to treat psychosocial deficits following early-life stress, such as autistic spectrum disorders-like signs (Yamasue and Domes, 2018). Indeed, skin to skin contact, favoring OT release (Hardin et al., 2020), has been proven to relieve pain and to have beneficial consequences on the social and behavioral outcomes of preterm birth (Johnston et al., 2003; Charpak et al., 2017). Our working hypothesis is that OT levels could be reduced following NMS, possibly because of an alteration of maternal care, and could impact the proper development of chloride homeostasis and GABAergic function. In good agreement with this idea, early-life stress in humans has been associated with reduced endogenous OT in the cerebrospinal fluid (Heim et al., 2009). A link between OT and chloride homeostasis has been demonstrated in the past as OT stimulates the upregulation of KCC2 exporters optimizing the inhibitory action of GABAergic neurons (Leonzino et al., 2016). In a mouse model, the effect of OT on the GABAergic shift in hippocampal and neocortical neurons at birth has been shown to be mimicked and occluded by BTB, suggesting a possible involvement of NKCC1 (Khazipov et al., 2008). In our study, we could not link the NMS phenotype and associated deficit in OT with an altered expression of KCC2 in the spinal cord but showed a transient decrease in KCC2 levels until P45 in the cerebrum,

suggesting that OT is indeed a critical determinant of chloride transporter function and chloride homeostasis.

Moreover, NKCC1 expression and function has sometimes been shown to be altered in the spinal cord following inflammation, in particular in animal models of inflammatory pain (Zhang et al., 2008). In our study, we found that several pro-inflammatory cytokines are upregulated as well as the inducible Cox-2 favoring the synthesis of prostaglandins E2. Together with the downregulation of anti-inflammatory IL-10, it is then tempting to speculate that this “subinflammatory state” is tonically maintaining high levels of NKCC1, being responsible for the NMS phenotype. This working hypothesis is further supported by several reports indicating that NKCC1 expression and activity is definitely regulated by inflammatory processes including those driven by microglia (Morales-Aza et al., 2004; Hasbargen et al., 2010; Huang et al., 2014; Weidenfeld and Kuebler, 2017). It is thus worth mentioning that OT possesses potent anti-inflammatory properties and is involved in the development, function and regulation of the immune system (Li et al., 2016). Recently, a study has demonstrated that intrathecal injection of OT relieved bone cancer pain symptoms in a rat model by suppressing the upregulation of several proinflammatory cytokines in the spinal cord (Mou et al., 2020). Administration of carbetocin, a selective agonist of the OT receptor, has also been shown to reduce microglial activation, providing a long-term neuroprotection following perinatal brain injury (Mairesse et al., 2019), highlighting the benefits of OT signaling in the limitation of neuro-inflammatory processes. Of course, the main limit of our study is that the direct link between OT, inflammatory factors, and NKCC1 expression has not been investigated so far. Further studies would then be required to evaluate the effect of a neonatal OT treatment on inflammatory factors levels in the different brain areas, as well as the activation state of astrocytes and microglia. Another possibility would be to evaluate the impact of adult and/or

neonatal treatments of NMS animals with anti-inflammatory factors on their later behavior and chloride transporters levels.

After NMS, we propose that neonatal OT deficits impair chloride homeostasis favoring the activity of NKCC1, indirectly via upregulation of inflammatory mediators. Further experiments will be necessary to identify the detailed molecular pathway involved in nerve cells of newborns. This proposal is strongly supported by the neonatal effect of BTB and OT on pain hypersensitivity, anxiety and sociability. Further experiments would thus be necessary to assess chloride balance in supraspinal regions in which OT acts on anxiety and social behavior, such as the amygdala or the bed nucleus of the stria terminalis.

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Disclosures

The authors have no conflict of interest to declare.

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Figure legends

Figure 1: Changes in relative expression of spinal transcripts coding for NKCC1 and KCC2 in CTRL and NMS animals as measured with RT-qPCR from P14 to P100 in the cerebrum (A; n = 6 per group) and spinal cord (B; n = 4 per group), and the corresponding ratio of NKCC1/KCC2 expression. **A.** NMS was associated with a significant increase in cerebral NKCC1 mRNA expression at all time points measured and to a decrease in KCC2 at P24 and P45. **B.** NMS was associated with a significant increase in spinal NKCC1 mRNA expression at all time points measured and no change in spinal KCC2 mRNA expression.

Statistical significance was assessed with Sidak's multiple comparison post-hoc test, illustrated as follows: $p < 0.005$ (*) or $p < 0.001$ (***) for CTRL vs NMS and $p < 0.005$ (#), $p < 0.01$ (##) or $p < 0.001$ (###) for intragroup comparison vs preceding time point.

Figure 2: Effect of a single i.t. BTD injection at adulthood (90 μ g/20 μ L) on mechanical nociception and OT analgesia induced by swim stress (n = 8 per group). **A.** Intrathecal BTD in NMS animals induced a rapid increase in mechanical nociceptive thresholds as evaluated with calibrated forceps. **B.** Intrathecal BTD in NMS animals restored OTergic stress-induced analgesia (SIA).

Statistical significance was assessed with Tukey's multiple comparison test, illustrated as follows: $p < 0.01$ (**) or $p < 0.001$ (***).

Figure 3: Effect of a neonatal treatment with BTD (0.5mg/kg, s.c.) in NMS animals and VU0463271 (2mg/kg, s.c.) in CTRL animals on anxiety-like symptoms and social interactions at adulthood. **A.** Neonatal BTD treatment in NMS animals restores CTRL values on the time spent in the open arms of an elevated plus maze at P45, while VU0463271 induces anxiety-like symptoms in CTRL animals (CTRL: n = 17, CTRL+VU: n = 10, NMS: n = 13,

NMS+BTB: n = 15). Statistical significance was assessed with Tukey's multiple comparison test, illustrated as follows: $p < 0.01$ (**) or $p < 0.001$ (***). **B.** NMS-induced social preference deficit in the three-chamber test can be restored with neonatal BTB treatment, but neonatal VU0463271 failed to induce social interaction deficits in CTRL animals (CTRL: n = 22, CTRL+VU: n = 11, NMS: n = 14, NMS+BTB: n = 11). Statistical significance was assessed with Sidak's multiple comparison test, illustrated as follows: $p < 0.001$ (***).

Figure 4: Neonatal treatment with OT (1mg/kg, i.p.) from P2 to P12 can rescue NMS-induced anxiety-like symptoms and social interaction deficits. **A.** Neonatal OT treatment in NMS animals restores CTRL values on the time spent in the open arms of an elevated plus maze at P45 (CTRL: n = 17, NMS: n = 13, NMS+OT: n = 14). Statistical significance was assessed with Tukey's multiple comparison post-hoc test, illustrated as follows: $p < 0.01$ (**) or $p < 0.001$ (***). **B.** NMS induces a deficit in social preference in the three-chamber test, which can be restored with neonatal OT treatment (CTRL: n = 22, NMS: n = 14, NMS+OT: n = 14). Statistical significance was assessed with Sidak's multiple comparison post-hoc test, illustrated as follows: $p < 0.001$ (***).

Figure 5: Relative gene expression (gene of interest / housekeeping gene HPRT) of several inflammatory mediators in cerebrum of CTRL and NMS rats as measured by RT-qPCR: interleukin-1 α (**A**, n = 6 per group), interleukin-1 β (**B**, n = 6 per group), interleukin-6 (**C**, n = 6 per group), tumor necrosis factor α (**D**, n = 6 per group), cyclooxygenase-2 (**E**, n = 6 per group) and interleukin-10 (**F**, n = 6 per group). Statistical significance was assessed with Tukey's multiple comparison post-hoc test, illustrated as follows: $p < 0.01$ (**) or $p < 0.001$ (***) for CTRL vs NMS and $p < 0.05$ (#), $p < 0.01$ (##) or $p < 0.001$ (###) for intragroup comparison vs preceding time point.

Figure 6: Relative gene expression (gene of interest / housekeeping gene HPRT) of several inflammatory mediators in the spinal cord of CTRL and NMS rats as measured by RT-qPCR: interleukin-1 α (**A**, n = 4 per group), interleukin-1 β (**B**, n = 5 per group), interleukin-6 (**C**, n = 5 per group), tumor necrosis factor α (**D**, n = 4 per group), cyclooxygenase-2 (**E**, n = 5 per group) and interleukin-10 (**F**, n = 5 per group). Statistical significance was assessed with Tukey's multiple comparison post-hoc test, illustrated as follows: p < 0.01 (**) or p < 0.001 (***) for CTRL vs NMS and p < 0.05 (#), p < 0.01 (##) or p < 0.001 (###) for intragroup comparison vs preceding time point.

Figure 7: Proposed mechanism to explain the behavioral impairment detected at adulthood following neonatal maternal separation. The over-expression of pro-inflammatory factors and deficit in oxytocin in the neonatal period is suggested to dysregulate the ratio between NKCC1 and KCC2 transporter expression/function in favor of NKCC1. This may lead to an imbalance in chloride homeostasis in spinal and supraspinal nervous structures.

Figure 1

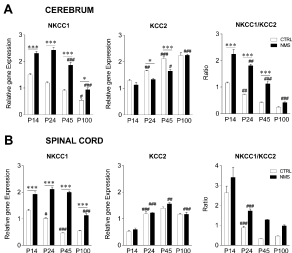


Figure 2

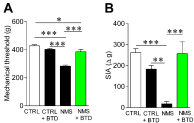


Figure 3

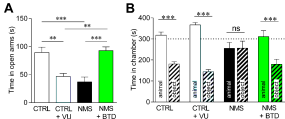


Figure 4

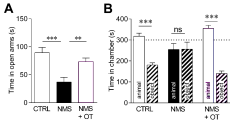


Figure 5

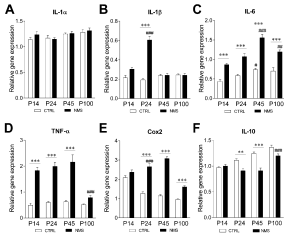


Figure 6

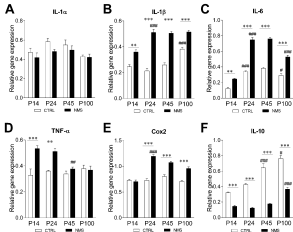


Figure 7

