



**HAL**  
open science

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

Géraldine Gazzo, Meggane Melchior, Andréa Caussaint, Clémence Gieré,  
Vincent Lelièvre, Pierrick Poisbeau

## ► To cite this version:

Géraldine Gazzo, Meggane Melchior, Andréa Caussaint, Clémence Gieré, Vincent Lelièvre, et al.. Overexpression of chloride importer NKCC1 contributes to the sensory-affective and sociability phenotype of rats following neonatal maternal separation. *Brain, Behavior, and Immunity*, 2021, 92, pp.193-202. 10.1016/j.bbi.2020.12.010 . hal-03352638

**HAL Id: hal-03352638**

<https://hal.science/hal-03352638v1>

Submitted on 9 Mar 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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

Géraldine Gazzo, Meggane Melchior, Andréa Caussaint, Clémence Gieré, Vincent Lelièvre and Pierrick Poisbeau

Centre National de la Recherche Scientifique and University of Strasbourg, Institut des Neurosciences Cellulaires et Intégratives, 67000 Strasbourg, France.

### *Corresponding author:*

Prof. Pierrick Poisbeau, Institute of Cellular and Integrative Neuroscience, CNRS UPR-3212, 8 allée du Général Rouvillois, 67000 Strasbourg, France.

Tel: (+33) 3 88 45 67 27- Email: [poisbeau@inci-cnrs.unistra.fr](mailto:poisbeau@inci-cnrs.unistra.fr)

**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

Abstract: 247 words

Main text: 5185 words (including figure legends, acknowledgments and disclosure)

Figures: 7 figures and 1 supplementary table. Colors to be used in online version only.

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<sub>A</sub> 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- $\alpha$  (TNF-  $\alpha$ ) (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<sub>A</sub> 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 cylindrical 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 OTerpic analgesia

Rats were exposed to a forced swim stress protocol known to induce recruitment of OTerpic descending controls of pain (Wotjak et al., 1998; Melchior et al., 2018). Animals were placed in a Plexiglas™ 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/ $\beta$ -mercaptoethanol preparation using ultraturax and stored at  $-80^{\circ}\text{C}$ . 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 (TURBO<sup>TM</sup> 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  $95^{\circ}\text{C}$ , 20 seconds at  $60^{\circ}\text{C}$ , and 20 seconds at  $72^{\circ}\text{C}$ ). 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 % ( $\pm 2$  %), 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  $\mu$ 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  $\mu$ g / 20  $\mu$ 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:  $1.49 \pm 0.05$ ; NMS:  $2.30 \pm 0.07$ ) to P100 (CTRL:  $0.54 \pm 0.05$ ; NMS:  $0.94 \pm 0.07$ ). 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:  $1.66 \pm 0.03$ ; NMS:  $1.34 \pm 0.04$ ) to P45 (CTRL:  $2.12 \pm 0.07$ ; NMS:  $1.65 \pm 0.06$ ), but not at P14 (CTRL:  $1.30 \pm 0.05$ ; NMS:  $1.13 \pm 0.1$ ) nor P100 (CTRL:  $2.25 \pm 0.10$ ; NMS:  $2.25 \pm 0.04$ ). 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:  $1.30 \pm 0.05$ ; NMS:  $1.92 \pm 0.09$ ) to P100 (CTRL:  $0.54 \pm 0.03$ ; NMS:  $1.13 \pm 0.07$ ). 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 (90 $\mu$ g/20 $\mu$ L) 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  $\pm$  8.18 g to 383.13  $\pm$  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  $\pm$  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  $\pm$  18.65 g; NMS: 17.88  $\pm$  11.66 g; NMS after BTD i.t.: 257.25  $\pm$  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:  $89.43 \pm 9.54$  s; NMS:  $36.92 \pm 8.76$  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+BTB:  $92.91 \pm 6.53$  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  $255.3 \pm 27.92$  s, time spent with new objects  $256.2 \pm 33.55$  s, preference ratio  $1.27 \pm 0.17$ ). This was in sharp contrast with the control group (time spent with the new animal  $318.3 \pm 14.56$  s, time spent with the new object  $180.9 \pm 10.48$  s, preference ratio  $1.99 \pm 0.20$ ; 2wANOVA, condition x chamber,  $F_{(3,108)} = 8.936$ ,  $p < 0.0001$ ). Interestingly, neonatal BTB 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  $311.4 \pm 27.63$  s, time spent with new objects  $179.9 \pm 23.02$  s, preference ratio of  $2.2 \pm 0.44$  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:  $46.83 \pm 5.39$  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  $366.9 \pm 10.82$  s, time spent with new objects  $144.2 \pm 9.91$  s, preference ratio  $2.7 \pm 0.26$ ).

### **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 OTerpic 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:  $89.43 \pm 9.54$  s; NMS+OT:  $73.53 \pm 6.44$  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  $355.4 \pm 14.33$  s, time spent with new objects  $140.1 \pm 12.16$  s, preference ratio  $2.9 \pm 0.40$ ).

### **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 $\alpha$  (IL-1 $\alpha$ ) 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 $\beta$  (IL-1 $\beta$ ), 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- $\alpha$  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 $\alpha$  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 $\beta$  (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- $\alpha$  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, BTM 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 BTM 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 BTM 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.

## **Acknowledgments**

We thank Stéphane Doridot and Edouard Gottschalk for providing animal care at the Chronobiotron facility and our past collaborators, Pierre-Eric Juif and Adrien Lacaud, who contributed to the project at its early stage. This work was funded by Centre National de la Recherche Scientifique, Université de Strasbourg and Fédération pour la Recherche sur le Cerveau (FRC 2019). Dr Gazzo and Dr Melchior were supported by a PhD scholarship from the French Ministère de la Recherche et de l'Enseignement Supérieur at the time of the study. PP is a senior fellow of the Institut Universitaire de France. We thank the following research programs of excellence for their support: FHU Neurogenycs, Fondation Université de Strasbourg through the Strasbourg Pain Initiative and French National Research Agency (ANR) through the Programme d'Investissement d'Avenir (contract ANR-17-EURE-0022, EURIDOL Graduate school of pain).

## **Disclosures**

The authors have no conflict of interest to declare.

## References

- Bolton JL, Molet J, Ivy A, Baram TZ, 2017. New insights into early-life stress and behavioral outcomes. *Curr Opin Behav Sci* 14:133-139.
- Bonin RP, De Koninck Y, 2013. Restoring ionotropic inhibition as an analgesic strategy. *Neurosci Lett* 557 Pt A:43-51.
- Charpak N, Tessier R, Ruiz JG, Hernandez JT, Uriza F, Villegas J, Nadeau L, Mercier C, Maheu F, Marin J, Cortes D, Gallego JM, Maldonado D, 2017. Twenty-year Follow-up of Kangaroo Mother Care Versus Traditional Care. *Pediatrics* 139.
- Chen SR, Zhu L, Chen H, Wen L, Laumet G, Pan HL, 2014. Increased spinal cord Na(+)-K(+)-2Cl(-) cotransporter-1 (NKCC1) activity contributes to impairment of synaptic inhibition in paclitaxel-induced neuropathic pain. *J Biol Chem* 289:31111-31120.
- Chomczynski P, Sacchi N, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.
- Chung EK, Zhang XJ, Xu HX, Sung JJ, Bian ZX, 2007. Visceral hyperalgesia induced by neonatal maternal separation is associated with nerve growth factor-mediated central neuronal plasticity in rat spinal cord. *Neuroscience* 149:685-695.
- Cordero-Erausquin M, Coull JA, Boudreau D, Rolland M, De Koninck Y, 2005. Differential maturation of GABA action and anion reversal potential in spinal lamina I neurons: impact of chloride extrusion capacity. *J Neurosci* 25:9613-9623.
- Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y, 2003. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* 424:938-942.
- Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y, 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438:1017-1021.
- Doyon N, Vinay L, Prescott SA, De Koninck Y, 2016. Chloride Regulation: A Dynamic Equilibrium Crucial for Synaptic Inhibition. *Neuron* 89:1157-1172.
- Eto K, Ishibashi H, Yoshimura T, Watanabe M, Miyamoto A, Ikenaka K, Moorhouse AJ, Nabekura J, 2012. Enhanced GABAergic activity in the mouse primary somatosensory cortex is insufficient to alleviate chronic pain behavior with reduced expression of neuronal potassium-chloride cotransporter. *J Neurosci* 32:16552-16559.
- Furukawa M, Tsukahara T, Tomita K, Iwai H, Sonomura T, Miyawaki S, Sato T, 2017. Neonatal maternal separation delays the GABA excitatory-to-inhibitory functional switch by inhibiting KCC2 expression. *Biochem Biophys Res Commun* 493:1243-1249.
- Galan A, Cervero F, 2005. Painful stimuli induce in vivo phosphorylation and membrane mobilization of mouse spinal cord NKCC1 co-transporter. *Neuroscience* 133:245-252.
- Gibson JR, Bartley AF, Hays SA, Huber KM, 2008. Imbalance of neocortical excitation and inhibition and altered UP states reflect network hyperexcitability in the mouse model of fragile X syndrome. *J Neurophysiol* 100:2615-2626.
- Goncalves JT, Anstey JE, Golshani P, Portera-Cailliau C, 2013. Circuit level defects in the developing neocortex of Fragile X mice. *Nat Neurosci* 16:903-909.
- Gottschalk MG, Domschke K, 2018. Oxytocin and Anxiety Disorders. *Curr Top Behav Neurosci* 35:467-498.
- Hardin JS, Jones NA, Mize KD, Platt M, 2020. Parent-Training with Kangaroo Care Impacts Infant Neurophysiological Development & Mother-Infant Neuroendocrine Activity. *Infant Behav Dev* 58:101416.
- Hasbargen T, Ahmed MM, Miranpuri G, Li L, Kahle KT, Resnick D, Sun D, 2010. Role of NKCC1 and KCC2 in the development of chronic neuropathic pain following spinal cord injury. *Ann N Y Acad Sci* 1198:168-172.

- Heim C, Young LJ, Newport DJ, Mletzko T, Miller AH, Nemeroff CB, 2009. Lower CSF oxytocin concentrations in women with a history of childhood abuse. *Mol Psychiatry* 14:954-958.
- Holland FH, Ganguly P, Potter DN, Chartoff EH, Brenhouse HC, 2014. Early life stress disrupts social behavior and prefrontal cortex parvalbumin interneurons at an earlier time-point in females than in males. *Neurosci Lett* 566:131-136.
- Hu D, Yu ZL, Zhang Y, Han Y, Zhang W, Lu L, Shi J, 2017. Bumetanide treatment during early development rescues maternal separation-induced susceptibility to stress. *Sci Rep* 7:11878.
- Huang LQ, Zhu GF, Deng YY, Jiang WQ, Fang M, Chen CB, Cao W, Wen MY, Han YL, Zeng HK, 2014. Hypertonic saline alleviates cerebral edema by inhibiting microglia-derived TNF-alpha and IL-1beta-induced Na-K-Cl Cotransporter up-regulation. *J Neuroinflammation* 11:102.
- Hyde TM, Lipska BK, Ali T, Mathew SV, Law AJ, Metitiri OE, Straub RE, Ye T, Colantuoni C, Herman MM, Bigelow LB, Weinberger DR, Kleinman JE, 2011. Expression of GABA signaling molecules KCC2, NKCC1, and GAD1 in cortical development and schizophrenia. *J Neurosci* 31:11088-11095.
- Janssen SP, Gerard S, Rajmakers ME, Truin M, Van Kleef M, Joosten EA, 2012. Decreased intracellular GABA levels contribute to spinal cord stimulation-induced analgesia in rats suffering from painful peripheral neuropathy: the role of KCC2 and GABA(A) receptor-mediated inhibition. *Neurochem Int* 60:21-30.
- Johnston CC, Stevens B, Pinelli J, Gibbins S, Filion F, Jack A, Steele S, Boyer K, Veilleux A, 2003. Kangaroo care is effective in diminishing pain response in preterm neonates. *Arch Pediatr Adolesc Med* 157:1084-1088.
- Ju LS, Yang JJ, Gravenstein N, Seubert CN, Morey TE, Sumners C, Vasilopoulos T, Yang JJ, Martynyuk AE, 2017. Role of environmental stressors in determining the developmental outcome of neonatal anesthesia. *Psychoneuroendocrinology* 81:96-104.
- Juif PE, Salio C, Zell V, Melchior M, Lacaud A, Petit-Demouliere N, Ferrini F, Darbon P, Hanesch U, Anton F, Merighi A, Lelievre V, Poisbeau P, 2016. Peripheral and central alterations affecting spinal nociceptive processing and pain at adulthood in rats exposed to neonatal maternal deprivation. *Eur J Neurosci* 44:1952-1962.
- Kaila K, Price TJ, Payne JA, Puskarjov M, Voipio J, 2014. Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nat Rev Neurosci* 15:637-654.
- Khazipov R, Tyzio R, Ben-Ari Y, 2008. Effects of oxytocin on GABA signalling in the foetal brain during delivery. *Prog Brain Res* 170:243-257.
- Lelievre V, Hu Z, Byun JY, Ioffe Y, Waschek JA, 2002. Fibroblast growth factor-2 converts PACAP growth action on embryonic hindbrain precursors from stimulation to inhibition. *J Neurosci Res* 67:566-573.
- Lemonnier E, Degrez C, Phelep M, Tyzio R, Josse F, Grandgeorge M, Hadjikhani N, Ben-Ari Y, 2012. A randomised controlled trial of bumetanide in the treatment of autism in children. *Transl Psychiatry* 2:e202.
- Leonzino M, Busnelli M, Antonucci F, Verderio C, Mazzanti M, Chini B, 2016. The Timing of the Excitatory-to-Inhibitory GABA Switch Is Regulated by the Oxytocin Receptor via KCC2. *Cell Rep* 15:96-103.
- Lewis CM et al., 2003. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet* 73:34-48.
- Li T, Wang P, Wang SC, Wang YF, 2016. Approaches Mediating Oxytocin Regulation of the Immune System. *Front Immunol* 7:693.

- Luis-Delgado OE, Barrot M, Rodeau JL, Schott G, Benbouzid M, Poisbeau P, Freund-Mercier MJ, Lasbennes F, 2006. Calibrated forceps: a sensitive and reliable tool for pain and analgesia studies. *J Pain* 7:32-39.
- Lukas M, Bredewold R, Landgraf R, Neumann ID, Veenema AH, 2011. Early life stress impairs social recognition due to a blunted response of vasopressin release within the septum of adult male rats. *Psychoneuroendocrinology* 36:843-853.
- Luscher B, Shen Q, Sahir N, 2011. The GABAergic deficit hypothesis of major depressive disorder. *Mol Psychiatry* 16:383-406.
- Mairesse J, Zinni M, Pansiot J, Hassan-Abdi R, Demene C, Colella M, Charriaut-Marlangue C, Rideau Batista Novais A, Tanter M, Maccari S, Gressens P, Vaiman D, Soussi-Yanicostas N, Baud O, 2019. Oxytocin receptor agonist reduces perinatal brain damage by targeting microglia. *Glia* 67:345-359.
- Melchior M, Juif PE, Gazzo G, Petit-Demouliere N, Chavant V, Lacaud A, Goumon Y, Charlet A, Lelievre V, Poisbeau P, 2018. Pharmacological rescue of nociceptive hypersensitivity and oxytocin analgesia impairment in a rat model of neonatal maternal separation. *Pain* 159:2630-2640.
- Merner ND, Chandler MR, Bourassa C, Liang B, Khanna AR, Dion P, Rouleau GA, Kahle KT, 2015. Regulatory domain or CpG site variation in SLC12A5, encoding the chloride transporter KCC2, in human autism and schizophrenia. *Front Cell Neurosci* 9:386.
- Merner ND, Mercado A, Khanna AR, Hodgkinson A, Bruat V, Awadalla P, Gamba G, Rouleau GA, Kahle KT, 2016. Gain-of-function missense variant in SLC12A2, encoding the bumetanide-sensitive NKCC1 cotransporter, identified in human schizophrenia. *J Psychiatr Res* 77:22-26.
- Miletic G, Miletic V, 2008. Loose ligation of the sciatic nerve is associated with TrkB receptor-dependent decreases in KCC2 protein levels in the ipsilateral spinal dorsal horn. *Pain* 137:532-539.
- Moloney RD, Stilling RM, Dinan TG, Cryan JF, 2015. Early-life stress-induced visceral hypersensitivity and anxiety behavior is reversed by histone deacetylase inhibition. *Neurogastroenterol Motil* 27:1831-1836.
- Morales-Aza BM, Chillingworth NL, Payne JA, Donaldson LF, 2004. Inflammation alters cation chloride cotransporter expression in sensory neurons. *Neurobiol Dis* 17:62-69.
- Mou X, Fang J, Yang A, Du G, 2020. Oxytocin ameliorates bone cancer pain by suppressing toll-like receptor 4 and proinflammatory cytokines in rat spinal cord. *J Neurogenet*:1-7.
- Nomura H, Sakai A, Nagano M, Umino M, Suzuki H, 2006. Expression changes of cation chloride cotransporters in the rat spinal cord following intraplantar formalin. *Neurosci Res* 56:435-440.
- Okada-Ogawa A, Nakaya Y, Imamura Y, Kobayashi M, Shinoda M, Kita K, Sessle BJ, Iwata K, 2015. Involvement of medullary GABAergic system in extraterritorial neuropathic pain mechanisms associated with inferior alveolar nerve transection. *Exp Neurol* 267:42-52.
- Paternain L, Martisova E, Milagro FI, Ramirez MJ, Martinez JA, Campion J, 2012. Postnatal maternal separation modifies the response to an obesogenic diet in adulthood in rats. *Dis Model Mech* 5:691-697.
- Plotsky PM, Meaney MJ, 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Brain Res Mol Brain Res* 18:195-200.
- Poisbeau P, Grinevich V, Charlet A, 2018. Oxytocin Signaling in Pain: Cellular, Circuit, System, and Behavioral Levels. *Curr Top Behav Neurosci* 35:193-211.
- Price TJ, Cervero F, de Koninck Y, 2005. Role of cation-chloride-cotransporters (CCC) in pain and hyperalgesia. *Curr Top Med Chem* 5:547-555.



- Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K, 1999. The K<sup>+</sup>/Cl<sup>-</sup> co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397:251-255.
- Romijn HJ, Hofman MA, Gramsbergen A, 1991. At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? *Early Hum Dev* 26:61-67.
- Sibille E, Morris HM, Kota RS, Lewis DA, 2011. GABA-related transcripts in the dorsolateral prefrontal cortex in mood disorders. *Int J Neuropsychopharmacol* 14:721-734.
- Stil A, Liabeuf S, Jean-Xavier C, Brocard C, Viemari JC, Vinay L, 2009. Developmental up-regulation of the potassium-chloride cotransporter type 2 in the rat lumbar spinal cord. *Neuroscience* 164:809-821.
- Sullivan CR, Funk AJ, Shan D, Haroutunian V, McCullumsmith RE, 2015. Decreased chloride channel expression in the dorsolateral prefrontal cortex in schizophrenia. *PLoS One* 10:e0123158.
- Sung KW, Kirby M, McDonald MP, Lovinger DM, Delpire E, 2000. Abnormal GABA<sub>A</sub> receptor-mediated currents in dorsal root ganglion neurons isolated from Na-K-2Cl cotransporter null mice. *J Neurosci* 20:7531-7538.
- Talge NM, Neal C, Glover V, Early Stress TR, Prevention Science Network F, Neonatal Experience on C, Adolescent Mental H, 2007. Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? *J Child Psychol Psychiatry* 48:245-261.
- Tao R, Li C, Newburn EN, Ye T, Lipska BK, Herman MM, Weinberger DR, Kleinman JE, Hyde TM, 2012. Transcript-specific associations of SLC12A5 (KCC2) in human prefrontal cortex with development, schizophrenia, and affective disorders. *J Neurosci* 32:5216-5222.
- Tata DA, Markostamou I, Ioannidis A, Gkioka M, Simeonidou C, Anogianakis G, Spandou E, 2015. Effects of maternal separation on behavior and brain damage in adult rats exposed to neonatal hypoxia-ischemia. *Behav Brain Res* 280:51-61.
- Tobon AL, Newport DJ, Nemeroff A, 2018. The Role of Oxytocin in Early Life Adversity and Later Psychopathology: a Review of Preclinical and Clinical Studies. *Current Treatment Options in Psychiatry* 5:401-415.
- Toda H, Boku S, Nakagawa S, Inoue T, Kato A, Takamura N, Song N, Nibuya M, Koyama T, Kusumi I, 2014. Maternal separation enhances conditioned fear and decreases the mRNA levels of the neurotensin receptor 1 gene with hypermethylation of this gene in the rat amygdala. *PLoS One* 9:e97421.
- Tyzio R, Holmes GL, Ben-Ari Y, Khazipov R, 2007. Timing of the developmental switch in GABA(A) mediated signaling from excitation to inhibition in CA3 rat hippocampus using gramicidin perforated patch and extracellular recordings. *Epilepsia* 48 Suppl 5:96-105.
- Tyzio R, Nardou R, Ferrari DC, Tsintsadze T, Shahrokhi A, Eftekhari S, Khalilov I, Tsintsadze V, Brouchoud C, Chazal G, Lemonnier E, Lozovaya N, Burnashev N, Ben-Ari Y, 2014. Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. *Science* 343:675-679.
- Valencia-de Ita S, Lawand NB, Lin Q, Castaneda-Hernandez G, Willis WD, 2006. Role of the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter in the development of capsaicin-induced neurogenic inflammation. *J Neurophysiol* 95:3553-3561.
- Verriotis M, Chang P, Fitzgerald M, Fabrizi L, 2016. The development of the nociceptive brain. *Neuroscience* 338:207-219.

- Wang C, Shimizu-Okabe C, Watanabe K, Okabe A, Matsuzaki H, Ogawa T, Mori N, Fukuda A, Sato K, 2002. Developmental changes in KCC1, KCC2, and NKCC1 mRNA expressions in the rat brain. *Brain Res Dev Brain Res* 139:59-66.
- Watanabe M, Fukuda A, 2015. Development and regulation of chloride homeostasis in the central nervous system. *Front Cell Neurosci* 9:371.
- Weidenfeld S, Kuebler WM, 2017. Cytokine-Regulation of Na(+)-K(+)-Cl(-) Cotransporter 1 and Cystic Fibrosis Transmembrane Conductance Regulator-Potential Role in Pulmonary Inflammation and Edema Formation. *Front Immunol* 8:393.
- Weinstock M, 2001. Alterations induced by gestational stress in brain morphology and behaviour of the offspring. *Prog Neurobiol* 65:427-451.
- Wotjak CT, Ganster J, Kohl G, Holsboer F, Landgraf R, Engelmann M, 1998. Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. *Neuroscience* 85:1209-1222.
- Yamasue H, Domes G, 2018. Oxytocin and Autism Spectrum Disorders. *Curr Top Behav Neurosci* 35:449-465.
- Young LJ, Barrett CE, 2015. Neuroscience. Can oxytocin treat autism? *Science* 347:825-826.
- Zhang W, Liu LY, Xu TL, 2008. Reduced potassium-chloride co-transporter expression in spinal cord dorsal horn neurons contributes to inflammatory pain hypersensitivity in rats. *Neuroscience* 152:502-510.

## **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 $\mu$ g/20 $\mu$ 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+BTD: 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 BTD treatment, but neonatal VU0463271 failed to induce social interaction deficits in CTRL animals (CTRL: n = 22, CTRL+VU: n = 11, NMS: n = 14, NMS+BTD: 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 $\alpha$  (**A**, n = 6 per group), interleukin-1 $\beta$  (**B**, n = 6 per group), interleukin-6 (**C**, n = 6 per group), tumor necrosis factor  $\alpha$  (**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 $\alpha$  (**A**, n = 4 per group), interleukin-1 $\beta$  (**B**, n = 5 per group), interleukin-6 (**C**, n = 5 per group), tumor necrosis factor  $\alpha$  (**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

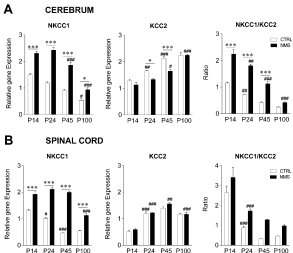


Figure 2

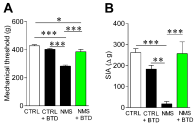


Figure 3

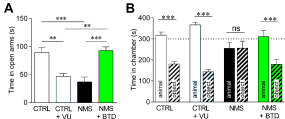




Figure 4

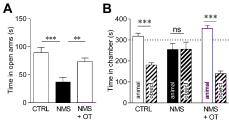


Figure 5

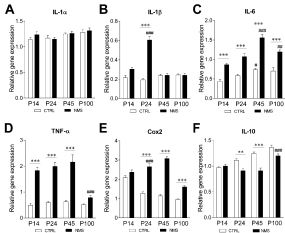


Figure 6

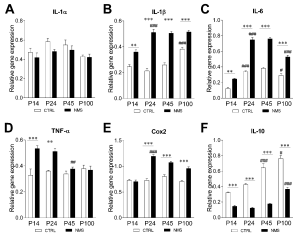


Figure 7

