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Acceleration of GABA-switch after early life stress changes mouse prefrontal glutamatergic transmission

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ABSTRACT

Early life stress (ELS) alters the excitation-inhibition-balance (EI-balance) in various rodent brain areas and may be responsible for behavioral impairment later in life. The EI-balance is (amongst others) influenced by the switch of GABAergic transmission from excitatory to inhibitory, the so-called "GABA-switch".

Here, we investigated how ELS affects the GABA-switch in mouse infralimbic Prefrontal Cortex layer 2/3 neurons, using the limited-nesting-and-bedding model. In ELS mice, the GABA-switch occurred already between postnatal day (P) 6 and P9, as opposed to P15–P21 in controls. This was associated with increased expression of the inward chloride transporter NKCC1, compared to the outward chloride transporter KCC2, both of which are important for the intracellular chloride concentration and, hence, the GABA reversal potential (Erev). Chloride transporters are not only important for regulating chloride concentration postsynaptically, but also presynaptically. Depending on the Erev of GABA, presynaptic GABA$_A$ receptor stimulation causes a depolarization or hyperpolarization, and thereby enhanced or reduced fusion of glutamate vesicles respectively, in turn changing the frequency of miniature postsynaptic currents (mEPSCs). In accordance, bumetanide, a blocker of NKCC1, shifted the Erev GABA towards more hyperpolarized levels in P9 control mice and reduced the mEPSC frequency. Other modulators of chloride transporters, e.g. VU0463271 (a KCC2 antagonist) and aldosterone -which increases NKCC1 expression-did not affect postsynaptic Erev in ELS P9 mice, but did increase the mEPSC frequency. We conclude that the mouse GABA-switch is accelerated after ELS, affecting both the pre- and postsynaptic chloride homeostasis, the former altering glutamatergic transmission. This may considerably affect brain development.

1. Introduction

The balance between excitatory and inhibitory transmission (the EI-balance) in the brain is supposed to be extremely important for correct development. In the prenatal stage of rodents, maturation of GABA transmission precedes that of glutamatergic transmission (Behar et al., 1996; Tyzio et al., 1999). In this early period, during which GABA is important for development, its transmission is mainly excitatory (Cherubini et al., 1991; Ebihara et al., 1995; Leinekugel et al., 1995). Gradually, glutamatergic transmission appears and GABA transmission becomes inhibitory. In rodents, the switch from excitation to inhibition of GABA appears to take place around postnatal day 15 (Cancedda et al., 2007; Wang and Kriegstein, 2008, 2011; Le Magueresse and Monyer, 2013; Peerboom and Wierenga, 2021), starting in the caudal part of the brain and then gradually spreading to more rostral parts (Rakhade and Jensen, 2009). In females, the switch takes place somewhat earlier than in males (Galanopoulou, 2008). Disturbances of the balance during maturation appear to be a risk factor for the development of some psychiatric disorders (Cancedda et al., 2007; Ben-Ari, 2017; Deidda et al., 2015; Tyzio et al., 2014; Rakhade and Jensen, 2009). For instance, in some studies using an animal model for autism spectrum disorders, the switch even does not take place at all (Tyzio et al., 2014).

The switch from excitation to inhibition by GABA depends on the expression of chloride transporters. The sodium/potassium/chloride cotransporter NKCC1, responsible for an active inward transport of chloride, is expressed in high amounts early in development, whereas the expression of the potassium/chloride cotransporter KCC2, which is responsible for an active efflux of chloride, is initially low but gradually

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increases during development (Fig. 1A and B) (Ben-Ari et al., 2012). Thus, the ratio of the expression of NKCC1 compared to KCC2 proteins (Ben-Ari et al., 2007) is important for the intracellular Cl⁻ concentration, and changes from high to low(er) during development. A high intracellular Cl⁻ concentration shifts the reversal potential (Erev) of GABA towards more positive values than the resting membrane potential, causing an efflux of Cl⁻ when GABA$_A$ receptors are activated, and thus depolarization. When the intracellular Cl⁻ concentration declines during development (due to higher expression of KCC2), the Erev of GABA shifts towards a more negative value than the resting membrane potential, causing GABA to be an inhibitory transmitter (Fig. 1A).

During development, the ratio between the glutamatergic and GABAergic transmission and thus the EI-balance varies but stabilizes early in adulthood (Rakhade and Jensen, 2009; Sanchez and Jensen, 2001). Therefore, it comes as no surprise that early life adversity affects this balance. In a recent study, we demonstrated that the EI-balance in mouse medial prefrontal cortex (mPFC) neurons is decreased, primarily due to a change in the glutamatergic transmission. In a short period after early life stress (ELS), in this case induced by the limited bedding and nesting model (Rice et al., 2008), the frequency, but not the amplitude, of AMPA-receptor mediated miniature excitatory postsynaptic currents (mEPSCs) turned out to be decreased (Karst et al., 2020). The GABAergic mIPSC frequency and amplitude were not affected after ELS. Overall, this means that the EI-balance is decreased directly after ELS. Normally the EI-balance gradually moves towards a lower level of excitability during maturation, but in ELS-exposed mice, this shift was already discerned at a very early age. Our conclusion was that ELS accelerates the maturation of the EI-balance. This fits with other examples showing that ELS accelerates some aspects of development (Bath et al., 2016, 2017; Karst et al., 2020; Derks et al., 2016). For instance, Bath et al. (2016) reported an acceleration in the NR2a:NR2b subunit ratio one week after ELS and an acceleration in the expression of Parvalbumin (PV) in fast spiking interneurons in the hippocampus.

Our earlier method (whole cell patch clamp recording (Karst et al., 2020),) did not allow investigation of a shift in in the Erev of GABA, given the fixed intracellular and extracellular Cl⁻ concentrations with this method. Nevertheless, an ELS-induced change in the GABA-switch could play a role in affecting the EI-balance. To determine the Erev of GABA we therefore now used the perforated patch clamp technique. Others have already shown that ELS can affect the expression of the Cl⁻ cotransporters, NKCC1 and KCC2 (Galanopoulou, 2008; Furukawa et al., 2017; Veerawatananan et al., 2015; Hu et al., 2017). The published data, though, is contradictory, most likely due to the variation in ELS models (Galanopoulou, 2008). demonstrated that maternal separation of rat pups at P4–P6 for 6h daily, increased the expression of KCC2 in CA1 pyramidal neurons. The expression of NKCC1 was not changed, but the effect of bumetanide on the Erev of GABA was less effective in ELS rats compared to controls. This could result from an altered phosphorylation of NKCC1. Changes in phosphorylation of chloride transporters and thus effectiveness is a phenomenon already described before in chronically stressed mice (MacKenzie and Maguire, 2015) (Tsukahara et al., 2016).

GABA$_A$ receptors are not only located postsynaptically, but also presynaptically in several brain structures. Presynaptic axonal GABA$_A$ receptors have been found in the retina, calyx of Held, posterior pituitary, cerebral cortex, hippocampus and cerebellar molecular layer interneurons (MacDermott et al., 1999; Trigo et al., 2008). These presynaptically located receptors are reported to have a functional interaction with NKCC1 (Fig. 1B) (Jang et al., 2001). At high axonal Cl⁻ concentration, determined by a higher expression of presynaptic NKCC1's, GABA$_A$ receptor activation results in an increase of the mEPSCs frequency due to depolarization of the synaptic nerve terminals (Jang et al., 2001). reported an increase of glutamate release due to an increase of Ca^{2+} via high threshold Ca^{2+} channels activated by depolarization via presynaptic GABA$_A$ activation (Fig. 1B1).

In this study we explored the effect of ELS on the GABA-switch in ELS mice during the first three postnatal weeks, i) by determining the Erev of
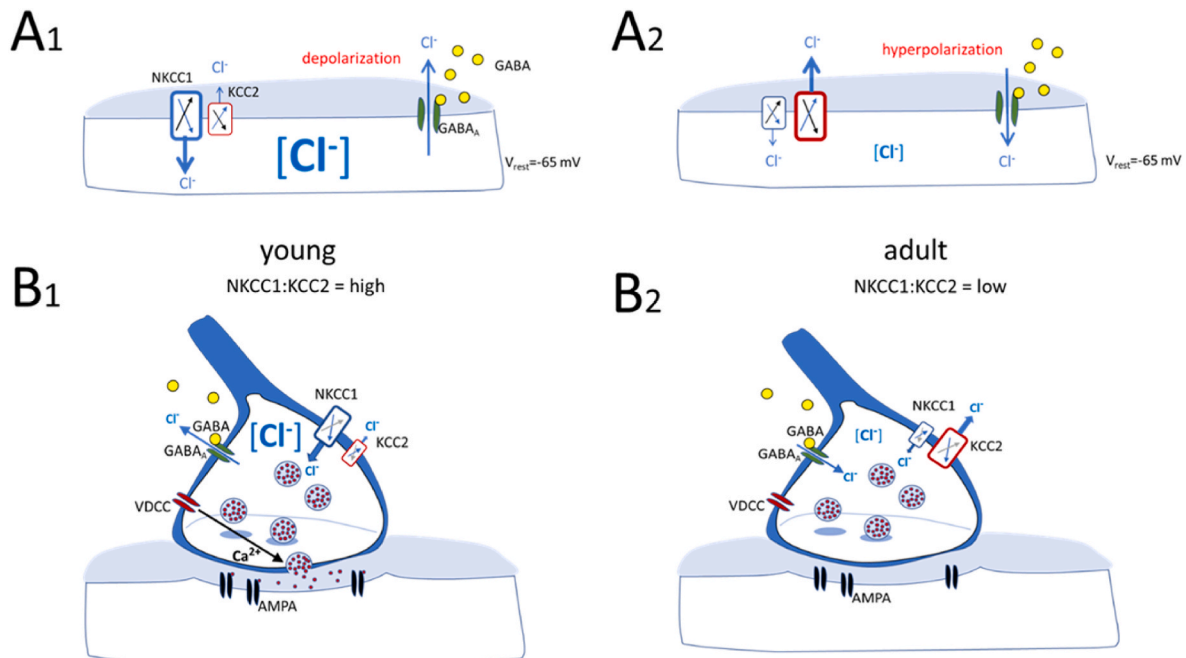
Fig. 1. A. In young rodents (A1), the inward chloride transporter, NKCC1, is expressed at relatively higher levels than in adult animals (A2), resulting in a higher intracellular chloride concentration. Therefore, GABA stimulation of young rodents causes a depolarization of neurons. When NKCC1 expression decreases and KCC2 expression increases with aging, intracellular chloride concentration decreases. GABA activation then results in a hyperpolarization. This phenomenon of GABA changing from being excitatory to inhibitory is called the "GABA-switch". B. In the presynaptic nerve terminal, the chloride transporters are also responsible for a depolarization or hyperpolarization of the membrane. In young rodents (B1), where the intracellular chloride concentration is high, GABAa receptor activation causes a depolarization. Depolarization will activate voltage dependent calcium channels. The increased influx of calcium will then promote fusion of glutamate vesicles, resulting in an increased frequency of mEPSCs. In adult neurons (B2), presynaptic chloride concentration is assumed to be low. GABAa activation then results in hyperpolarization and suppression of vesicle release.

GABA using perforated patch clamp; and ii) the expression of the chloride co-transporters. We also studied a possible involvement of the presynaptic GABA$_A$-induced depolarization in the attenuated glutamate mEPSC frequency after ELS at P9, as was reported before (Karst et al., 2020).

2. Material and methods

2.1. Animals

The experiments were approved by the Dutch Central Committee Animal experiments (centrale commissie dierproeven, CCD), project #AVD1150020184927. Animals were housed at reversed day-night cycle (lights on: 20:00–08:00 h), with temperature of 22 ± 2 °C and humidity of approximately 65%. Food (standard chow) and water were provided ad libitum. The described methodology adheres to the ARRIVE guidelines (Kilkenny et al., 2010) and Gold Standard Publication Checklist (Hooijmans et al., 2010) to improve the quality of reporting on animal studies.

2.2. Early life stress

All experiments were carried out with C57/Bl6 mice. After birth the litter was randomly assigned to either the control condition (standard housing) at postnatal day (P) 2, or the experimental conditions where a limited amount of nesting and bedding material (LBN) was available between P2 and P9 (Rice et al., 2008; Naninck et al., 2015). Both, standard housed and LNB mice were weighted before placing them in a new standard housed or LNB cage respectively, and then left undisturbed until P9 (or the start of the experiment when tested at P3 or P6). To prevent potential effects of differences in the number of pups per litter, all litters were culled to 6 pups, with at least 2 pups being of the same sex. Starting at P9, all mice were housed with the dams under standard conditions. For electrophysiological recordings, mice were decapitated at either P3, P6, P9, P15 or P21 of age. To investigate developmental changes in expression of specific mRNAs, we examined mice at P9, P15, P21 and adult mice (between 10 and 12 weeks of age).

All mice were directly taken from the home cage when they entered the experiment (e.g. for slice preparation); this also pertained to mice tested at P9, so that potential (stress) effects of transferring mice to other housing conditions were avoided.

2.3. Electrophysiology

2.3.1. Slice preparation

For electrophysiological experiments, the brain was quickly removed between 8:30 and 9 a.m. and stored in ice cold artificial cerebrospinal fluid (ACSF) containing: 120 mM choline chloride, 3.5 mM KCl, 0.5 mM CaCl2, 6 mM MgSO4, 1.25 mMNaH2PO4, 25 mM D-glucose and 25 mM NaHCO3. Trunk blood was collected and centrifuged for 10 min at 4000 rpm; plasma was frozen at -20 °C and corticosterone levels were measured afterwards with a RIA kit (MP Biomedicals Inc). Coronal slices of 350 µm thickness were made with a vibratome (Leica VT 1000S) and placed in ACSF containing: 120 mM NaCl, 3.5 mM KCl, 1.3 mM MgSO4, 1.25 mM NaH2PO4, 2.5 mM CaCl2, 25 mM D-glucose and 25 mM NaHCO3 and heat shocked at 32 °C for 20 min. The storage bath was then placed at room temperature and after recovery for at least 1 h, slices were used for the recordings. One slice at a time was transferred to the recording bath, continuous perfused with ACSF at 32 °C. Cells were visible with an upright microscope (Zeiss Aksioskop) with infrared DIC, a 40× water immersion objective, a 10 × video lens and a microscopy camera (Qimaging, Rolera bolt). All electrophysiological recordings were made from pyramidal-shaped neurons of the infralimbic (IL) mPFC layer 2 or 3.

2.3.2. Reversal potential recordings

Perforated patch clamp recordings: The pipette solution was composed as follows: 150 mM KCl and 10 mM Hepes, (295 mOSM; pH 7.3 adjusted with KOH) and stored at 4°C. Just before the recordings Gramicidin (80 µg/l) was added to the pipette solution and used for only one day. The Gramicidin stock solution contained 16 mg/ml DMSO and was stored at 5 °C. After establishing a gigaseal from a ILmPFC layer 2 or 3 pyramidal neuron, the series resistance of the membrane is slowly decreasing due to the perforation of the membrane within the tip of the pipette by gramicidin. From that moment on, we stimulated the neuron via puffing a GABA agonist Muscimol (10 µM, 100 µsec) from a glass pipette with a Picospritzer II (CV General Valve corporation, USA) in the close vicinity of the neuron. Chloride currents can be recorded when perforation is achieved and the patch clamp electrode is able to control the cellular voltage. To determine the Erev of GABA we stimulated the neurons with 10 µM Muscimol at several different holding potentials. GABA$_A$ receptors were stimulated from a holding potential of -120 mV and then with incrementing steps of 20 mV to $+20$ mV.

2.3.3. mEPSC and mIPSC recordings

The pipette solution to record mEPSCs and mIPSCs with was composed as follows: 120 mM Cs methanesulfonate, 17.5 mM CsCl, 10 mM Hepes, 5 mM BAPTA, 2 mM MgATP, 0.1 mM GTP, 5 mM QX314 (295 mOSM; pH 7.3 adjusted with CsOH). Miniature postsynaptic currents mediated by glutamate and GABA were recorded in neurons in the presence of 0.5 µM TTX (Latoxan, France). The mEPSCs (AMPA receptor-mediated currents) were recorded at a holding potential (Vh) of -65 mV, i.e. the reversal potential for chloride; and subsequently the GABAergic mIPSCs at Vh of $+10$ mV, which is the reversal potential for glutamate (Fig. 1A). All data was stored and afterwards the events were detected with a template search and analyzed with Clampfit 10.7 to determine the mean frequency and amplitude.

2.4. Expresssion of genes with qPCR

Animals were decapitated at 10 a.m. (2 h after the dark phase started). The brains were dissected and put in 0.9% NaCl solution on ice. To isolate the mPFC from the brains, 1 mm thick transversal slices were made from the brain, using a 1 mm matrix, and the tissue containing the mPFC was isolated. Brain samples were immediately put on dry ice, and stored at -80 °C until further processing. RNA was isolated using Trizol (Life Technologies) to break down cells and cell components while preserving RNA integrity during homogenization. Chloroform was added to separate RNA from other components into a water phase that was collected after spinning other components down. Iso-propanol (VWR, USA) was used to concentrate the RNA into a pellet, followed by 75% ethanol to wash the RNA pellet. The pellet was resuspended with miliQ water and stored at -80 °C until cDNA synthesis. Purity and concentration of RNA isolates were determined using a nanodrop spectrophotometer, right before cDNA synthesis. cDNA was made using a Quantitec kit (Qiagen Benelux B·V) resulting in 0.5 µg cDNA in a 10 µL solution.

Next, qPCR was performed using Taqman probes and primers for NKCC1 and KCC2 (Bonapersona et al., 2019); GAPDH was used as internal control (reference gene). Amplification cycles (40 cycles) were performed in volumes of 5 µL, consisting of 4 µL Taqman® Fast Advanced Mastermix (Applied Biosystems) and 1 µL containing 5 ng cDNA. Amplistar 384-well skirted plates were used, sealed with Amplistar adhesive clear plate seals type 2. Amplification cycle thermal conditions were as follows: 2 min 50 °C, 10 min 95 °C, followed by 40 cycles of 15 s 95 °C and 1 min 60 °C. The threshold was set automatically for control value determination, used to calculate the relative gene expression.

Relative gene expression was calculated using the Pfaffl method. This method takes the primer efficiencies into account, correcting for the differences in efficiency of the gene of interest and reference gene.

2.5. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 23.0. Data are presented as mean ± SEM, with all individual datapoints visible as well. The primary question was whether the GABA Erev and the expression of NKCC1 and KCC2 are changed in an age-dependent manner (main effect of age) and if so, whether this is influenced by ELS (interaction between age and ELS). To test this, we applied two-way ANOVA, with the factors age and condition. Significant interactions were followed up by post-hoc Bonferroni analysis; or by two tailed Students t-test, in case the condition was significantly different. For those ages where a significant interaction effect was observed, we tested whether the presynaptic glutamate release and GABA Erev could be manipulated with agonists and antagonists of the chloride transporters NKCC1 and KCC2; the effectiveness of drug treatment was tested for significance with a one way ANOVA and post-hoc LSD analysis. The effect of GABAa agonist and antagonist treatment on the presynaptic glutamate release was tested with a paired two tailed Students t-test, comparing pretreatment values with those during treatment.

3. Results

3.1. ELS accelerates the GABA shift

As reported in our previous paper (Karst et al., 2020), the EI-balance in IL-mPFC layer 2/3 neurons was decreased at the end of the ELS period, i.e. at P9, mainly due to a decrease in the frequency of the glutamatergic AMPA mEPSs. This was confirmed in the present study, showing a marked reduction of the mEPSC frequency at the end of the ELS period, compared to control (p = 0.0003, Students t-test; Fig. 2). The frequency of the mIPSCs was not affected after ELS (p = 0.54, not shown).

However, excitatory versus inhibitory influences also depend on the Erev GABA, which can be reliably determined using the perforated patch clamp technique, which (different from the whole cell recording mode) leaves the intracellular chloride concentration undisturbed (Fig. 3). In control (CNT) mice, the Erev shifted from -53.3 ± 4.8 mV at P9 and -56.9 ± 5.7 mV at P15, to -72.0 ± 7.2 mV at P21 (Fig. 3C), indicating that the switch takes place between P15 and P21. However, in ELS mice the GABA-switch already took place at P9 (Fig. 3C), i.e. in ELS mice the Erev was -74.8 ± 4.6 mV at P9. A two-way ANOVA for condition (ELS) and age showed that ELS caused a significant effect on Erev ($F_{(1,74)} = 5.541$; $p < 0.05$) and that the effect depended on age ($F_{(2,74)} = 3.654$; $p < 0.05$). Posthoc analysis (Bonferroni) revealed that in ELS P9 the Erev

is significantly different from CNT P9 ($p < 0.01$).

To examine when exactly the GABA-switch takes place in ELS mice, we also recorded the Erev in ELS and CNT mice at P3 and P6. In ELS mice at P3, the Erev in the IL-PFC neurons was shifted to inhibitory potentials of -79.8 ± 4.0 mV (Fig. 4), but -to our surprise-also in CNT mice (Erev -82.6 ± 2.9 mV). A possible explanation for the early switch in mice at P3 could be that the procedure at P2, i.e. weighing and handling the pups, was stressful and induced effects that persisted for at least 24 h. To examine this possibility, we also measured the Erev in P3 pups that were not disturbed at P2. A One-way ANOVA test for these three groups at P3 revealed significant differences ($F_{(2, 27)} = 5.587$; $p = 0.01$). Indeed, in the P3 undisturbed mice, the Erev was much more positive (-63.0 ± 5.8 mV) and significantly different from CNT P3 mice (Students t-test $p = 0.005$), confirming our hypothesis that the handling procedure at P2 exerts effects that last for at least a day.

Follow-up recordings in P6 CNT and ELS mice revealed that the Erev in both groups was again restored to a depolarizing current with Erev of -57.9 and -61.0 mV, respectively. These values were significantly different from the P3 CNT and P3 ELS mice, tested with a One-way ANOVA ($F_{(4, 50)} = 4.503$; $p = 0.004$). Overall, this means that the switch in ELS mice takes place between P6 and P9, and is thus accelerated compared to controls.

3.2. Potential role of postsynaptic chloride homeostasis

The switch from excitation to inhibition depends on the chloride concentration in the neuron. Therefore, we predicted that the expression of the transporters responsible for the intracellular chloride concentration, would be affected in P9 ELS mice. We therefore measured the expression of mRNA for NKCC1 and KCC2 with the qPCR technique. For these experiments, we pooled the samples of P9 and P15 to increase the amount of tissue. A two way ANOVA test for age and expression of NKCC1 or KCC2 showed a significant effect for condition ($F_{(1,86)} = 4610$; $p < 0.05$, CNT versus ELS) of NKCC1 expression. The results depicted in Fig. 5 demonstrate that in ELS compared to control mice a decrease in the NKCC1 mRNA expression is observed in the pooled samples of P9 and P15 ($p = 0.028$, Students t-test), but not at other ages. We conclude that the decrease in the NKCC1 mRNA expression -if translated to the protein level-might have caused a decrease in the intracellular Cl^- concentration in ELS mice, which could be responsible for the acceleration of the GABA switch. No changes were observed for the KCC2 expression.

To study whether a reduction in NKCC1 expression could be responsible for a shift in the Erev and therefore the GABA switch, we
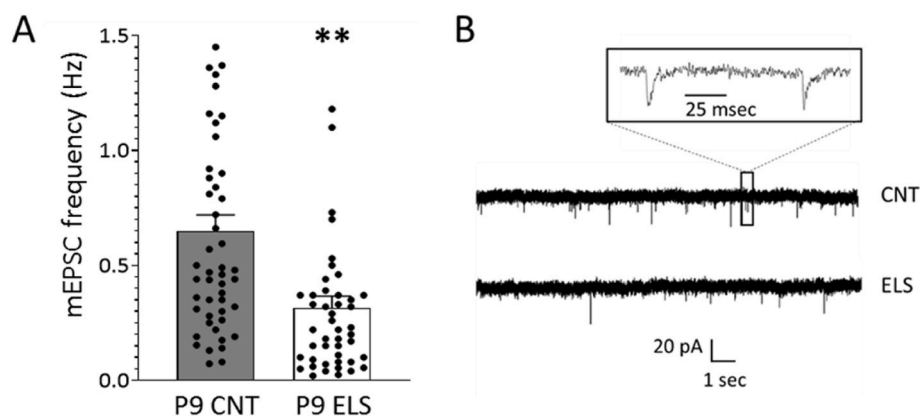
Fig. 2. The frequency of mEPSCs in IL-PFC neurons of layer 2/3 is reduced in mice at postnatal day P9, i.e. at the end of the limited nesting and bedding procedure from P2–P9 (**A**). **B.** Typical examples of mEPSC recordings of neurons in slices from a control (CNT) and early life stressed (ELS) mouse. The inset shows a detail of a mEPSC. (** indicates $p < 0.01$).
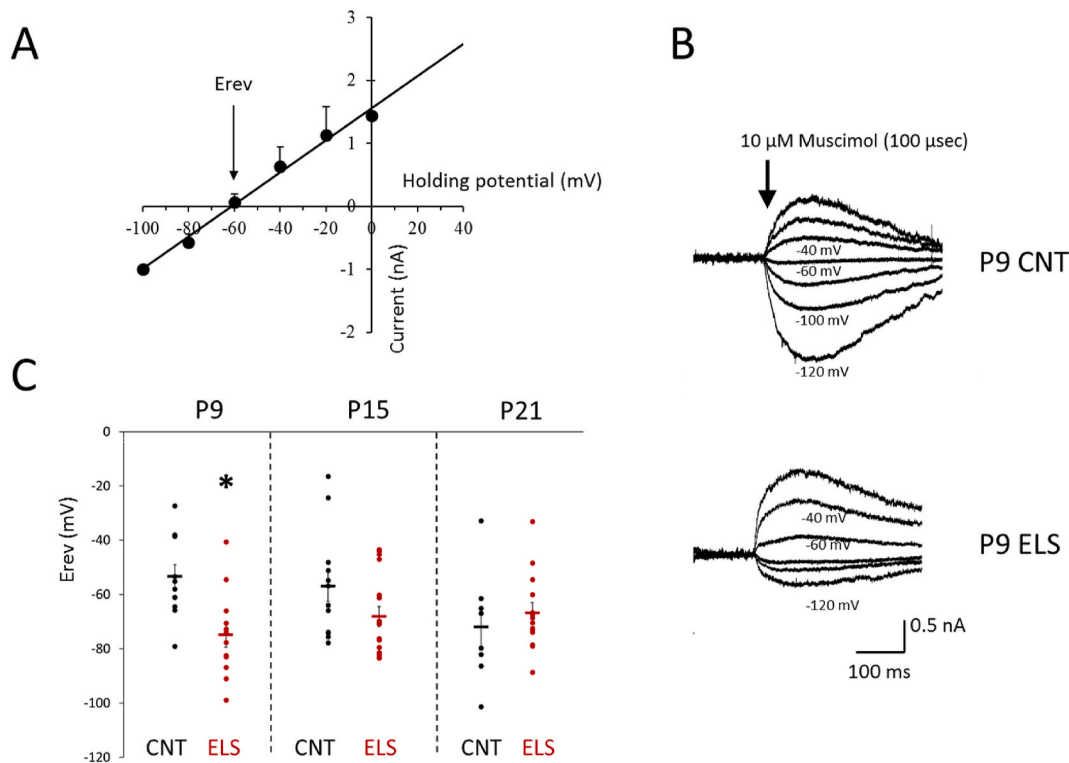
Fig. 3. GABA transmission is already shifted from depolarization to hyperpolarization at P9 in ELS mice, With perforated patch clamp recordings, GABA$_A$ mediated chloride currents are evoked by activating the GABA$_A$ receptors with a picrospritzer puffing muscimol (10 μM, duration 100 μsec) to the neuron at holding potentials from −120 to 0 mV. In **A** the amplitudes of the evoked GABA$_A$ currents are plotted at the different holding potentials in P15 CNT mice. The reversal potential (Erev) for GABA is indicated by an arrow. **B** shows examples of a recording from a P9 CNT and a P9 ELS mouse, clearly showing a shift of the Erev to hyperpolarization in the latter. (**C**) In control mice the Erev GABA switches from a depolarizing potential (approximately −55 mV) at P9 and P15 to a hyperpolarizing potential at P21 (−75 mV). In the ELS mice the Erev GABA$_A$ is already hyperpolarizing at P9.(* indicates p < 0.05).
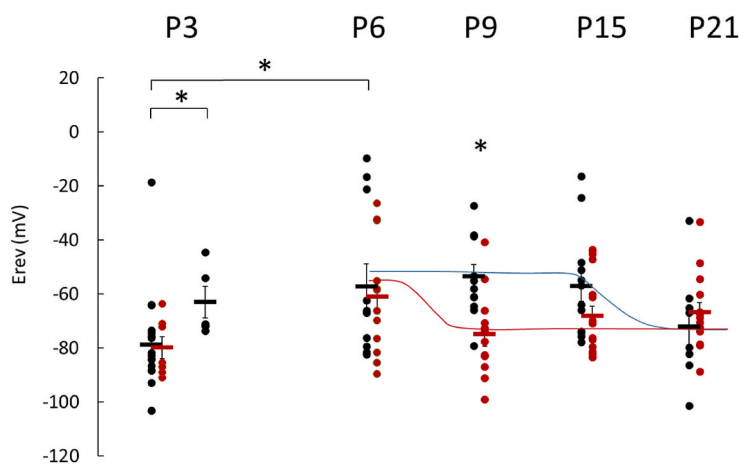
Fig. 4. Erev GABA is plotted per age in control (CNT) and early life stressed mice (ELS). The GABA-switch, i.e. when GABA switches from excitatory to inhibitory, appears in the CNT mice between P15 and P21 (black line), whereas in the ELS mice it already appears between P6 and P9 (red line). The red and black lines are supposititious lines for the effect of ELS on Erev between P6 and P21. The hyperpolarizing Erev GABA at P3 is probably caused by the (stressful) handling procedure at P2, weighing and handling the mice. In an undisturbed nest, Erev GABA of the IL-mPFC neurons are depolarizing, as was expected. (* indicates p < 0.05).

treated slices from P9 mice before and during recording with bumetanide, an antagonist of the NKCC1 transporter (Fig. 6A1). From Fig. 6B it is obvious that bumetanide shifts the Erev in a hyperpolarizing direction (p < 0.01, Students t-test).

An important question is if we also could "rescue" the ELS effect at P9. Therefore, an agonist of NKCC1 or antagonist of KCC2 was necessary. To this end we tested the effect of i) the mineralocorticoid aldosterone; ii) corticosterone in the presence of a glucocorticoid receptor antagonist; and iii) the KCC2 antagonist VU0463271. A one-way ANOVA test showed that none of the manipulations of the chloride transporters affected Erev in ELS mice (F(3,7592) = 1.9, p = 0.136).

More specifically, aldosterone, a mineralocorticoid receptor (MR) agonist, was earlier reported to activate NKCC1 expression (Ding et al., 2014; Bazard et al., 2020). However, in our hands, treatment with aldosterone did not affect the Erev in slices of ELS mice (Fig. 6B). Another way to activate MRs and thus possibly NKCC1 function, is by using a combination of corticosterone (100 nM), a mixed agonist of MRs and glucocorticoid receptors (GRs), and 500 nM RU38486 (Mifepristone), a GR antagonist. Also this way of stimulating the MRs did not change the Erev of GABA (Fig. 6B). We next explored an alternative route to elevate the chloride concentration, by blocking KCC2. Application of VU0463271, an antagonist of KCC2, was reported by others to increase the chloride concentration (Dzhala and Staley, 2021; Sivakumaran et al., 2015). However, we were not able to obtain a shift in the
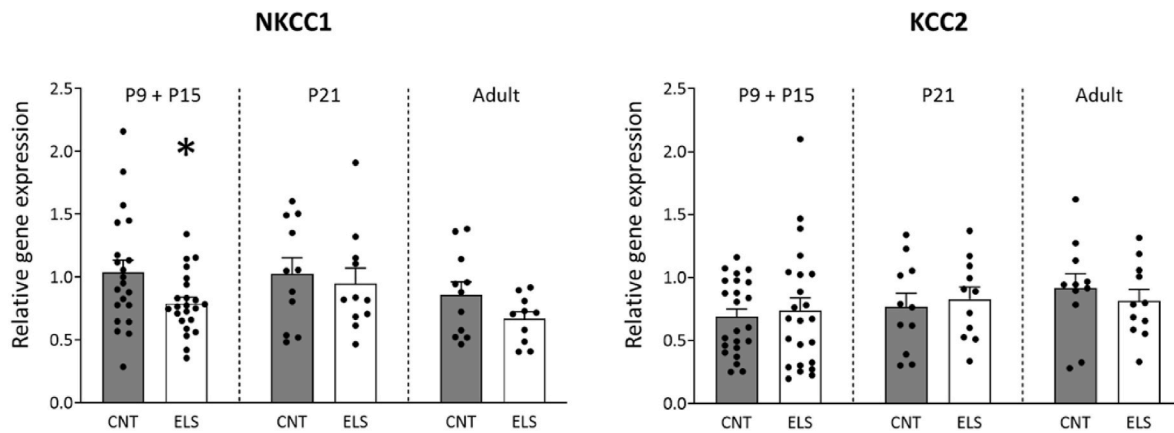
Fig. 5. The mRNA expression (mean ± SEM) of NKCC1 and KCC2 with qPCR shows that ELS causes a reduction of the mRNA expression for NKCC1, but not KCC2 in the pooled samples of the mPFC of P9 and P15 mice. (* indicates p < 0.05).

GABA Erev in slices of ELS P9 mice during VU0463271 treatment (Fig. 6B).

3.3. Potential role of presynaptic chloride homeostasis

Presynaptic chloride homeostasis may also play an important role in transmitter release, regulated by GABA$_A$ receptors. To test whether the decrease in frequency of mEPSCs at P9 in ELS mice might be caused by a decrease in axon-terminal Cl$^-$ concentration, we treated the slices with agents manipulating the chloride transporters (Fig. 6A1). In a first series of experiments, we found a reduction in the mEPSCs frequency when the slices of CNT P9 mice were treated with Bumetanide, a NKCC1 blocker (Fig. 6C) (p < 0.05, Student's t-test).

Next, we tested whether we could increase the mEPSC frequency by increasing the intracellular Cl$^-$ concentration with a treatment potentially increasing NKCC1 expression (aldosterone, RU486/Cort) or a KCC2 antagonist (VU0463271), as described in the previous section. With a one way ANOVA (F(3,0.239) = 3.105, p = 0.038) we showed that all treatments resulted in a significant increase in the mEPSC frequency (Fig. 6C; post-hoc LSD, p < 0.05). Therefore, the reduction of the NKCC1 expression in ELS P9-15 mice might cause a reduction in the mEPSC frequency (at least in part) by acting via presynaptic GABA$_A$ receptors.

We thus provided indirect evidence that the presynaptic Cl$^-$ concentration differs between P9 ELS and CNT mice, which will reveal a change in the mEPSC frequency in the presence of GABA. As was shown by (Holter et al., 2010), tonic release of GABA commonly takes place in the maturing rodent. If the difference in mEPSC frequency is indeed due to a difference in presynaptic Cl$^-$ concentration, a GABA$_A$ receptor antagonist, bicuculline, is expected to reduce the mEPSC frequency in CNT P9 mice. Conversely, the GABA$_A$ receptor agonist muscimol is predicted to increase the mEPSC frequency, comparable to what was published by (Jang et al., 2001, 2005). By contrast, in P9 ELS mice -where GABA activation is already supposed to be inhibitory-the GABA$_A$ agonist would decrease and an antagonist would increase mEPSC frequency, i.e. the exact opposite of what is expected in control mice. As shown in Fig. 7 for CNT P9 mice, muscimol indeed increased (p = 0.024, paired Students t-test) whereas bicuculline decreased the mEPSC frequency (p = 0.039), as was hypothesized. In contrast to our expectation, GABA$_A$ activation with muscimol did not give a further decline in the frequency in P9 ELS mice, nor did inhibition of the GABA$_A$ receptor result in an increase of the mEPSC frequency.

4. Discussion

Here, we showed that ELS accelerates the "GABA switch" in IL-mPFC neurons, i.e. the phenomenon that GABA turns from being excitatory to inhibitory, which is explained by a shift of the Erev for GABA towards a more hyperpolarized value. This switch normally takes place between postnatal week 2 and 4 (Rinetti-Vargas et al., 2017; Kahle et al., 2015; Y. K. Ben-Ari et al., 2012) (Peerboom and Wierenga, 2021)), but in ELS mice it is shifted to a much younger age, at some moment between P6 and P9. Interestingly, the Erev turned out to be quite sensitive to the "state" of the animal over the past 24 h, as transferring mice to another cage at P2 (as opposed to leaving the litters undisturbed) caused a temporary shift of Erev GABA towards more hyperpolarized values. This phenomenon, though, cannot explain the difference observed at P9, since both control and ELS mice were left undisturbed for >24 h prior to the experiment.

The accelerated GABA switch after ELS fits well with the hypothesis that ELS causes a general acceleration in development (Bath et al., 2016, 2017; Derks et al., 2016; Karst et al., 2020). For instance, in an earlier study by our group (Karst et al., 2020) we showed that the EI balance, in that case represented by the ratio between the frequency of excitatory mEPSCs and inhibitory mIPSCs, in mice after ELS was already at the level comparable with adult mice at P9. Also, Bath et al. (2016) reported an acceleration in the NMDA receptor subunit NR2a:NR2b ratio a week after ELS and an acceleration in the expression of Parvalbumin (PV) in fast spiking interneurons in the hippocampus. The accelerated GABA switch was accompanied by decreased NKCC1 mRNA expression. We found only partial evidence that altered NKCC1 expression may indeed underlie the reduced excitatory transmission in layer 2/3 IL-mPFC neurons after ELS, through post- and presynaptic mechanisms.

The excitatory GABAergic transmission in the prenatal and first postnatal weeks is important for a proper development of the brain. It mediates proliferation, migration and synapse maturation (Cancedda et al., 2007; Wang and Kriegstein, 2011; Wang and Kriegstein, 2008; Le Magueresse et al., 2013; Peerboom and Wierenga, 2021). Disturbances of the GABA-switch have been associated with psychiatric disorders such as autism and schizophrenia, but also individuals with Down's syndrome (Ben-Ari, 2017; Deidda et al., 2015). Under these conditions morphological changes in the brain have been demonstrated (Ben-Ari et al., 2012; Ben-Ari et al., 2007; Wang and Kriegstein, 2011). Compounds that affect the Erev of GABA endorse the importance of chloride homeostasis during development. Especially the chloride transporters NKCC1 and KCC2 are important regulators. Treatment of animals with an antagonist of the NKCC1, causing a decrease of intracellular chloride and a shift in the Erev of GABA towards hyperpolarized values, has permanent consequences for behavior and causes morphological changes in several brain structures (Wang and Kriegstein, 2008, 2011). However, in our study where ELS was shown to cause a shift of the Erev for GABA, we did not observe changes in the morphology in the mPFC (Karst et al., 2020) even though the NKCC1 mRNA expression was attenuated. This is largely in line with the results of a study by (Farrell et al., 2016): They showed that in female but not in male rats there was
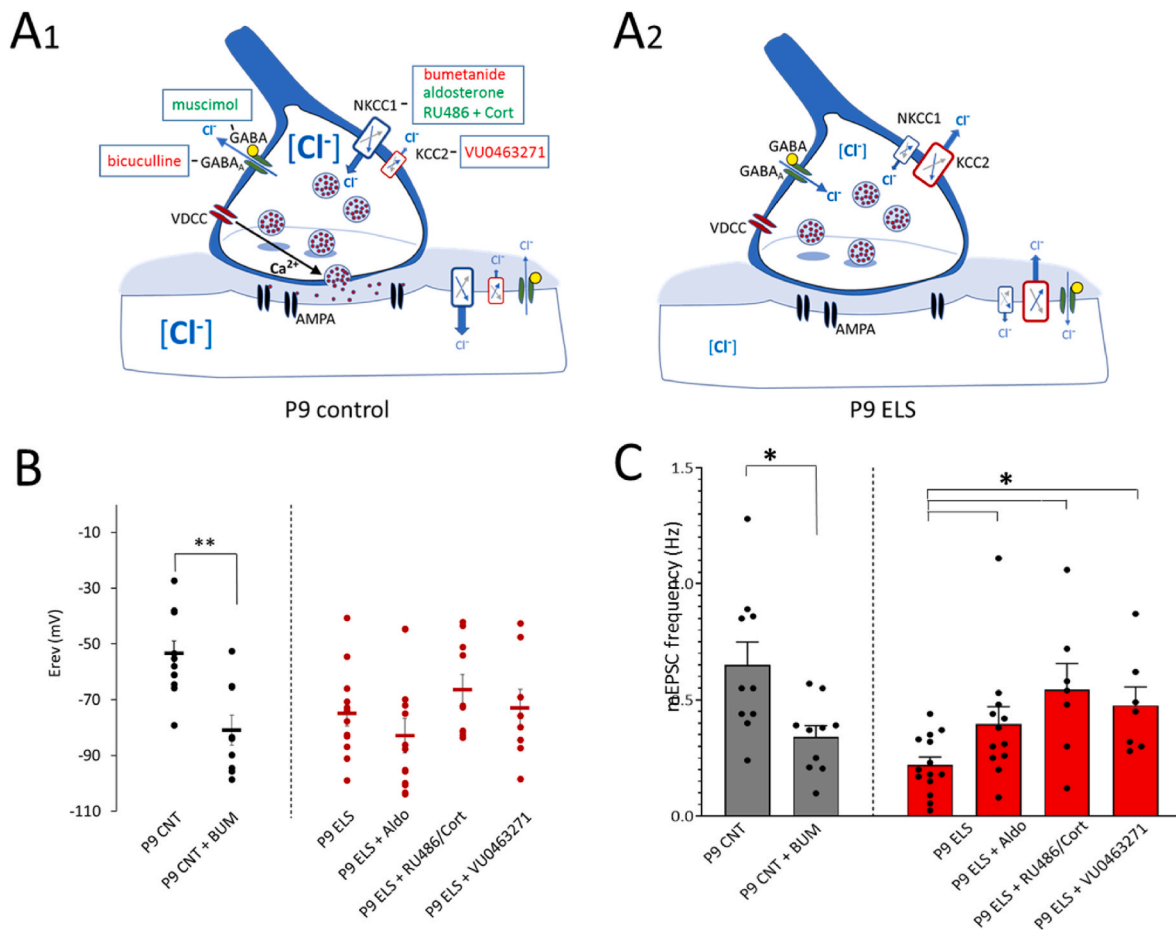
Fig. 6. A. Hypothetical model describing changes in Cl⁻ household after ELS. In P9 CNT mice (**A1**), stimulation of GABA$_A$ receptors (here depicted as channels in grey) causes depolarization of the postsynaptic neuron, as well as the presynaptic nerve terminal. Depolarization occurs due to an efflux of the high intracellular Cl⁻ concentration. At this age, the expression of the NKCC1 inward Cl⁻ transporter is high compared to the KCC2 outward Cl⁻ transporter. Presynaptic depolarization by GABA causes activation of the voltage dependent Ca^{2+} channels (VDCC, here in red), resulting in an influx of Ca^{2+}. Ca^{2+} enhances vesicle fusion leading to an increase of glutamate release. By contrast, in P9 ELS mice (**A2**) GABA$_A$ receptor stimulation causes a hyperpolarization, as is normally observed in neurons after the GABA switch has occurred between P15 and P21. The expression of NKCC1 is lower in P9 ELS mice, causing a lower intracellular pre- and postsynaptic Cl⁻ concentration. GABA$_A$ receptor stimulation then causes an influx of chloride, resulting in a hyperpolarization of the membrane. Hyperpolarization does not cause voltage-dependent Ca^{2+} activation, preventing vesicle release. All agonists (green) and antagonists (red) used for GABA$_A$ receptor and Cl⁻ transporters are depicted in A1. **B.** Bumetanide (BUM) blocks NKCC1 and causes a reduction of the intracellular chloride concentration in IL-mPFC neurons in P9 CNT mice, thereby shifting the Erev in hyperpolarizing direction. Neither aldosterone, which was earlier shown to increase the expression of NKCC1, nor VU0463271, an antagonist of KCC2, shifted the Erev of GABA in depolarizing direction by increasing the Cl⁻ concentration. **C.** In P9 CNT mice, bumetamide (BUM) reduces glutamate release, as is reflected by a reduction in mEPSC frequency. Remarkably, the reduced frequency in P9 ELS PFC neurons is rescued by activation of NKCC1 with aldosterone (MR agonist). Also, activation of the MR by blocking the GR with RU38486 and activating MR with corticosterone rescued the reduced mEPSC frequency. Blocking KCC2 with VU0463271, which would result in an increase of presynaptic Cl⁻, also increased the mEPSC frequency in IL-mPFC neurons of P9 ELS mice. (* indicates $p < 0.05$, **$p < 0.01$).

an increased number of infralimbic apical dendritic branches and increased length, and a decreased thin spine density at P40 after maternal separation between P2–P21 (4 h a day). As we currently only used male mice, we cannot exclude that morphological changes in mPFC neurons do occur after ELS in female mice. The reason that we restricted the current study to male mice was based on an extensive behavioral meta-analyses study of Bonapersona et al. (2019), reporting that the effects of ELS in female animals are in the same direction as that of male animals but far less robust. In accordance, most studies where we examined female rodents after ELS did not reveal significant effects (Krugers et al., 2012; Loi et al., 2017; Arp et al., 2016).

Of note, the observed reduction in NKCC1 mRNA expression after ELS was based on tissue pooled from P9 and P15 mice, to obtain sufficient material (given the very small size of the mPFC at that age), and hence power, for a meaningful comparison with controls. This differed from the electrophysiological experiments, where the two age groups were examined separately, with significant effects of condition at P9 but

not P15. This discrepancy in design can be considered as a limitation of the current study. While pooling might have artificially "amplified" potential differences by increasing power, one could also argue that it potentially diluted the difference between control and ELS conditions, since the electrophysiological analyses revealed a significant difference between Erev GABA in control and ELS mice at P9 but not P15. Given that, on average, the Erev GABA was highly comparable for P9 and P15 control mice (∼-55 mV) and quite different from the value observed at P21 (−72 mV), potential dilution may not have played a role, though. Another limitation is that we only examined mRNA expression and not protein levels or posttranslational modifications of the transporters, such as phosphorylation. Importantly, though, a blocker of the NKCC1 transporter, bumetanide, clearly affected the Erev at P9, supporting a direct relationship between the expression of NKCC1 and Erev.

In support of the notion that pre- and postsynaptic Cl⁻ concentration in general forms an important target for the effect of ELS is the observation that the effect of ELS on Erev GABA can be mimicked by treating
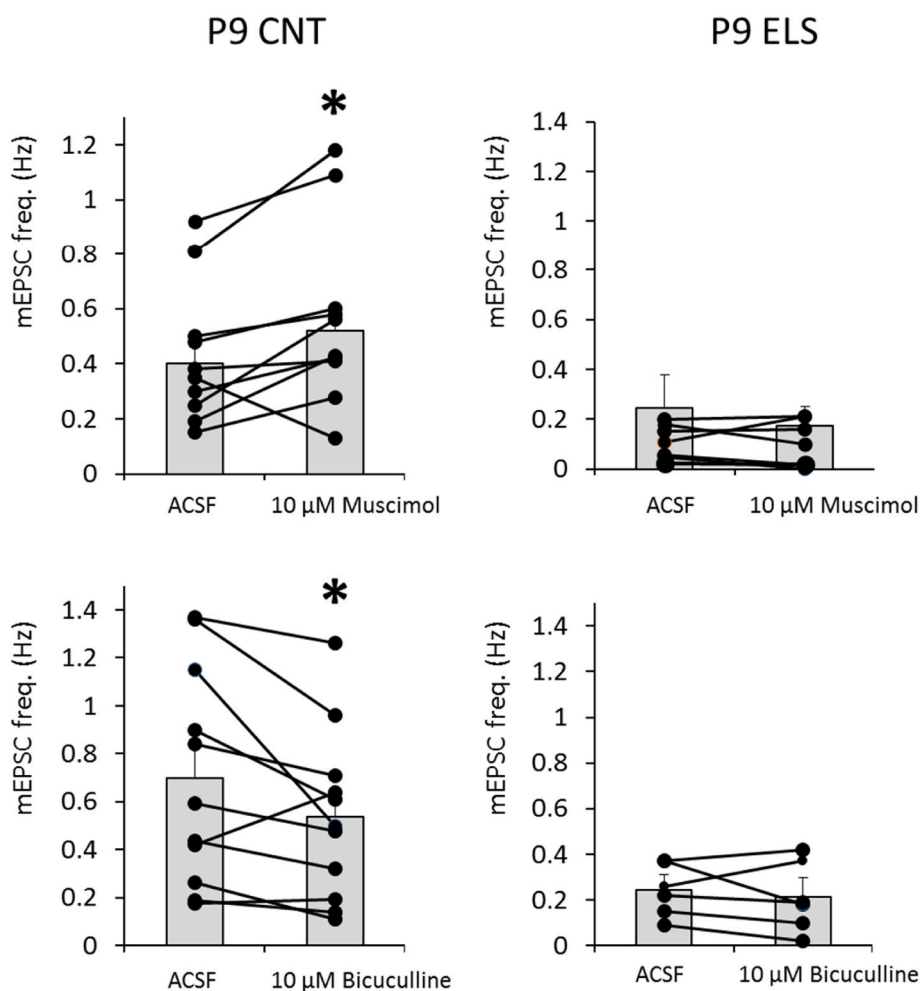
Fig. 7. During wash in of a $GABA_A$ agonist, muscimol, the frequency of mEPSCs increases in IL PFC neurons of P9 CNT acute slices. Most likely, presynaptic $GABA_A$ activation with muscimol causes a depolarization of the presynaptic terminals, resulting in an increase of spontaneous release of glutamate. By contrast, blocking $GABA_A$ receptors with bicuculline reduces the release of glutamate, seen as a reduction of the mEPSC frequency during wash in. Thus, the tonic $GABA_A$ induced glutamate release here, is prevented with bicuculline. (* indicates $p < 0.05$, paired Students t-test).

CNT P9 slices with bumetanide. After bumetamide treatment, the Erev GABA was similar to that seen in ELS P9 mice, and the frequency of the mEPSCs in ILmPFC neurons was also attenuated to the level of ELS P9 animals. Although KCC2 antagonists were reported to cause the opposite effect on the Erev of GABA and on glutamate release -by increasing the intracellular Cl^- concentration-, we were only partly able to replicate these findings. Aldosterone (Ding et al., 2014; Bazard et al., 2020) and VU0463271 (Sivakumaran et al., 2015) are both known to increase the Cl^- influx by activating NKCC1 and blocking KCC2, respectively. We hypothesized that both modulators are possible candidates to rescue the effect of ELS on the GABA switch. However, in our hands, these modulators were not able to shift the Erev GABA towards more depolarized levels. A possible explanation could be that aldosterone exerts different effects in peripheral tissue than in brain cells, as the reported effect of aldosterone on NKCC1 was obtained from epithelial cells of the colon (Ding et al., 2014; Bazard et al., 2020). On the other hand, both agents were able to rescue the presynaptic, axonal effects of ELS: The decreased frequency of the mEPSCs in P9 ELS mice was changed to control level in the presence of aldosterone and VU0463271. Bumetanide also affected the mEPSC frequency in P9 CNT mice; it attenuated the frequency. Therefore, we demonstrated that, at least during brain maturation, the presynaptic Cl^- concentration in the ILmPFC is most likely an important factor that determines the $GABA_A$ receptor mediated release of glutamate. It is well known that in spinal cord neurons presynaptic control of chloride via NKCC1 plays a role in pain regulation (Wei et al., 2013). Besides in the spinal cord, axonal $GABA_A$ receptors have also been found in the retina, calyx of Held, posterior pituitary, cerebral cortex, hippocampal and cerebellar molecular layer interneurons (MacDermott et al.,

1999; Trigo et al., 2008). Jang et al. (2001), 2002 and 2005 demonstrated that $GABA_A$ receptor-mediated presynaptic depolarization is also present at central synapses and facilitates spontaneous neurotransmitter release in 9–15 days old Wistar rats. At a young age, presynaptic NKCC1 activity was reported to regulate glutamate release via its induced high level of chloride. High presynaptic concentrations of chloride, present in young mice, cause a depolarization after GABAergic stimulation. Depolarization subsequently activates high voltage dependent Ca^{2+} channels, resulting in an influx of calcium, which in turn stimulates vesicle release (Zorrilla de San Martin et al., 2017). In young animals, a high concentration of GABA is present and induces a tonic activation. Given the effectiveness of bicuculline in our hands, tonic release of GABA appears to be at least one factor determining the mEPSC frequency in young control mPFC cells too. Conversely, an increase of the mEPSC frequency after treatment of P9 ELS tissue with bicuculine did not occur, nor a decrease in mEPSCs frequency when $GABA_A$ receptors were activated with muscimol. A possible explanation for the latter is that the presynaptic chloride concentration in these animals causes an Erev that resembles resting membrane potential, and thus will not cause a potential shift when GABA receptors are stimulated. Differences in the pre- and postsynaptic expression or activity of the chloride transporters, causing a difference in the Erev of the presynaptic terminal or neuron, could also explain the differences between the pre- and postsynaptisch effects. However, to our knowledge, no publications exist that speak in favor of such a mechanism.

All in all, from this and our previous study we conclude that ELS (using the limited bedding and nesting model), causes an attenuation of the excitatory tone in the IL-mPFC area at the end of the first and in the

second postnatal week. In the present study we demonstrate that an accelerated shift of the GABA-switch from excitatory to inhibitory transmission, potentially via decreased NKCC1 expression, may contribute to the decreased excitatory tone, via pre- and postsynaptic mechanisms. While the acceleration only pertains to a brief early developmental window, altered glutamatergic transmission may affect the morphological maturation of IL-mPFC cells at a critical moment in time and hence exert long-lasting consequences for the neuronal circuits and the behavior for which these circuits are essential. Behavioral studies confirm this notion (Sun et al., 2021) (Usui et al., 2021; Bercum et al., 2021; Reincke and Hanganu-Opatz, 2017). If similar mechanisms occur in humans, this may predispose individuals with a vulnerable genetic and/or environmental load to eventually precipitate psychopathology.

CRediT authorship contribution statement

Henk Karst: Conceptualization, Methodology, Data creation, Writing – original draft, Writing – review & editing, Supervision. **Wouter J. Droogers:** Data creation, Writing – review & editing. **Nelleke van der Weerd:** Data creation, Formal analysis. **Ruth Damsteegt:** Resources, Project administration. **Nicky van Kronenburg:** Animal management. **R. Angela Sarabdjitsingh:** Conceptualization, Methodology. **Marian Joëls:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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