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General anaesthetics do not impair developmental expression of the KCC2 potassium-chloride cotransporter in neonatal rats during the brain growth spurt

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Editor's key points

- Anaesthetic-induced developmental neurotoxicity correlates with changes in GABAergic neurotransmission determined by KCC2 expression.
- The effects of three general anaesthetics on KCC2 expression were determined in neonatal rats.
- Expression of KCC2 was not altered, and is unlikely to contribute to the neurodevelopmental effects of anaesthesia.

Background. The developmental transition from depolarizing to hyperpolarizing γ-aminobutyric acid-mediated neurotransmission is primarily mediated by an increase in the amount of the potassium-chloride cotransporter KCC2 during early postnatal life. However, it is not known whether early neuronal activity plays a modulatory role in the expression of total KCC2 mRNA and protein in the immature brain. As general anaesthetics are powerful modulators of neuronal activity, the purpose of this study was to explore how these drugs affect KCC2 expression during the brain growth spurt.

Methods. Wistar rat pups were exposed to either a single dose or 6 h of midazolam, propofol, or ketamine anaesthesia at postnatal days 0, 5, 10, or 15. KCC2 expression was assessed using immunoblotting, immunohistochemistry, or quantitative polymerase chain reaction analysis up to 3 days post-exposure in the medial prefrontal cortex.

Results. There was a progressive and steep increase in the expression of KCC2 between birth and 2 weeks of age. Exposure to midazolam, propofol, or ketamine up to 6 h at any investigated stages of the brain growth spurt did not influence the expression of this cotransporter protein.

Conclusion. I.V. general anaesthetics do not seem to influence developmental expression of KCC2 during the brain growth spurt.

Keywords: anaesthesia, paediatric; brain, GABA; neonates; toxicity, neurotoxicity

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Ionic plasticity is a fundamental and unique feature of γ-aminobutyric acid (GABA)-mediated signalling via the ionotropic $GABA_A$ receptor.[1] This implies that, depending primarily on the transmembrane chloride (Cl^-) electrochemical gradient, exposure to GABA can induce either hyperpolarization or depolarization of postsynaptic neurones. In the adult brain, because of low intracellular Cl^- concentrations, activation of $GABA_A$ receptors results in Cl^- influx and a related membrane hyperpolarization. In contrast, as intracellular Cl^- concentrations are higher in immature neurones, GABA has a depolarizing and sometimes even excitatory action during development, including the initial phases of the brain growth spurt.[2 3] The functional transition of $GABA_A$ receptor-mediated neurotransmission from depolarizing to hyperpolarizing is linked to the developmental upregulation of the potassium-chloride cotransporter KCC2, a neurone-specific plasmalemmal ion transporter that mediates K^+-gradient fuelled electrically neutral Cl^- extrusion from cells.[1 4] KCC2 expression increases during the 2nd postnatal week in the rodent cerebral cortex,[1 5 6] and from the 30th gestational week in humans.[7 8]

Despite the physiological importance of the KCC2-mediated ontogenetic E_{GABA} shift, our knowledge about the molecular mechanisms underlying progressive upregulation of this cation-chloride cotransporter during the brain growth spurt remains elusive. An intriguing and influential hypothesis suggested that presynaptically released GABA acts as a self-limiting trophic factor in a negative feedback loop where depolarizing postsynaptic $GABA_A$ receptor responses and consequent Ca^{2+} transients are required for the developmental upregulation of KCC2.[9] However, subsequent studies showed that developmental KCC2 upregulation takes place in hippocampal and midbrain cultures in the

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complete absence of $GABA_A$ receptor signalling.[10 11] During embryonic development in mice lacking, the vesicular inhibitory amino acid transporter (Viaat), KCC2 mRNA and protein levels were unaffected, despite the absence of GABAergic synaptic transmission.[12] Finally, experiments on NKCC1 knock-out mice which lack depolarizing GABAergic signalling showed no alteration in the expression of KCC2.[13 14] There are also data indicating that growth factor signalling via tyrosine kinase receptors (Trk) modulates KCC2 levels. Overexpression of brain-derived neurotrophic factor (BDNF) during embryonic development increases KCC2 mRNA levels in the mouse forebrain,[15] but whether this is a specific effect or reflects a general acceleration of neuronal maturation is not known. On the other hand, interictal-like neuronal activity patterns down-regulate KCC2 via the BDNF-TrkB pathway in hippocampal slice cultures, and a similar phenomenon has also been observed in both *in vivo* and *in vitro* animal models of epilepsy.[16–18]

General anaesthetics are powerful modulators of GABAergic and glutamatergic neurotransmission, and thus of neuronal activity.[19] Importantly, these drugs also influence BDNF-TrkB signalling pathways.[20–22] An appealing hypothesis is that KCC2 expression could be significantly influenced by general anaesthetics. This would imply that anaesthesia exposure during the brain growth spurt would result in an early non-physiological increase in neuronal KCC2 levels. This, in turn, might entrain a premature E_{GABA} shift in developing neuronal networks. Given the fundamental and clinical importance of this issue, the present work was designed to study this possibility. By focusing on well-defined time points during the brain growth spurt, we systematically evaluated the effects of midazolam, propofol and ketamine anaesthesia on KCC2 expression in the rat medial prefrontal cortex (mPFC) up to 72 h after drug administration.

Methods

The experimental protocol was conducted according to the guidelines of the Swiss Federal Veterinary Office and was approved by the Cantonal Veterinary Office, Geneva, Switzerland. Animals were group-housed and bred in the animal facilities of the University of Geneva Medical School under light- (12 h light/dark cycle) and temperature-controlled (20–24°C) conditions. Food and water were available at libitum. Every effort was made to minimize the number of animals used and their suffering. 0- to 15-day-old Wistar rats (males and females) were used for all experiments.

Anaesthesia procedures

According to the experimental protocol, general anaesthesia was induced by intraperitoneal (i.p.) injection of either midazolam (25 mg kg^{-1}), propofol (40 mg kg^{-1}) or ketamine (30 mg kg^{-1}) on postnatal day (PND) 0, 5, 10, or 15. Depending on the developmental stage, these drugs induced sedation (i.e. loss of the righting reflex) for 45 and 60 min. In the 6 h-long anaesthesia groups, after these aforementioned initial dosing regimens, animals then received an hourly i.p. injection (four in total) of midazolam (12.5 mg kg^{-1}), propofol (20 mg kg^{-1}), or ketamine (15 mg kg^{-1}). Control sham-treated animals received i.p. injections of physiological saline at equivalent volumes and frequency in each experimental protocol. All pups underwent the same maternal separation and handling as anaesthetized animals, and were kept in individual cages for the duration of the experimental procedure. Body temperature was monitored and maintained at 37–38°C by means of a heating pad (Harvard Apparatus, Holliston, MA, USA). In experiments where pups were euthanized at 24 or 72 h after drug exposure, they were replaced beside their respective mother once fully recovered from anaesthesia.

Immunohistochemistry

Animals were euthanized by an overdose of pentobarbital (120 mg kg^{-1} i.p.) and perfused transcardially with ice cold 4% paraformaldehyde (pH 7.4) solution. Brains were then removed and postfixed for 4 h in 4% paraformaldehyde. Of note, 40 μm-thick coronal sections of the left hemisphere were then cut on a vibratome in ice-cold phosphate-based saline solution (PBS, pH 7.4). For immunolabelling of KCC2, coronal sections were incubated with rabbit anti-KCC2 antibody (Millipore, Billerica, MA, USA; 1:2000 dilution) for 24 h at ambient temperature in PBS containing sucrose (5%), bovine serum albumin (2%), Triton X-100 (1%), and sodium azide (0.1%). Slices were then rinsed in PBS solution and incubated for an additional 24 h with an Alexa-conjugated secondary antibody (Molecular Probes, Carlsbad, CA, USA 1:1000). Slices were mounted and coverslipped using Immunomount (Thermo Scientific, Pittsburgh, PA, USA).

Immunoblotting

After decapitation, the prefrontal cortex was rapidly microdissected on ice and subsequently frozen in liquid nitrogen. Protein extraction from samples was carried out in RIPA buffer (100 mM Tris-Cl; 300 mM NaCl pH: 7.4, 10% NP-40, 10% sodium deoxycholate) together with a protease inhibitor cocktail (Complete mini, Roche, Basel, Switzerland). After homogenization with a syringe and sonication, lysates were centrifugated at 10 000 *g* for 20 min at 4°C, supernatants were collected and protein quantified using the Bradford method (Protein Assay, Biorad, Hercules, CA, USA). Soluble proteins were then denatured for 5 min at 95°C with sodium dodecyl sulphate and β-mercaptoethanol. Depending on the developmental stage, 10–30 μg of protein were equally loaded on a 7.5% polyacrylamide gel. After 90 min of migration at 120 V, proteins were transferred to a nitrocellulose membrane at 40 V for 1 h. Subsequent to blotting, the membrane was incubated with Ponceau stain to ensure proper blotting and an equal quantity of protein loaded. After washing the Ponceau stain, the membrane was blocked with phosphate buffered saline with tween (PBST)-5% milk for 1 h in room temperature, then it was incubated overnight at 4°C in a rabbit polycolonal IgG against KCC2 (Millipore, 1:10 000 dilution). Membranes were

subsequently washed and incubated with a secondary antibody (polyclonal goat anti-rabbit immunoglobulins/HRP, DakoCytomation, Denmark, 1:2000 dilution), exposed to luminol reagent (Roche Diagnostics) for 4 min and exposed to a photographic film (Amersham Hyperfilm ECL, Buckinghamshire, UK). Membranes were then treated with a stripping buffer (Restore Western Blot, Thermo Scientific) for 1 h at room temperature, blocked with PBST-5% milk for 1 h and incubated with anti β-tubulin antibody (purified anti-mouse β-tubulin, Covance, Princeton, NJ, USA) for 1 h followed by a secondary antibody (polyclonal rabbit anti-mouse immunoglobulin/HRP, DakoCytomation, Denmark, 1:2000 dilution) for 30 min. Protein intensities were quantified using NIH Image J (Bethesda, MD, USA) and KCC2 expression was normalized to β-tubulin.

Real-time quantitative polymerase chain reaction

Total RNA of each sample was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quality was verified by Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). The following primer sequences were used for KCC2: forward 5′-GATGCACCTCAC CAAGAA C-3′, reverse 5′-CTGGCTTCTCCTCG TTGT-3′. 0.5 μg RNA were converted to cDNA using TAKARA reverse transcription. Than 2-fold dilution series were subjected to SYBR Green PCR assays using triplicates. Relative gene expression was calculated as described by geNorm, and average quantities were normalized to three control (housekeeping) genes for each sample as previously described.[23]

Statistics

All mean values are given with the standard error of mean (SEM). Normality was tested for each distribution (D'Agostino and Pearson test), and α was set to 5% for all tests. Two-tailed Student's test were used for testing statistical significance ($P<0.05$) between groups using Prism software, Version 5.0a (GraphPad software, Inc., La Jolla, CA, USA).

Results

We first explored the developmental time course of KCC2 expression in the rat mPFC during the first two weeks of postnatal life. There was a progressive increase of KCC2 immunoreactivity during this period (Fig. 1A). These observations were extended by immunoblot analysis of KCC2 levels in extracts from the mPFC at distinct developmental time points (Fig. 1B and C). These experiments revealed only very faint amounts of KCC2 at PND 0, and the expression levels increased approximately 4-fold by PND 5 [391 (114)% of PND 0]. A steep, >10-fold increase in the amount of KCC2 was then observed between PND 5 and 10 [3950 (1560)% of PND 0], and this was followed by a further 3-fold increment between PND 10 and 15 [14 800 (2680)% of PND 0]. These results, in line with previous work,[24] thus demonstrate a large increase in the expression of KCC2 during the early postnatal period in the rat mPFC.

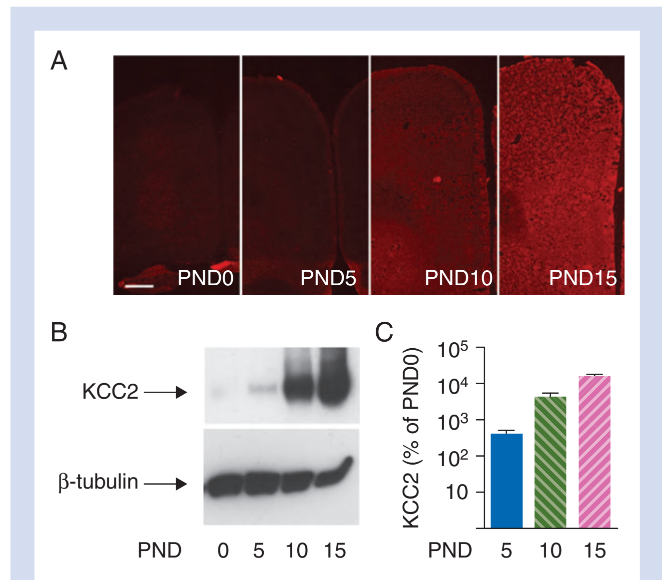

Fig 1 Developmental expression of KCC2 in the rat medial prefrontal cortex during the early postnatal period. (A) Immunohistochemistry reveals a progressive increase in the amount of KCC2 between PND 0 and 15. (B) Representative immunoblots of samples from medial prefrontal cortex tissue demonstrating the developmental increase in KCC2 expression. (C) Quantitative analyses of immunoblotting. Six animals from three independent litters were used. KCC2 levels are normalized to those of β-tubulin and results are expressed as mean (SEM) on a logarithmic scale. PND, postnatal day. Scale bar in (A): 400 μm.

To examine whether and how general anaesthesia-induced manipulation of neuronal activity and synaptic transmission affects developmental expression of KCC2 in the mPFC during the early postnatal period, newborn rats were exposed to either a single shot or a 6 h anaesthesia protocol at PND 0, 5, 10, and 15 using midazolam, propofol, or ketamine. The effects of these treatments on KCC2 levels were then assessed by immunoblot (midazolam and ketamine) or quantitative polymerase chain reaction (qPCR) analysis (propofol) 6, 24, and 72 h after drug exposure. The most extensive analysis on KCC2 expression was carried out using midazolam as this drug is not only considered as a prototype of $GABA_A$ receptor potentiating drugs, but is also widely used in other fields of medicine. Exposing animals at PND 0 to a single dose of midazolam (25 mg kg^{-1} i.p.) induced loss of the righting reflex for ~45 min. Analyses of KCC2 levels 6 h after drug exposure revealed a large interindividual heterogeneity in responses to this treatment (Fig. 2A). At this stage, when only scarce amounts of KCC2 can be detected in the cerebral cortex, midazolam-induced changes in KCC2 expression varied between 50 and 585% compared with the mean of control values, a variability that was several fold higher compared with the variability between control saline-treated animals (values ranging between 50 and 202% of the mean of control). The magnitude of the apparent variability after midazolam administration was somewhat decreased at later

post-exposure time points but remained larger than in control groups (Fig. 2B and C). Importantly, statistical analysis revealed no significant differences between control and single dose midazolam-treated groups either at 6 h (P=0.13), 24 h (P=0.54), or 72 h (P=0.33) after drug injection. When midazolam anaesthesia was maintained for 6 h at PND 0, many animals displayed respiratory depression after a few hours and some died. Given these observations together with the technical difficulties to perform reliable arterial blood sampling in this age group in order to determine adequacy of blood gas values, we thus decided to omit the 6 h protocol at this age. In contrast, as previously demonstrated, we could safely deliver a 6 h anaesthetic at later developmental stages.[25 26] As seen in Figures 3–5, the range of interindividual variability after midazolam exposure was comparable with interindividual variability between controls,

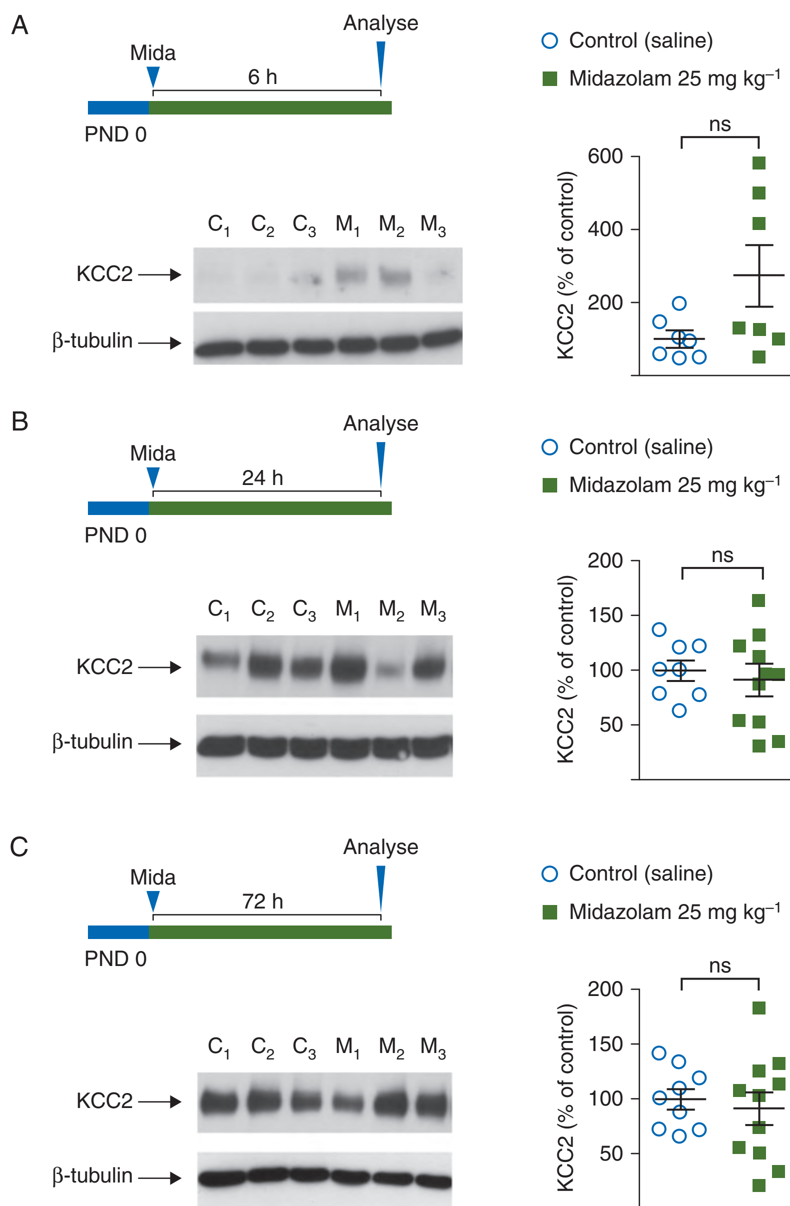

Fig 2 Effect of a single dose midazolam exposure at postnatal day 0 on KCC2 protein levels at 6 h (A), 24 h (B), and 72 h (C) after drug administration. Upper left panels describe the experimental protocol; lower left panels show representative immunoblots; right panels display the quantitative analysis of the related immunoblots. At each time point, at least seven animals from three independent litters were used for quantitative analysis. Each single dot represents KCC2 levels from one animal. Results were normalized to the internal control β-tubulin and are expressed as mean (SEM). After verification for normal distribution (D'Agostino and Pearson omnibus normality test), statistical significance between control and drug exposed groups were checked using the two-tailed Student's t-test. PND, postnatal day; C, control (saline injected) animals; M, midazolam injected animals; ns, non-significant difference between groups.

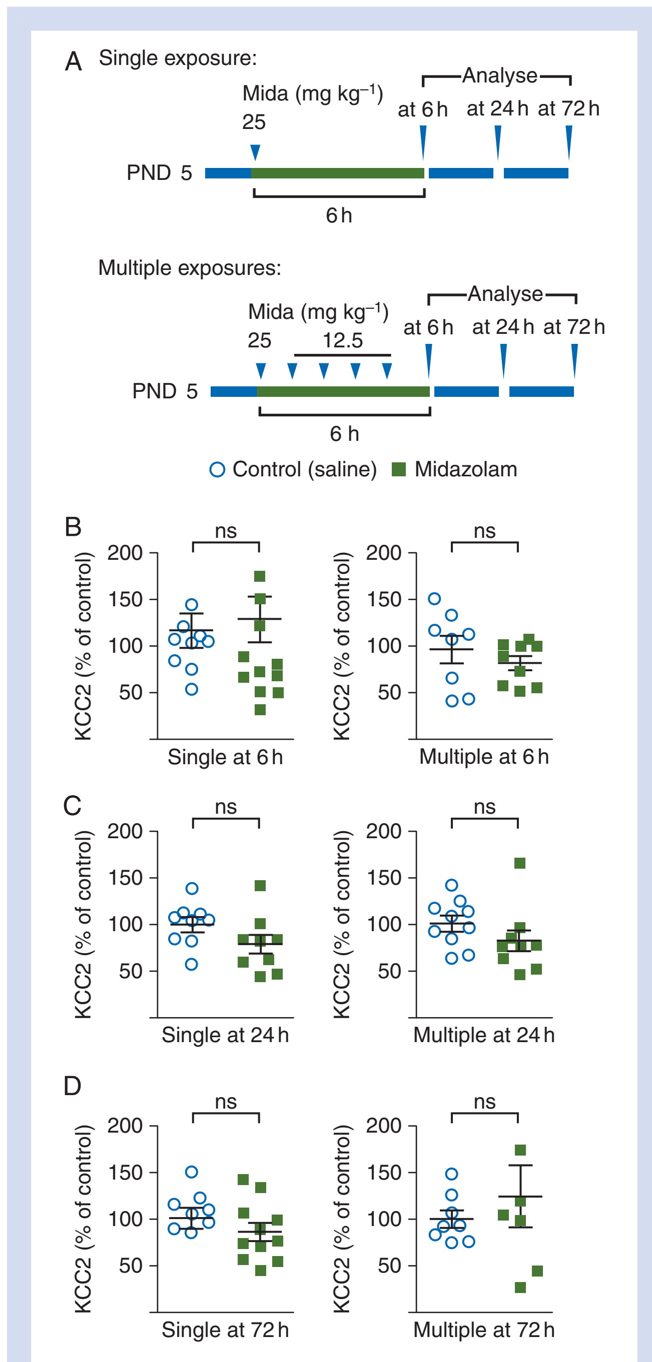

Fig 3 Effect of midazolam exposure at postnatal day 5 on KCC2 protein levels. (A) Experimental protocols. From (B–D) Quantitative analyses of immunoblots after single dose (left panels) or 6 h (right panels) midazolam exposure at 6 h (B), 24 h (C), and 72 h (D) after drug administration. At each time point, at least seven animals from three independent litters were used for quantitative analysis. Each single dot represents KCC2 levels from one animal. Results were normalized to the internal control β-tubulin and are expressed as mean (SEM). After verification for normal distribution (D'Agostino and Pearson omnibus normality test), statistical significance between control and drug exposed groups was checked using the two-tailed Student's *t*-test. PND, postnatal day; C, control (saline injected) animals; M, midazolam injected animals; ns, non-significant difference between groups.

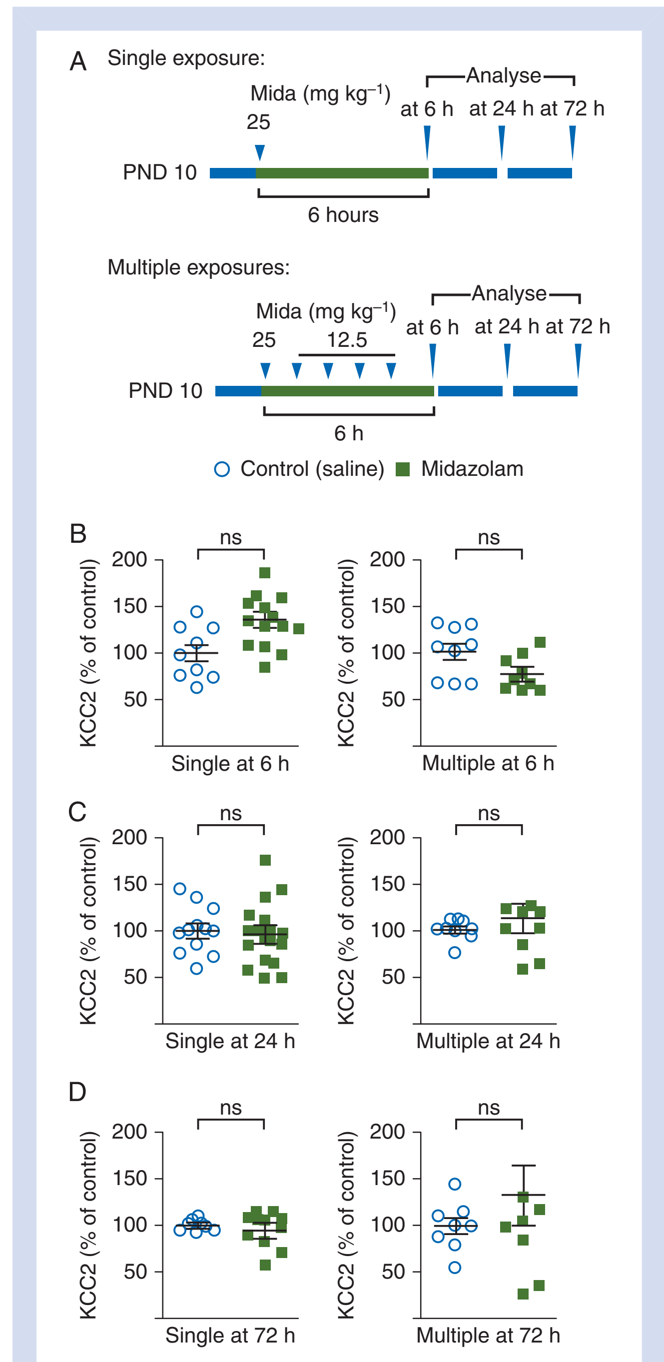

Fig 4 Effect of midazolam exposure at postnatal day 10 on KCC2 protein levels. (A) Experimental protocols. From (B–D) Quantitative analyses of immunoblots after single dose (left panels) or 6 h (right panels) midazolam exposure at 6 h (B), 24 h (C), and 72 h (D) after drug administration. At each time point, at least seven animals from three independent litters were used for quantitative analysis. Each single dot represents KCC2 levels from one animal. Results were normalized to the internal control β-tubulin and are expressed as mean (SEM). After verification for normal distribution (D'Agostino and Pearson omnibus normality test), statistical significance between control and drug exposed groups was checked using the two-tailed Student's *t*-test. PND, postnatal day; C, control (saline injected) animals; M, midazolam injected animals; ns, non-significant difference between groups.

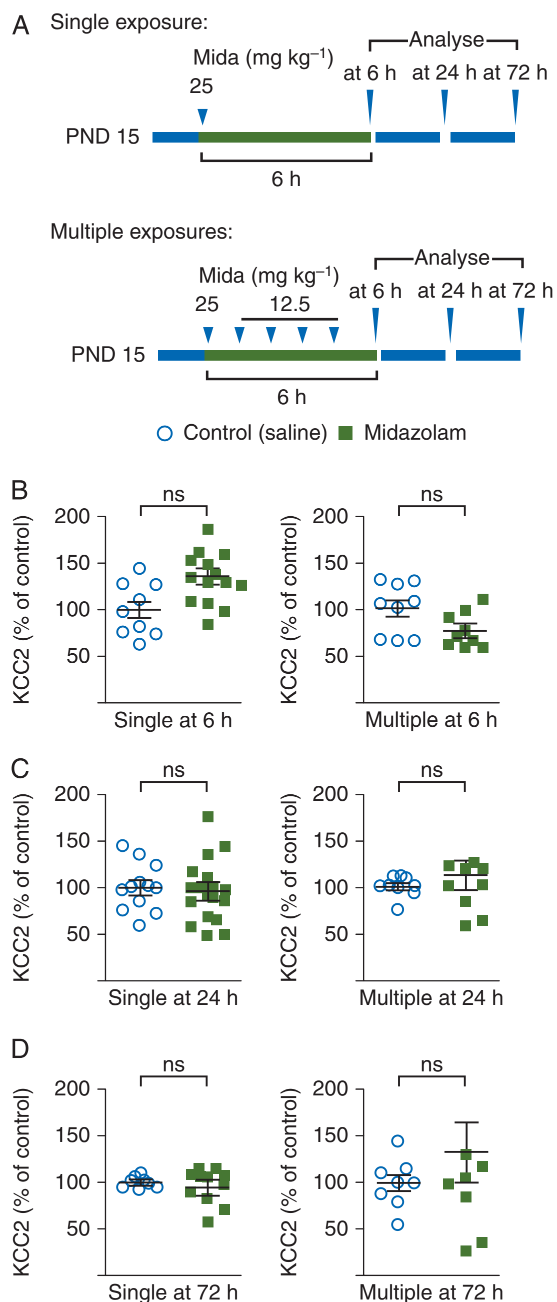

Fig 5 Impact of midazolam exposure at postnatal day 15 on KCC2 protein levels. (A) Experimental protocols. (B–D) Quantitative analyses of immunoblots after single dose (left panels) or 6 h (right panels) midazolam exposure at 6 h (B), 24 h (C), and 72 h (D) after drug administration. At each time point, at least seven animals from three independent litters were used for quantitative analysis. Each single dot represents KCC2 levels from one animal. Results were normalized to the internal control β-tubulin and are expressed as mean (SEM). After verification for normal distribution (D'Agostino and Pearson omnibus normality test), statistical significance between control and drug exposed groups was checked using the two-tailed Student's *t*-test. PND, postnatal day; C, control (saline injected) animals; M, midazolam injected animals; ns, non-significant difference between groups.

and neither single dose nor 6 h midazolam-based anaesthesia protocols revealed significant alterations in KCC2 levels up to 3 days after exposure at PND 5, 10, or 15. Altogether, these findings indicate that exposure up to 6 h to the $GABA_A$ receptor potentiator midazolam does not induce significant changes in KCC2 expression patterns during the postnatal period during which KCC2 levels reach near-adult values.[4]

To evaluate if other general anaesthetics exert comparable effects on KCC2 expression as midazolam, we next focused on propofol and investigated the impact of this drug on KCC2 expression at the mRNA level using quantitative PCR. In line with data obtained using midazolam, these experiments revealed no effect of either a single or a 6 h propofol exposure on KCC2 mRNA levels at PND 5, 10, and 15 when tested 6 h post-exposure (Fig. 6). Finally, we investigated how anaesthetics-induced blockade of NMDA receptors using ketamine affects KCC2 levels. As seen in Figure 7, up to a 6 h exposure to ketamine at any developmental stage tested did not affect KCC2 expression. Altogether, these findings demonstrate that anaesthesia-induced modulation of neuronal activity and neurotransmission *per se* did not influence developmental expression levels of KCC2 during the brain growth spurt.

Discussion

In agreement with previous observations,[1] we found an important developmental increase in the amount of the cation-chloride cotransporter KCC2 in the rat medial prefrontal cortex during the first 2 weeks of postnatal life. Exposure to midazolam, propofol, or ketamine for up to 6 h at any investigated stages of the brain growth spurt, however, did not influence the expression of this cotransporter either at the mRNA or at the protein level. These *in vivo* results thus indicate that general anaesthetic-induced potentiation of $GABA_A$ receptors or blockade of NMDA receptors does not induce KCC2 expression. They also suggest that transient alterations in neural activity do not modulate the level of expression of this cotransporter during early postnatal development in rat.

In addition to driving functional connectivity in the adult brain, neurotransmitters also play a fundamental role during the development of the central nervous system.[27] Amongst them, GABA is of particular relevance as this neurotransmitter and its receptors are present from early stages of development, and have been implicated in progenitor proliferation and cell migration.[28–30] Importantly, $GABA_A$ receptor-mediated membrane depolarization is considered to be a key regulator during early stages of both excitatory and inhibitory synaptogenesis in developing neurones.[30–32] Ectopic expression of KCC2 in immature neurones induces a premature shift of GABAergic responses from excitation to inhibition.[33–35] Interestingly, this genetic manipulation increases the expression of functional GABAergic synapses in cultured hippocampal neurones and in the Xenopus tadpole visual system.[31 33] We have recently demonstrated that premature expression of KCC2 in pyramidal neurones

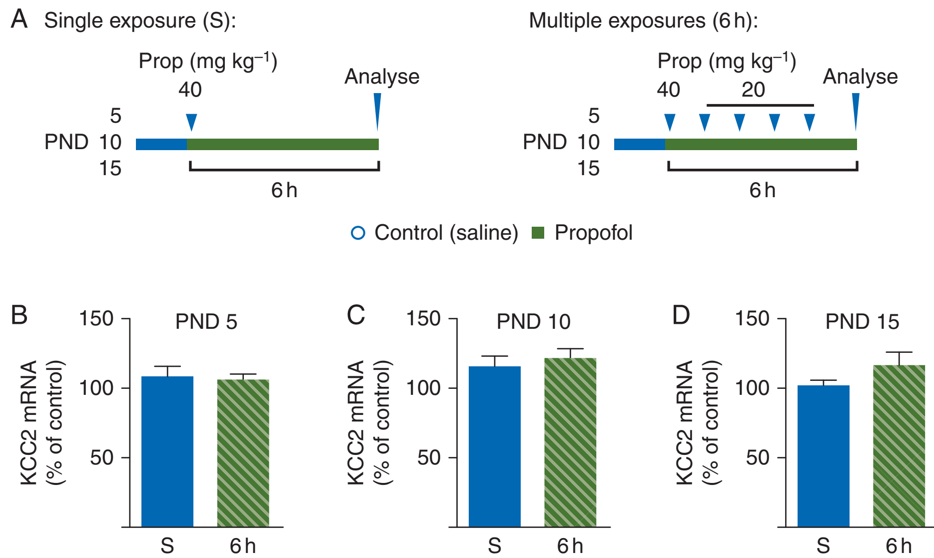

Fig 6 Effect of propofol exposure on KCC2 mRNA levels during the early postnatal period. (A) Experimental protocols. (B–D) Quantitative PCR analysis of KCC2 mRNAs at PND 5 (B), PND 10 (C), and PND 15 (D) after a single dose (S) or a 6 h (6 h) propofol treatment. Six animals from three independent litters were used for quantitative PCR analysis. After verification for normal distribution (D'Agostino and Pearson omnibus normality test), statistical significance between control and drug exposed groups was checked using the two-tailed Student's *t*-test.

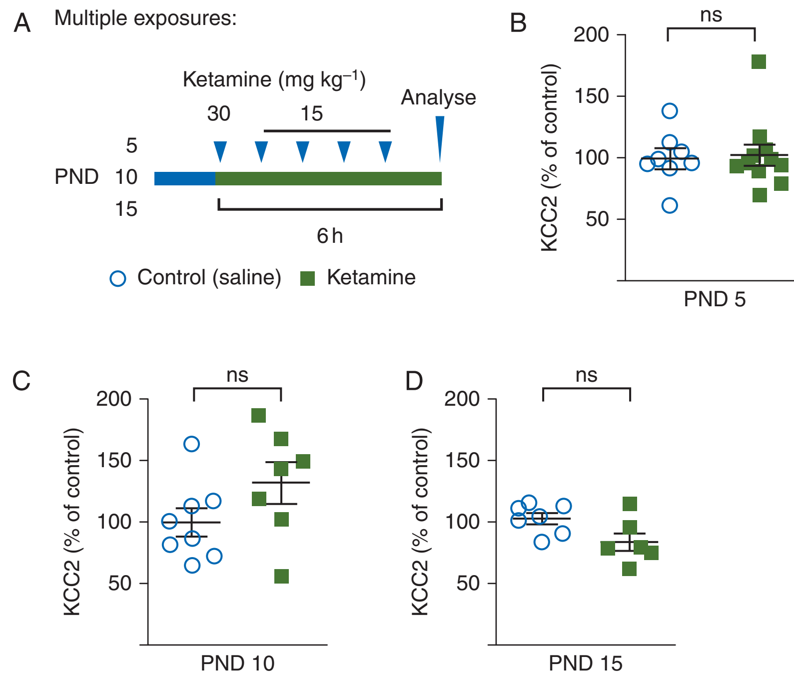

Fig 7 Impact of a 6 h ketamine exposure on KCC2 expression. (A) Experimental protocols. From B–D: quantitative analyses of immunoblots after drug exposure at PND 5 (B), PND 10 (C), and at PND 15 (D). At each time point, at least seven animals from three independent litters were used for quantitative analysis. Each single dot represents KCC2 levels from one animal. Results were normalized to the internal control β-tubulin and are expressed as mean (SEM). After verification for normal distribution (D'Agostino and Pearson omnibus normality test), statistical significance between control and drug exposed groups was checked using the two-tailed Student's *t*-test. PND, postnatal day; ns, non-significant difference between groups.

of the cerebral cortex leads to increased dendritic spinogenesis and a related increase in spontaneous excitatory activity of these cells, an effect that was not related to the ion transporter function of KCC2.[36 37] Altogether, these experimental findings suggest that events leading to pathophysiological alterations of KCC2 levels would have fundamental impact on the functions of developing neuronal circuitries. In line with this possibility, recent clinical observations suggest that alterations in KCC2 expression might contribute to schizophrenia and to the development of affective disorders.[38 39]

The hypothesis that $GABA_A$-acting general anaesthetics might impair the expression of KCC2 during development was motivated by several lines of previous experimental work (see Introduction). Interestingly, propofol-induced impairment of long-term potentiation in hippocampal slice cultures has been shown to be correlated with sustained expression of KCC2.[40] We have recently demonstrated that both general anaesthetics and ectopic expression of KCC2 induce increased dendritic spinogenesis in the cerebral cortex.[25 37 41 42] These latter observations raised the intriguing possibility that general anaesthetic-induced dendritic spinogenesis is mediated by KCC2, most probably through the involvement of GABAergic and neurotrophic signalling pathways. However, our present results, showing no effect of midazolam, propofol, and ketamine on KCC2 expression at any stage of the brain growth spurt, clearly demonstrate that this is not the case. Our results are in line with previous observations which were mainly done in cultures,[10–14] and give further arguments suggesting that neither ionotropic glutamatergic nor GABAergic neurotransmission is required for the postnatal developmental expression of KCC2. It should, however, be pointed out that while physiological neuronal activity which is attenuated or abolished by anaesthetics does obviously not influence the rates of KCC2 transcription and translation, pathophysiological activity leads to down-regulation of KCC2 transcription in mature neurones[16–18] and to trafficking of KCC2 protein to the membrane in immature neurones.[43] It is also important to note that in our study we evaluated only three currently used i.v. anaesthetics. Thus, further studies would be necessary to determine whether volatile anaesthetics exert similar effects. This issue could be of potential relevance especially in light of data suggesting increased seizure activity after sevoflurane exposure in neonatal brain.[44] Also, as in clinical practice it is more common to use a combination of anaesthetics, it will be necessary to elucidate the impact of such paradigms on KCC2 expression. Finally, as our study focused on the medial prefrontal cortex, we cannot exclude that anaesthetics might modulate KCC2 expression in other brain regions.

In conclusion, i.v. general anaesthetics do not influence the postnatal expression of KCC2 during the brain growth spurt. Most importantly as general anaesthetics are powerful modulators of neuronal activity, our work also suggests that KCC2 gene expression is not influenced in cortical neurones in an activity-dependent manner during normal development.

Declaration of interest

None declared.

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