RAPID REPORT

Early expression of KCC2 in rat hippocampal cultures augments expression of functional GABA synapses

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The development of GABAergic synapses is associated with an excitatory to inhibitory shift of the actions of GABA because of a reduction of $[Cl^-]_i$. This is due to a delayed postnatal expression of the K^+–Cl^- cotransporter KCC2, which has low levels at birth and peaks during the first few postnatal weeks. Whether the expression of the cotransporter and the excitatory to inhibitory shift have other consequences on the operation of $GABA_A$ receptors and synapses is not yet known. We have now expressed KCC2 in immature neurones at an early developmental stage and determined the consequences on the formation of GABA and glutamate synapses. We report that early expression of the cotransporter selectively enhances GABAergic synapses: there is a significant increase of the density of $GABA_A$ receptors and synapses and an increase of the frequency of GABAergic miniature postsynaptic currents. The density of glutamate synapses and frequency of AMPA miniature postsynaptic currents are not affected. We conclude that the expression of KCC2 and the reduction of $[Cl^-]_i$ play a critical role in the construction of GABAergic networks that extends beyond the excitatory to inhibitory shift of the actions of GABA.

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During development, neuronal differentiation and assembly of neuronal networks are paralleled by major changes in the expression and function of the ionic channels and transporters that control neuronal activity (Ben-Ari, 2002). Some of the most dramatic developmental changes occur in $GABA_A$ receptor ($GABA_AR$)-mediated signalling. GABA is the principal inhibitory neurotransmitter in the adult brain. However, at early developmental stages, through the second postnatal week in rodent, GABA acting via chloride-permeable $GABA_AR$ channels exerts paradoxical depolarizing and excitatory action on the immature neurones (Ben-Ari *et al.* 1989; Leinekugel *et al.* 1999). The depolarizing action of GABA is due to the elevated intracellular chloride concentration in the immature neurones (Ben-Ari *et al.* 1989), which in turn is due to the prolonged postnatal development of the chloride homeostasis system, most notably the potassium–chloride cotransporter KCC2 (Rivera *et al.* 1999). KCC2 is not expressed in immature rat hippocampal neurones during the first postnatal week, and its expression during the second postnatal week is associated with a progressive negative shift in the reversal potential of the $GABA_AR$-mediated responses and switch in the action of GABA from excitatory to inhibitory (Ludwig *et al.* 2003; Stein *et al.* 2004; Rivera *et al.* 2005).

Several physiological roles for depolarizing and excitatory GABA have been demonstrated in the developing brain. Depolarization produced by GABA triggers sodium action potentials, activates voltage-gated calcium channels and facilitates the activity of NMDA channels via attenuation of the voltage-dependent magnesium block of NMDA receptors (Leinekugel *et al.* 1997). Depolarizing GABA is critically involved in the generation of giant depolarizing potentials (GDPs), the characteristic pattern of network activity in the immature cortex (Ben-Ari *et al.* 1989; Garaschuk *et al.* 2000; Ben-Ari, 2002). Growing evidence indicates that depolarizing GABA operates as a trophic factor in the developing brain that controls neuronal differentiation (Loturco *et al.*

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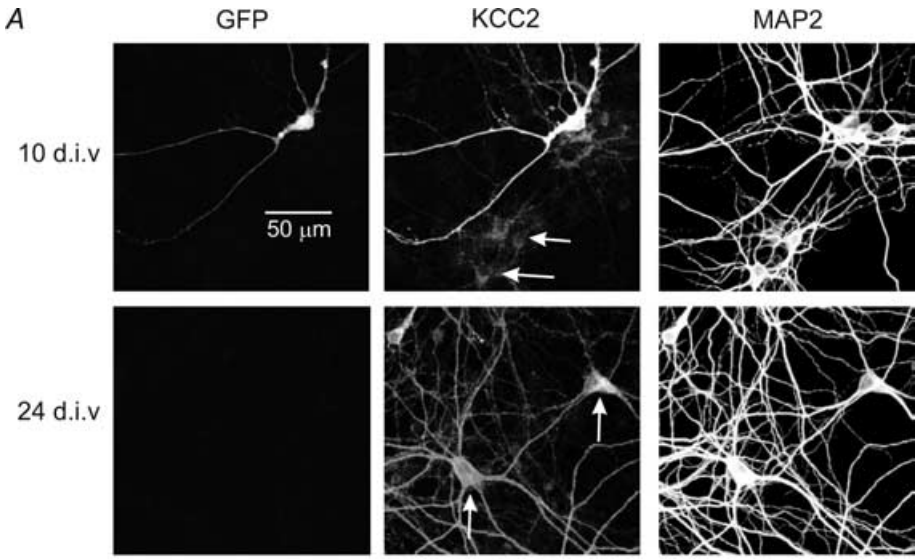

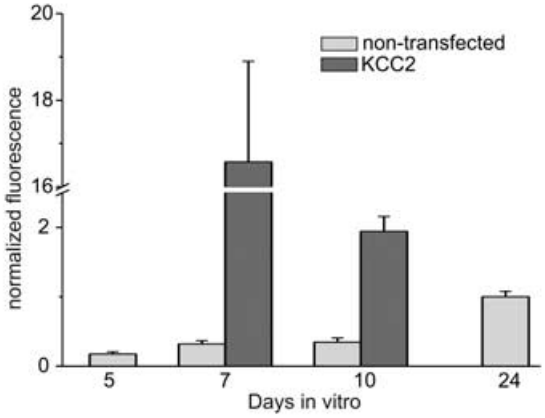

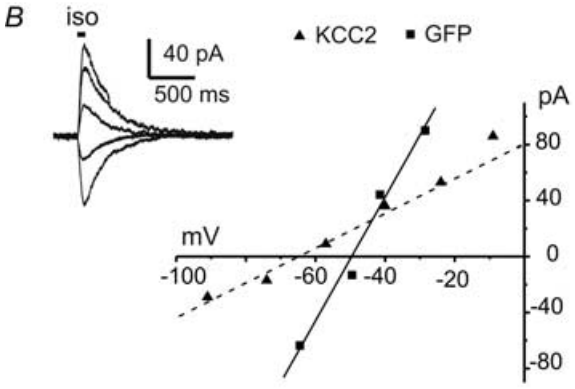

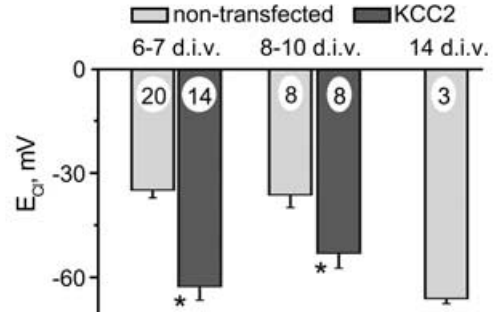

1995), neuronal growth (Barbin *et al.* 1993; Groc *et al.* 2002) and neuronal phenotype (Marty *et al.* 1996). The switch in the action of GABA from excitatory to inhibitory delineates not only the mode of function of the neuronal networks but also the trophic effects of GABA (Represa & Ben-Ari, 2005). Depolarizing GABA participates in induction of the long-term depression of GABAergic (Caillard *et al.* 1999) and glutamatergic (Pavlov *et al.* 2004) synapses in neonatal rat hippocampus. The blockage of $GABA_AR$ in hippocampus from newborn rats inhibits the formation of GABAergic synapses (Colin-Le Brun *et al.* 2004). Finally, in immature hippocampal cultures GABA autoregulates the transition from an excitatory to inhibitory function by means of modifying the expression level of KCC2 (Ganguly *et al.* 2001; but see Ludwig *et al.* 2003). However, little in known about whether a reciprocal relationship exists – namely that formation of GABAergic synapses may be controlled by the GABA switch. In the present work we studied this hypothesis by overexpressing KCC2 in dissociated neuronal cultures at developmental stages when they do not normally express the protein and functional synapses between neurones are not established yet. Expression of KCC2 resulted in a strong shift of the chloride equilibrium potential and potentiated formation of functional GABAergic – but not glutamatergic – synapses suggesting that expression of KCC2 and associated changes in chloride homeostasis and GABA functions may play an important role in formation of the inhibitory neuronal network.

Methods

Cell cultures and transfections

Neuronal hippocampal cultures were prepared from newborn (P0) Wistar rats that were rapidly decapitated after cervical dislocation. Hippocampi were dissected and cells were dissociated by treatment with 0.25% trypsin for 15 min at 37°C, and triturated through a fire-constricted Pasteur pipette in DNAse (0.1 mg ml^{-1}; Sigma). Neurones were plated onto poly-L-lysine-coated glass coverslips (14 mm diameter) at a density of 50 000 cells cm^{-2} in minimal essential medium (MEM; Invitrogen) supplemented with 10% NU serum, (BD Biosciences, Erembodegem, Belgium), 0.8% glucose 1 mM sodium pyruvate, 2 mM glutamine, and 10 IU ml^{-1} penicillin–streptomycin.

Five days after plating, the neurones were transfected with cDNAs using a Magnetofection kit (OZ Biosciences, Marseille, France) and Lipofectamine 2000 (Invitrogen) according to OZ Biosciences protocol. After transfection culture media was replaced with fresh MEM with 2% B27 supplement (Invitrogen). For most of the experiments, neurones were transfected with green fluorescent protein (pEGFP, BD Biosciences) or KCC2. cDNA encoding rat KCC2 (accession U55816) was subcloned into pIRES2-EGFP (BD Biosciences). In some experiments (as mentioned), red fluorescent protein (pDsRed2, BD Biosciences) was cotransfected with GFP and KCC2 (50/50%). All experiments with animals were approved by INSERM.

The probability of neurone transfection with exogenous KCC2 as well as morphology of neurones expressing KCC2 (KCC2 neurones) were similar to those transfected with GFP alone (GFP neurones) suggesting that the overexpression of KCC2 had no strong effect on neuronal viability and the development of dendrites (online supplemental material, Supplementary Fig. 1).

Immunocytochemistry

Neurones were fixed with 4% paraformaldehyde−4% sucrose in phosphate-buffer saline (PBS; pH 7.1), permeabilized with 0.30% Triton X-100 and blocked with 5% normal goat serum (room temperature). Primary antibodies were added for overnight at 4°C. Secondary fluorochrome-conjugated antibodies were applied for 2 h at room temperature.

As primary antibodies we used monoclonal anti-GAD67, anti-GAD65, anti-$GABA_AR$ β-chain (all Chemicon), polyclonal rabbit anti-VGLUT1 (Synaptic Systems, Gottingen, Germany) and rabbit anti-KCC2 (Hubner *et al.* 2001). Secondary antibodies included Cy3

Figure 1. Overexpression of functional KCC2 in hippocampal neurones in culture
A, illustration of the immunofluorescence of KCC2 (middle column) in neurones of different ages. Images in right column show immunoreactivity for the dendritic marker MAP2. Top row: 10-day-old culture that was transfected with KCC2 at 5 d.i.v. White arrows indicate KCC2 in non-transfected neurones. Bottom row: immunostainings of non-transfected 24 d.i.v. culture are shown for a comparison of the KCC2 levels at different time points. Plot shows mean level of exogenous and endogenous KCC2 in neurones at different time points. Level of endogenous KCC2 at all ages was determined in untransfected cultures. Data are normalized to the level of endogenous KCC2 at 24 D.I.V., 3–5 experiments per age, 10 neurones per experiment. *B*, chloride equilibrium potential (E_{Cl}) of neurones transfected with GFP or KCC2 + GFP (KCC2). E_{Cl} was measured using a gramicidin perforated patch and brief focal applications of isoguvacine (10 μM) as previously described (Tyzio *et al.* 2003). Traces illustrate responses to isoguvacine application at different holding potentials. Plot shows representative voltage–current relationships (VCRs) of isoguvacine responses. Note that VCRs measured for KCC2 neurones cross abscises at more negative potentials than VCRs of GFP neurones. Bars show mean E_{Cl} in neurones at different time points. Numbers on bars indicate the number of neurones studied in at least three independent experiments. * $P < 0.05$.

or Cy5 conjugated with goat anti mouse or goat antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA).

Identification of pyramidal neurones

For analysis of the density of presynaptic GAD and VGLUT clusters, pyramidal cells were selected based on their morphology (Benson *et al.* 1994) and negative staining of soma with a GAD67 antibody, a selective marker of interneurones localized in somato-dendritic regions and axonal terminals (Esclapez *et al.* 1994). Pyramidal neurones used for the analysis of $GABA_AR$ clusters and in electrophysiology experiments were selected based on their morphology. In addition, in a subset of the electrophysiology experiments, coverslips were fixed and immuno-labelled with GAD67 antibody (CY5 immunofluorescence) after recording was completed. Recorded neurones (neurones filled with biocytin) were identified using CY3 conjugated streptavidin (Jackson ImmunoResearch Laboratories). GAD67 staining was confirmed to be negative in 89% of neurones selected for recording and subsequently analysed ($n = 18$).

Image acquisition and analysis

Images were acquired with an Olympus Fluoview-500 confocal microscope (60×; NA 1.4 objective, zoom 1 or 3). To quantify the distribution of clusters on transfected neurones, we first focused on dendrites of neurones imaged with the fluorescent channel restricted to GFP. Fluorescence images of $GABA_AR$, GAD65 or GAD67 (CY3 fluorescence) and VGLUT1 (CY5 fluorescence) were then acquired. Cluster number and brightness overlapping with GFP-fluorescent neurone were analysed with the MetaMorph Imaging System (Universal Imaging, Westchester, PA, USA). All acquisitions and analysis were done blind. After analysis data were normalized to the mean of controls (GFP-transfected neurones) in every experiment.

Electrophysiological recordings

Electrophysiological recordings from neurones were performed 5 days after transfection. Neurones were continuously perfused with an extracellular solution containing (mM): 140 NaCl, 2.5 KCl, 20 Hepes, 20 D-glucose, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 0.001 tetrodotoxin, 0.05 AP-5, 0.001 strychnine, biocytin 0.2%, pH 7.4. Bicuculline at 10 μM or 10 μM CNQX were added to record mEPSCs or mIPSCs, respectively. To record whole cell responses of AMPAR and $GABA_AR$, 40 μM of AMPA or 10 μM of isoguvacine was applied by bath with an external solution containing, respectively, bicuculline or CNQX. Recording electrodes (4–6 MΩ) were filled with a solution containing (mM): 120 CsCl, 10 Hepes, 2.5 $MgCl_2$, 4 Na_2-ATP (adenosine triphosphate), 0.4 Na-GTP (guanosine triphosphate), 10 mM sodium phosphocreatine, 0.6 mM EGTA (pH 7.2). Recordings were made using an Axopatch-200A amplifier and pCLAMP acquisition software (Axon Instruments). Series resistance was compensated electronically. Since the amplitude of mIPSCs depends on the quality of intracellular perfusion with CsCl, we routinely measured E_{Cl} during bath applications of isoguvacine. In the analysis were included neurones showing E_{Cl} values close to +5 mV 5 min after membrane rupture. Data were low-pass filtered at 2 kHz and acquired at 10 kHz. Miniature postsynaptic currents were analyses using Mini Analysis software (Synaptosoft, Inc., Decatur, GA, USA).

The experiments for measuring the equilibrium potential of chloride were carried out using the perforated-patch whole cell recording technique as previously described (Tyzio *et al.* 2003). Micropipettes (5 MΩ) were filled with solution containing (mM): KCl 150, Hepes 10, Tris 10; 20 mg ml^{-1} gramicidin A (dissolved in DMSO) pH 7.2. Isoguvacine (10 μM) was focally applied to soma and proximal dendrites through a micropipette connected to a Picospritzer (pulse duration 50 ms, pressure 5 p.s.i.) (General Valve Corporation). E_{Cl} was determined as the point of crossing of voltage current relationship (VCR) of isoguvacine responses with abscises. All experiments were performed at 22–24°C.

Statistical analysis

All population data were expressed as the mean ± S.E.M. Student's *t* test (unless notified) was employed to examine the statistical significance of the differences between groups of data.

Results

To over-express KCC2 in neuronal cells we used a bicistronic construct of rat KCC2 and enhanced green fluorescent protein (GFP) separated by an internal ribosome entry site (IRES). As controls we carried out experiments on neurones from the same dishes that did not express exogenous KCC2 as well as on neurones from other dishes transfected with GFP alone. The transfection of neurones with KCC2 resulted in a strong increase of KCC2 immunoreactivity that remained at a high level for at least 5 days after transfection (Fig. 1*A*).

To directly test the functionality of the protein expression, we determined whether KCC2 over-expression modifies the chloride reversal potential (E_{Cl}). Patch clamp measurements with a gramicidin perforated patch that allows maintenance of an intact intracellular chloride

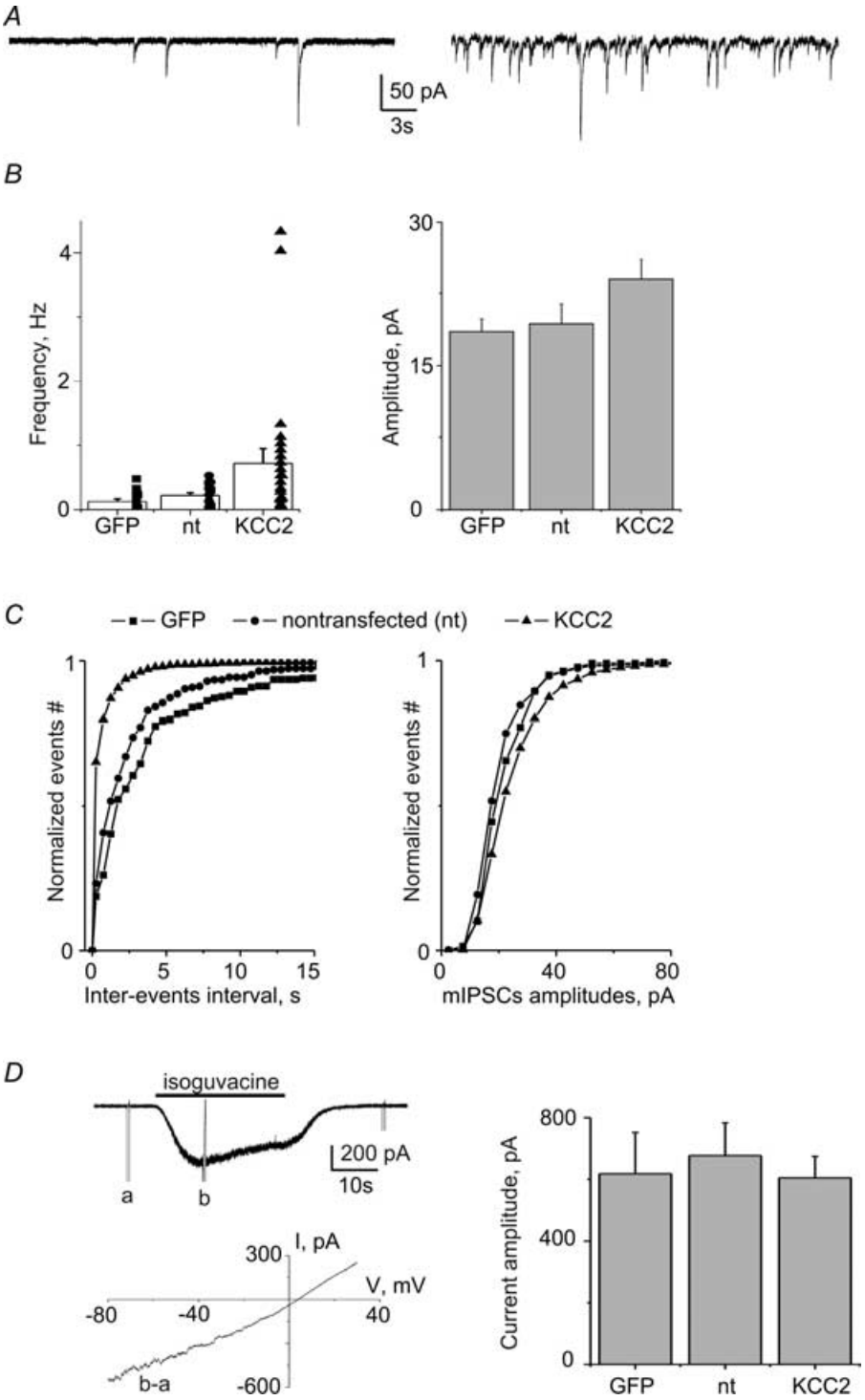

Figure 2. $GABA_A$R currents in neurones transfected with KCC2, non-transfected neurones from the same dishes and neurones transfected with GFP ($V_h = -60$ mV)
Data from 9 experiments (2–3 neurones per experiment). *A*, examples of miniature $GABA_A$ postsynaptic currents (mIPSCs) recordings in neurones transfected with GFP (left) and KCC2 (right). *B*, left, plot illustrates mean and point by point values of mIPSCs frequency. Right, plot shows mean mIPSCs amplitude. *C*, cumulative histograms of the distribution of interevent intervals (left) and amplitudes (right) of mIPSCs. Both interevent intervals and amplitudes in KCC2 neurones were significantly different from corresponding values in non-transfected or GFP transfected neurones. ($P < 0.01$, Kolmogorov-Smirnoff test). *D*, whole cell responses induced by bath application of 10 μM isoguvacine ($V_h = -60$ mV). Traces illustrate the response to GABA application (top) and VCR of this response (bottom). VCR of GABA response was obtained by subtraction of VCRs as indicated by corresponding letters on the top trace. Columns show the mean amplitude of whole cell $GABA_A$R responses measured at −60 mV.

concentration (Tyzio *et al.* 2003; Chavas & Marty, 2003) revealed that 24 h after transfection E_{Cl} was already strongly shifted towards negative values (Fig. 1*B*). The negative level of E_{Cl} in transfected neurones was sustained and was observed 3–5 days after transfection (Fig. 1*B*).

We then determined the physiological relevance of KCC2 over-expression. To do so, we measured the miniature postsynaptic currents of AMPA receptors and $GABA_A$ receptors (AMPAR and $GABA_AR$ – mEPSCs and mIPSCs, respectively) in pyramidal neurones transfected 5 days after plating (d.i.v., days *in vitro*) and then examined up to 5 d.i.v. after transfection. In agreement with previous studies (Burrone *et al.* 2002), in our cultures the formation of the majority of both AMPA and GABA synapses occurred from d.i.v. 7–10. At 5 d.i.v. (the day of transfection) only 3 out of 57 recorded neurones possessed mIPSCs and mEPSCs were not recorded in our sample of neurones. At 10 d.i.v. both mIPSCs and mEPSCs were observed in more than 90% of neurones. At this age neurones overexpressing KCC2 (KCC2 neurones) exhibited a striking difference in the frequency of mIPSCs as compared to either non-transfected neurones from the same dishes or neurones transfected with GFP only (GFP neurones) (Fig. 2*A*, *B* and *C*). The significance of this difference was confirmed by analysis of cumulative histograms of the interevent interval of mIPSCs (Fig. 2*C*). The frequency rise in KCC2 neurones went along with statistically significant increases in the amplitude of mIPSCs. The kinetic properties of mIPSCs in all neurone populations did not differ (not shown). Analysis of the amplitudes of whole cell responses to bath application of isoguvacine, an agonist of $GABA_AR$, revealed no

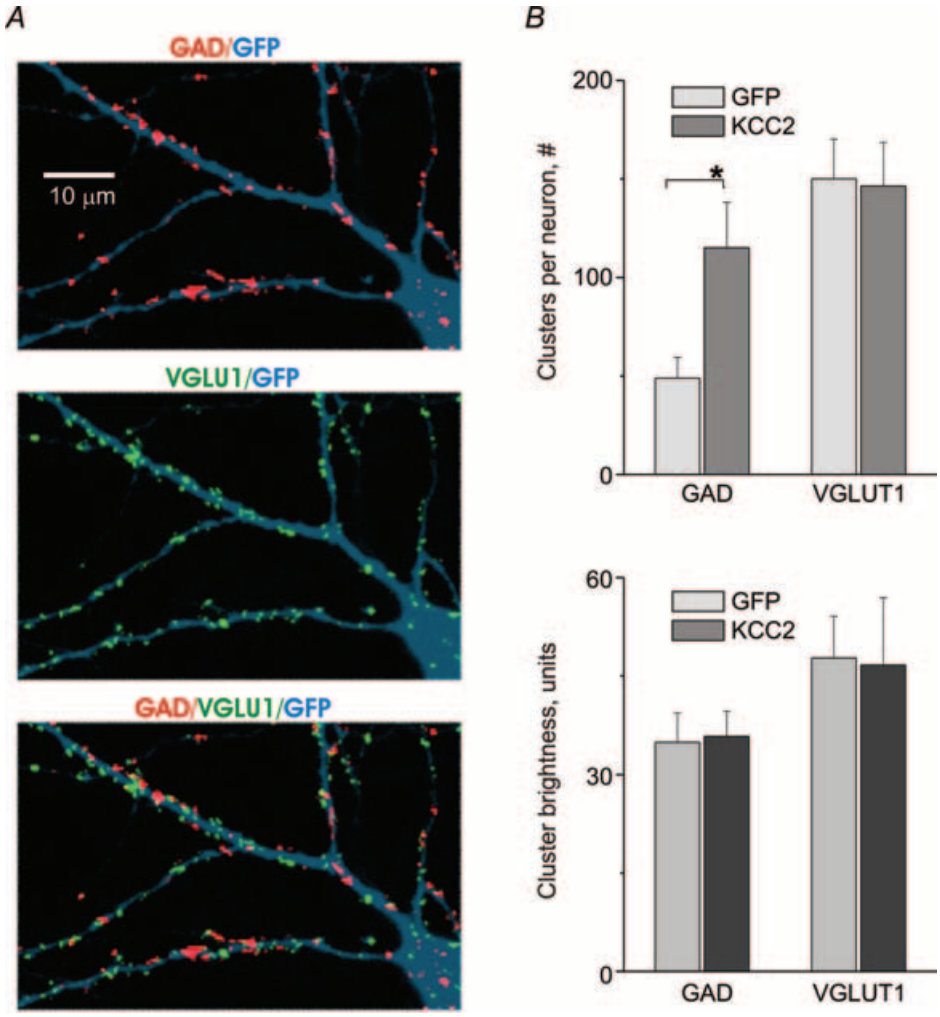

Figure 3. GABA and glutamate synapses on GFP and KCC2 neurones
A, examples of GABA and glutamate synapses visualized using a mixture of GAD65/GAD67 (red clusters) and VGLUT1 (green clusters) antibodies in GFP transfected neurone. GFP fluorescence is shown in blue for better visualization of the clusters. *B*, density and brightness of GAD and VGLUT1 clusters on GFP and KCC2 neurones (5 experiments, at least 6 neurones per experiment). * $P < 0.005$.

difference between KCC2, GFP and non-transfected neurones suggesting that KCC2-dependent modifications applied exclusively to synaptic $GABA_AR$ responses (Fig. 2*D*). Unlike mIPSCs, the properties of mEPSCs as well as amplitudes of whole cell responses to bath applications of AMPA were insensitive to the expression of KCC2 (Supplementary Fig. 2). Thus, the expression of KCC2 at an earlier developmental stage selectively enhances GABAergic synaptic activity.

Several hypotheses can be put forward to explain the higher frequency of mIPSCs, including the formation of new synapses. To verify if KCC2 modifies the number of GABAergic synapses, we studied the density of GABA synapses formed on transfected pyramidal neurones using a mixture of specific markers Gad65 and Gad67 (Esclapez *et al.* 1994). Glutamate synapses were visualized on the same neurone with VGLUT1 antibody (Fremeau *et al.* 2001). Figure 3 shows that the number of presynaptic GAD clusters (i.e. GABA synapses) visualized on KCC2 transfected neurones was significantly higher than those on neurones transfected with only GFP (Fig. 3). By contrast, the number of VGLUT1 clusters (marking glutamate synapses) was the same in both neurone populations. In further agreement with the electrophysiology data and GAD staining, immunostaining of $GABA_AR$ using anti-$GABA_AR$ β-chain monoclonal antibody showed significantly (6-fold) larger density of $GABA_AR$ clusters in KCC2 neurones (Fig. 4). Thus, the overexpression of KCC2 in developing neurones stimulates the clustering of $GABA_AR$ and the formation of synapses.

Discussion

The present results suggest that early expression of KCC2, at a time when GABA excites neurones because of a depolarizing chloride gradient, enhances GABAergic mechanisms. KCC2 overexpression increases the number of GABA synapses and the frequency and amplitude of mIPSCs. Interestingly, the amplitude of currents generated in the same neurones by exogenous applications of $GABA_AR$ agonist was not increased. Taken together these findings suggest a selective enhancement of synaptic events. In keeping with this, the increase in the density of $GABA_AR$ clusters might reflect the rearrangement of $GABA_AR$ from small extra-synaptic clusters (non-detectable in our experimental conditions) to larger synaptic clusters. Previously it has been reported that $GABA_AR$ clusters became larger during the emergence of presynaptic terminals (Christie *et al.* 2002). Notably, the increased density of postsynaptic $GABA_AR$ clusters (~6-fold) and the potentiation of mIPSCs (~7-fold, including 5.7-fold rise of frequency and 1.3-fold rise

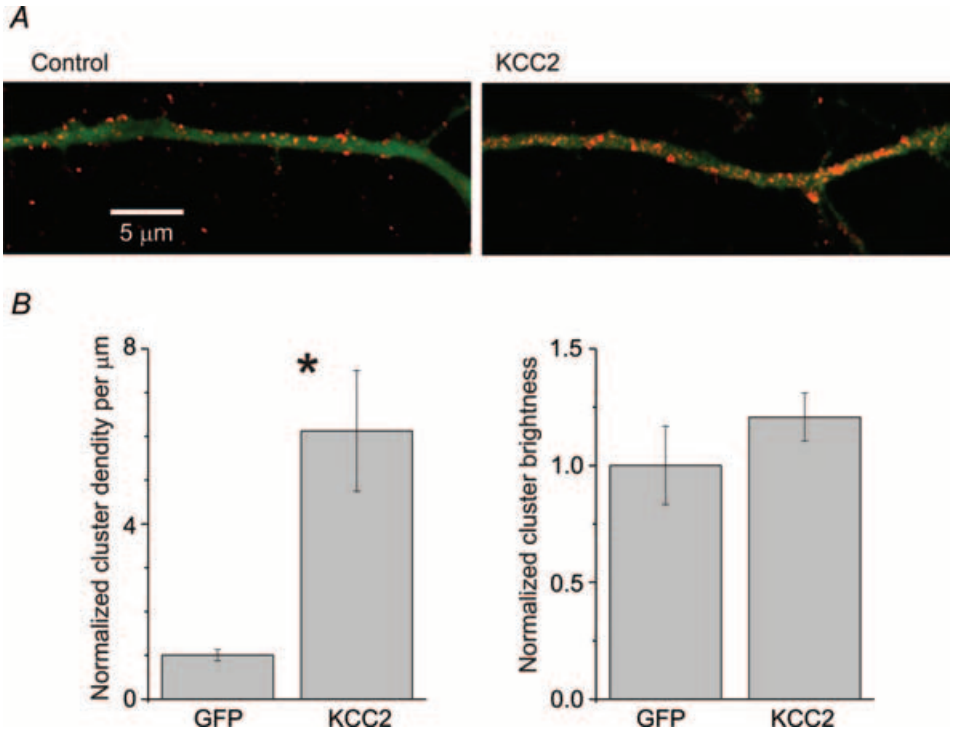

Figure 4. KCC2 increases density of $GABA_AR$ clusters
A, images of $GABA_AR$ clusters in dendrites of transfected neurones. Note the difference in density of clusters in GFP and KCC2 neurones. *B*, mean cluster density and brightness exhibited by $GABA_AR$ antibody labelling. Five experiments, at least 6 neurones per experiment. * $P < 0.05$.

of the amplitude) are higher than expected from the enhancement of GAD positive clusters (2.3-fold). This suggests that KCC2 might induce both the insertion of new $GABA_AR$ into existing synapses and formation of novel synaptic connections. A redistribution and aggregation of GABARs could be occurring through lateral membrane diffusion (Triller & Choquet, 2005) and/or internalization and reinsertion in the cell membrane (Kittler *et al.* 2000). The effect of KCC2 is specific since it only affects GABA synapses. Therefore, the developmental expression of KCC2 is not solely involved in the regulation of chloride and the shift of GABA action from excitatory to inhibitory: KCC2 might play an additional role in the regulation of synaptic plasticity.

The mechanisms that underlie the actions of KCC2 on GABAergic synapses are at present not known. KCC2 may act by modifying the excitability of transfected neurones by lowering the $[Cl^-]_i$, increasing the inhibitory function of GABA and modifying neuronal firing. This is, however, unlikely as similar manipulations of the resting membrane potential by overexpression of the inwardly rectifying potassium channel do not lead to a similar consequence (Burrone *et al.* 2002). Another possibility is that the intracellular chloride itself plays the role of second messenger and regulates in a concentration-dependent manner the level of expression of $GABA_AR$ in order to maintain chloride homeostasis. Finally, it is possible that by imposing an adult type of GABAergic operation, the transfection of KCC2 and the subsequent reduction of the intracellular chloride concentration reinforce GABAergic inhibitory function by expression of the new synapses to achieve the optimal balance between inhibitory and excitatory inputs.

Additional studies are needed to determine the physiological relevance and mechanism of the regulatory action of KCC2 and whether chloride or the cotransporter itself is responsible for the observed effects.

In conclusion, we have shown that the expression of exogenous KCC2 in immature neurones effectively decreases neuronal $[Cl^-]_i$. This leads to an increased formation of functional GABAergic synapses. This widens considerably the range of actions of KCC2 that is thus not restricted to regulation of ion homeostasis. Our results therefore open new vistas to study the physiological importance of the postnatal developmental expression of KCC2 and its role in the formation of the neuronal network.

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Supplemental material

The online version of this paper can be accessed at:
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http://jp.physoc.org/cgi/content/full/jphysiol.2005.089821/DC1
and contains supplemental material consisting of two figures:

Supplementary Fig. 1. Overexpression of the KCC2 does not modify the viability nor the morphology of neurons

Supplementary Fig. 2. AMPAR currents in transfected neurons

This material can also be found as part of the full-text HTML version available from http://www.blackwell-synergy.com