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ARTICLE *in* THE JOURNAL OF COMPARATIVE NEUROLOGY · FEBRUARY 2004

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Expression of the KCl Cotransporter KCC2 Parallels Neuronal Maturation and the Emergence of Low Intracellular Chloride

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ABSTRACT

Fast synaptic inhibition in the adult central nervous system (CNS) is mediated by GABA and glycine. During early development GABA acts as an excitatory neurotransmitter, which is deemed to be important for the maturation of the CNS. During development GABAergic responses undergo a switch from excitatory to inhibitory. This switch is correlated with upregulation of KCC2, the neuronal isoform of the potassium-chloride cotransporter family. KCC2 lowers the intraneuronal chloride concentration below its electrochemical equilibrium. KCC2 activity is thought to depend on phosphorylation by endogenous tyrosine kinases. Here, we analyzed the expression pattern of KCC2 during murine embryonic and postnatal development by in situ hybridization and Western blot analysis. KCC2 expression paralleled neuronal differentiation and preceded the decline of the GABA reversal potential (E_{GABA}) in spinal cord motoneurons and hippocampal pyramidal cells. The adult inhibitory response to GABA was established earlier in the spinal cord than in the hippocampus. Phosphorylated KCC2 protein was already present early in development when the functional GABA switch had not yet occurred. Thus, tyrosine-phosphorylation seems to be less important than the transcriptional upregulation of KCC2. J. Comp. Neurol. 468:57–64, 2004.

Indexing terms: GABA; K^+Cl^- cotransport; synaptic inhibition; expression; development

GABA and glycine are the main inhibitory neurotransmitters of the adult CNS but can also result in neuronal excitation in the immature CNS, as shown by Obata et al. (1978). Both mediate fast synaptic inhibition via $GABA_A$- and glycine-receptors, which are ligand-gated Cl^- channels. Therefore, the electrophysiological effect of GABA and glycine depends on the intracellular chloride concentration ($[Cl^-]_i$). The excitatory action of GABA in the immature CNS is believed to be important for the development of the nervous system, as it may exert a trophic action through membrane depolarization and an ensuing rise in intracellular Ca^{2+} (Cherubini et al., 1991; Yuste and Katz, 1991; LoTurco et al., 1995; Ben-Ari et al., 1997; Ben-Ari, 2002). GABA expression dominates differentiation in the embryonic rat neocortex and facilitates neurite outgrowth via $GABA_A$ autoreceptor chloride channels (Maric et al., 2001). Since glutamatergic synaptic transmission is first purely NMDA-receptor based, the depolarization by GABA may be necessary to provide the initial depolarization that is needed to relieve the voltage-dependent Mg^{2+}-block of NMDA-receptors (Ben-Ari et al., 1997).

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: HU 800/1-1 (C.A.H., T.J.J.); Grant number: JE 164/4-4 (C.A.H., T.J.J.).

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Received 26 March 2003; Revised 20 June 2003; Accepted 31 July 2003
DOI 10.1002/cne.10983
Published online the week of November 10, 2003 in Wiley InterScience (www.interscience.wiley.com).

In early stages the reversal potential for GABA (E_{GABA}) is more depolarized than the resting membrane potential, which indicates that in prenatal and early postnatal development the intracellular neuronal chloride concentration is above its electrochemical equilibrium (Owens et al., 1996; Ehrlich et al., 1999). $[Cl^-]_i$ can be changed by several transporters that couple the movement of Cl^- to those of K^+, Na^+, or HCO_3^-. The most important players are probably members of the cation-chloride cotransporter gene family. This family comprises $Na^+K^+2Cl^-$ cotransporters (NKCCs), which mainly raise $[Cl^-]_i$, and K^+Cl^- cotransporters (KCC1 through 4), which usually lower $[Cl^-]_i$. The resulting chloride gradient determines the response of a particular neuron to GABA. The importance of this gene family is demonstrated by human diseases like Bartter syndrome, Gitelman disease, or Andermann syndrome. The latter is caused by loss of function mutations in KCC3 and is characterized by a progressive polyneuropathy with or without agenesis of the corpus callosum (Howard et al., 2002). Disruption of KCC4 in mice leads to deafness and renal tubular acidosis (Boettger et al., 2002). The essential role of KCC2 in the developmental GABA switch was revealed in cultured hippocampal slices by oligo-antisense experiments (Rivera et al., 1999). We demonstrated that KCC2 is important for motor circuits at birth by a knockout mouse model (Hübner et al., 2001b).

The possible role of the excitatory GABA response for brain development and the link between KCC2 expression and the GABA response prompted us to investigate the time course of KCC2 expression in detail and to compare the distribution of KCC2 transcripts with the electrophysiological GABA response in the immature CNS.

MATERIALS AND METHODS

Animals

All studies were performed on C57BL/6 mice supplied by the central animal facility. Mice were killed by application of CO_2 as approved by the animal care and use committee.

In situ hybridization

Sense and antisense RNA probes labeled with (α-^{35}S)-UTP were generated with T7 polymerase from the cDNA corresponding to amino acids 63–229 according to the manufacturer's instructions (Ambion, Austin, TX). The probe was resuspended in a hybridization mix containing 50% formamide, 1 × Denhardt's solution, 4 × SSC, 5% dextran sulfate, 500 μg/ml yeast tRNA, and 10 mM DTT to a minimum of 2×10^6 cpm/ml. Ten μm cryosections were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4), acetylated, dehydrated, and subjected to in situ hybridization at 55°C for 18 hours. The slides were washed with 4 × SSC and subsequently treated with RNase A (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 20 μg/ml RNase A) for 30 min at 37°C. The slides were then washed and desalted. A 30-min high-stringency wash was performed in 0.1 × SSC at 55°C. Sections were dehydrated and exposed to high-resolution X-ray films (Kodak Biomax MR). Subsequently, the slides were dipped into Kodak NTB-2 nuclear track emulsion, developed for 3–6 weeks in Kodak Dektol, and stained with Giemsa (Sigma, St. Louis, MO). For whole-mount in situ hybridization KCC2 probes were labeled with digoxygenin-UTP (Boehringer Mannheim, Germany). Older developmental stages (E13.5 and E15.5) were cut into halves to facilitate the penetration of probes and antibodies.

Western blot

Spinal cords, brain stems, cerebella, hippocampi, and cortices of embryonal day (E) 15.5, E18.5, postnatal day (P) 3, P7, and P15 mice were dissected. The tissue was homogenized with a Teflon homogenizer in 0.154 M KCl on ice. Nuclei were removed by centrifugation at 1,000*g*. The supernatants were subsequently centrifuged at 132,000*g* for 30 min. The resulting membrane-enriched pellets were resuspended and protein content determined using the BCA method. Ten μg of total protein per lane were separated on reducing 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes with a tank transfer system. Western blots were probed with a rabbit antiserum against KCC2 (1:500), described previously (Hübner et al., 2001b) or rabbit anti-actin (Sigma) (1:2,000) as a loading control. Detection used a peroxidase coupled anti-IgG antibody (Roche Molecular Biochemicals, Nutley, NJ) and a chemiluminescence kit (Renaissance, DuPont, Rockville, MD).

Immunoprecipitation

Cortices of P3, P5, P9, and P30 mice were homogenized in ice-cold buffer containing 50 mM TRIS-HCl (pH 8.0), 300 nM okadaic acid, 10 mM Na orthovanadate, 10 mM NaF, 10 mM tetra-Na-pyrophosphate, 1.0% Nonidet P-40, and a protease inhibitor cocktail (Roche). The homogenate was centrifuged at 1,700*g* for 15 min, the supernatant collected, and the centrifugation repeated at 10,000*g* (30 min). The protein concentration of the resulting supernatants was determined using the BCA method and diluted with lysis buffer to a final protein concentration of 1 μg/μl. An aliquot of 1 ml was incubated with the antibody against KCC2 for 2 hours at 4°C. Protein-antibody-complexes were precipitated with 50 μl protein A sepharose (Pharmacia Biotech, Piscataway, NJ). The precipitates were washed three times with ice-cold PBS supplemented with protease- and phosphatase-inhibitors as described above. Finally, the sepharose beads were resuspended in 100 μl Laemmli buffer and incubated at 60°C for 10 min. Twenty μl of the eluted precipitates, 20 μl of the precipitation supernatants, and 20 μl of the protein lysates were separated on reducing 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes with a tank transfer system. Western blots were probed with a mouse monoclonal antibody against phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY, 1:1000). Detection was as described above.

Patch clamp studies

Spinal cord preparations were obtained as described previously (Suzue, 1984; Onimaru and Homma, 1987) and embedded in 1% agarose. The spinal cord was sliced horizontally (350 μm) with a vibratome (VT1000S, Leica). Hippocampal slices (350 μm) were prepared using standard methods. Slices were continuously superfused with 95% O_2/5% CO_2 gassed artificial cerebrospinal fluid (ASCF) that contained (in mM): 118 NaCl, 1 NaH_2PO_4, 25 $NaHCO_3$, 3 KCl, 1 $MgCl_2$, and 1.5 $CaCl_2$. Motoneurons or pyramidal cells were identified with DIC-infrared videomicroscopy by their location and their typical morphology.

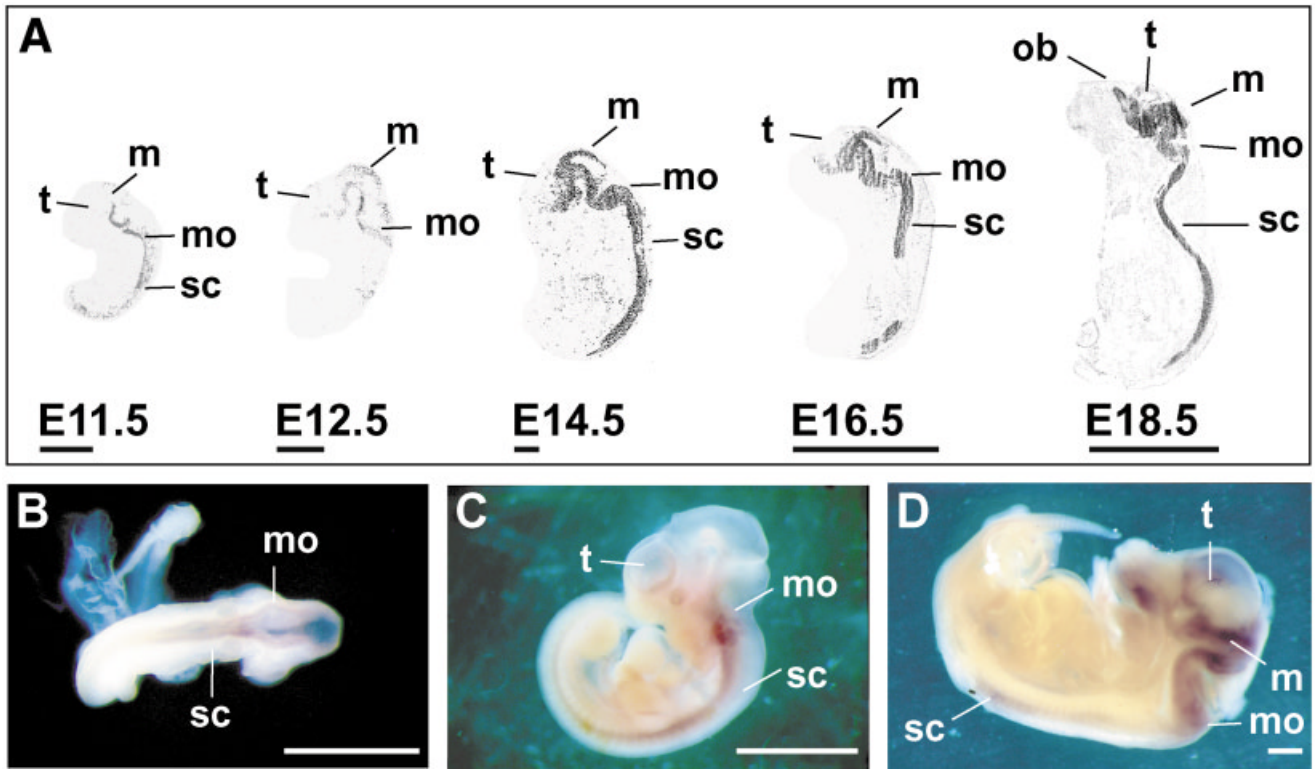

Fig. 1. Expression of KCC2 during embryonal development. **A:** Autoradiograms of parasagittal sections through mouse embryos at the stages indicated. The ventral surface is always to the left. **B–D:** Whole-mount in situ hybridizations at E10.5 (B), E11.5 (C), and E13.5 (D). Signals are restricted to the CNS throughout embryonal development and spread in a caudal-to-rostral fashion. Whole-mount signals appear violet. M, metencephalon; mo, medulla oblongata; ob, olfactory bulb; sc, spinal cord; t, telencephalon. Scale bars = 1 cm in A (E16.5 and E18.5); 1 mm in A (E11.5, E12.5, E14.5), B–D.

Electrodes (3–5 MΩ) were made form thick-wall borosilicate glass (Clark Electromedical, Reading, UK). Prior to recording, slices were bathed in gassed ASCF for at least 1 hour at 30°C. Perforated patch recordings were performed at room temperature. The progress of perforation was evaluated as a decrease of membrane resistance. Gramicidin (Sigma) was dissolved in DMSO (2 mg/ml) and diluted to a final concentration of 5–10 μg/ml with pipette solution (in mM): 140 KCl, 2 $MgCl_2$, 10 Hepes, 5 EGTA at pH 7.4). Chloride reversal potentials were determined using voltage ramps. The voltage clamp protocol consisted of stepping the membrane from a holding potential of −60 to +20 mV for 80 msec, then changing the voltage to −100 mV at a rate of 150 mV/sec. Recording was done with an Axopatch 200A amplifier and analyzed with pClamp 8.0 (Axon, Burlingame, CA). In all experiments 0.5 μM TTX (Tocris, St. Louis, MO) was added to block voltage-activated Na^+ currents. To determine E_{GABA}, 100 μM GABA was added to the bath.

Photograph production details

Low-power photomicrographs were taken using the Apozoom microscope (Wild Heerbrugg) and a Nikon F3 camera with an EPY 64T film (Kodak). For higher-resolution photomicrographs the Axiophot microscope and a photo system (Zeiss) was used with EPT 160T film. Photographs were scanned and processed using Adobe PhotoShop (San Jose, CA).

RESULTS

KCC2 transcripts are regulated during development

KCC2 transcripts were CNS-specific at all developmental stages of the mouse investigated. No transcripts were found in ganglia or other parts of the peripheral nervous system (Fig. 1). Detectable expression of KCC2 started at E10.5 and was restricted to the spinal cord and the immature brainstem, including the developing metencephalon (Fig. 1B). Around E10.5 the neuroepithelium of the neural tube starts to divide into the outer marginal, the intermediate, and the inner ependymal layer (Kaufman and Bard, 1999). No expression of KCC2 was observed in the neuroepithelium, where proliferation of neuronal precursor cells occurs (data not shown). Although KCC2 transcripts appeared in the basal telencephalon at E12.5 (Fig. 1A), the evolving hemispheres remained essentially unlabeled during embryonal development (Fig. 1A–D).

KCC2 transcripts parallel neuronal differentiation

KCC2 expression was restricted to neurons. Large fiber tracts like the corpus callosum or the anterior commissure remained unlabeled throughout development (Fig. 2J,K,N,O,R,S). At P15 the expression pattern was indistinguishable from adult mice (data not shown). Detailed analysis revealed that the distribution of KCC2 tran-

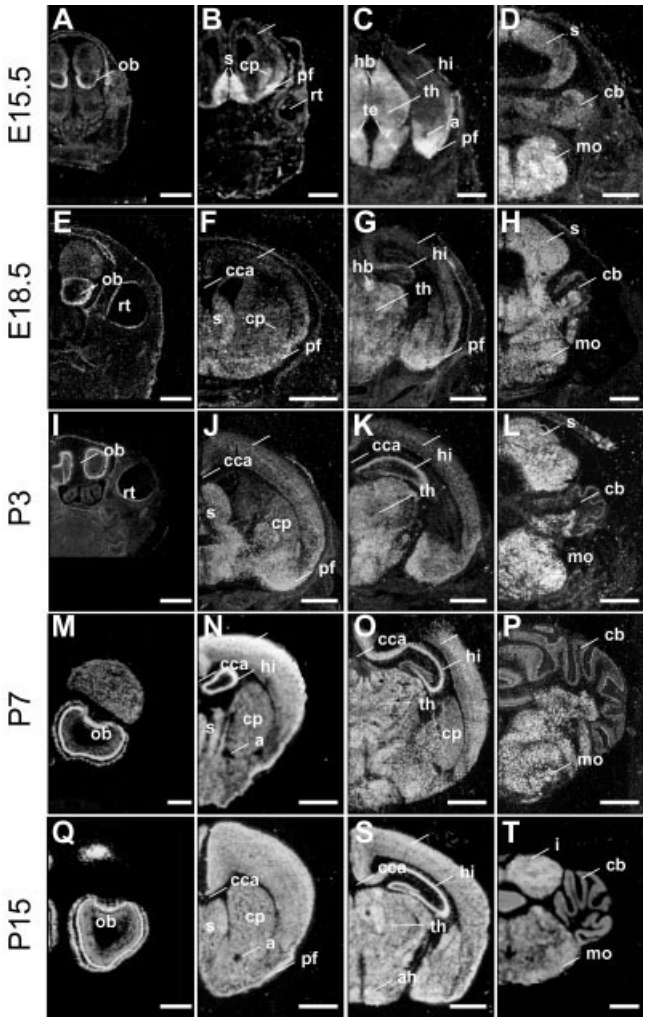

Fig. 2. KCC2 expression follows neuronal differentiation. Photoemulsion-dipped coronal sections through the head at E15.5, E18.5, P3, P7, and P15 are shown as darkfield photomicrographs. Photomicrographs of corresponding sectioning levels are disposed as columns. Signals (white) are detected only in areas of the brain where neuronal differentiation is taking place or is already complete. Fiber tracts do not exhibit any hybridization signals. ac, anterior commissure; ah, arcuate hypothalamic nucleus; cb, cerebellum; cc, cerebral cortex; cca, corpus callosum; cp, caudate putamen; hb, habenula; hi, hippocampus; if, inferior colliculus; mo, medulla oblongata; ob, olfactory bulb; pf, piriform cortex; rt, retina; s, septum; sc, superior colliculus; te, thalamic eminence; th, thalamus. Scale bars = 1 mm.

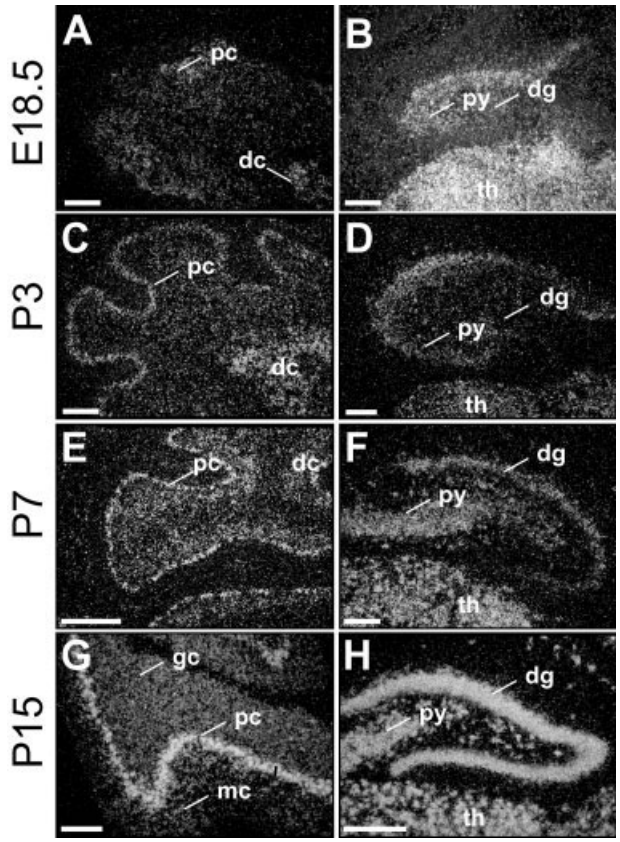

Fig. 3. KCC2 expression in the developing cerebellum and hippocampus. High-power magnifications of KCC2 expression in developing E18.5, P3, P7, P15 cerebella (**A,C,E,G**) and hippocampi (**B,D,F,H**) are shown. dc, deep cerebellar nuclei; dg, dentate gyrus; gc, granular layer; mc, molecular cell layer; pc, Purkinje cell layer; py, pyramidal cell layer; th, thalamus. Scale bars = 200 μm.

scripts closely paralleled the maturation of different brain regions (Figs. 2, 3).

Spinal cord and brainstem. Spinal cord motoneurons are among the first neurons to differentiate. We demonstrated previously that KCC2 transcripts are found in developing spinal cord motoneurons of the ventral horn and the medulla as early as E12.5 (Hübner et al., 2001b). At later stages the signal spread over the complete transverse section, including sensory nuclei, as shown at E15.5 for the medulla (Fig. 2D).

Cerebellum. The cerebellum develops around E11 as an evaginated thickening in the anterior part of the roof of the fourth ventricle, when Purkinje and granular cell precursors arise in the ventricular zone of the cerebellar neuroepithelium. Around E15, Purkinje cells start to differentiate, axon extensions are formed, and around birth dendrite formation and synaptogenesis takes place (Hatten et al., 1997). At E15.5 Purkinje cells already expressed KCC2 transcripts, as did cerebellar nuclei (Fig. 2D) and these signals increased towards E18.5 (Fig. 3A). Neurons of the granular and molecular layers differentiate later, when they start to migrate inward from the external cerebellar surface, a process occurring mainly during the first postnatal weeks (Paxinos, 1995). The first granular cells have finished their migration into the internal granular cell layer around P3. At P3, KCC2 transcripts could already be detected in granular cells (Figs. 2L, 3C). Hybridization signals of the Purkinje and granular cell layer further increased with ongoing maturation (Figs. 2L,P,T, 3E,G).

Thalamus. KCC2 expression also started early in the developing diencephalon. At E12 the thalamus, the largest of the diencephalic derivatives, starts to form. At E15.5, KCC2 transcripts were mainly found in the dorsolateral nuclei of the thalamus (Fig. 2C), where neuronal differentiation of thalamic neurons begins (Niimi et al., 1962). By contrast, the dorsomedian undifferentiated

parts were mainly unlabeled, but exhibited adult expression levels at E18.5 (Fig. 2G).

Telencephalon. The olfactory bulb is the first region of the telencephalon to differentiate. It has a characteristic laminar organization and two main types of neurons, projection neurons (mitral and tufted cells) and interneurons (granule cells). The oldest neurons are the mitral cells, which originate around E11. At E15, mitral cells already expressed KCC2 (Fig. 2A). The hybridization signals increased with further development. Tufted cells appear shortly later as mitral cells, whereas granule cells are mainly generated around birth and thereafter. They form a layer of increasing thickness beneath the mitral cell layer (Fig. 2M,Q), which expressed KCC2 at E18.5 (Fig. 2E,I). Some are interspersed between the glomeruli, which also showed intensive labeling for KCC2 (Fig. 2I,M,Q).

In the striatum, differentiation starts at E14 in the older ventrolateral parts with the incipient generation of medium-sized spiny neurons. At E15.5, signals were only found in these differentiated parts of the telencephalon (Fig. 2B). KCC2 expression also increased in parallel to the differentiation of cortical neurons (Fig. 2B,C,F,G,J,K). The roof of the telencephalic vesicles begins to stratify at E12, when cells from the mantle layer migrate into the overlying marginal zone to form the neopallial cortex, which will become the outer gray layer of the cerebral hemispheres (Kaufman and Bard, 1999). The basal older parts, such as the piriform cortex, finish their differentiation before the differentiation of the cortical plate is initiated and thus expressed KCC2 already at E15.5 (Fig. 2B,C). Signals spread over the complete neocortex during further development and reached adult levels during the first postnatal weeks (Fig. 2J,K,M–Q,R,S). Two interlocked C-shaped layers of the cortex form the hippocampus: the pyramidal cells of Ammon's horn (CA1–3) and the granule cells of the dentate gyrus. The oldest pyramidal cells are located in the CA3 field, where signals could be detected at E15.5 (Fig. 2C). KCC2 expression subsequently spread to CA1 at E18.5 (Fig. 3B). Expression of KCC2 was detectable in the granule cells of the dentate gyrus (Fig. 3F) during the first postnatal week, when the majority arise (Paxinos, 1995). Expression reached adult levels during the second postnatal week.

KCC2 protein levels in the developing brain

Western blots of equal protein amounts of dissected spinal cords, brainstems, cerebella, hippocampi, and cortices of various developmental stages were probed with an antiserum against KCC2. At E15.5 intense signals were detected in the caudal parts of the CNS (spinal cord and brainstem) and did not further increase beyond P3 (Fig. 4). KCC2 was detectable in E15.5 cerebellum and cortex preparations, whereas in the hippocampus significant protein amounts were detectable not earlier than perinatally. A drastic increase of KCC2 protein in the more rostral brain portions occurred in the first two postnatal weeks. Although this Western blot approach does not allow a subregional analysis, it demonstrates that the time course of protein expression closely paralleled the detection of KCC2 transcripts by in situ hybridization.

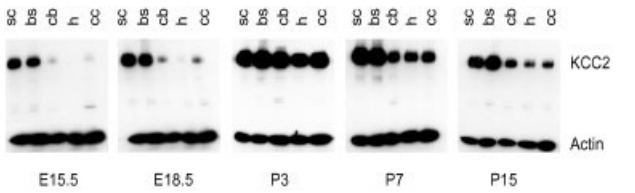

Fig. 4. The expression of the KCC2 protein increases in distinct brain areas during ongoing neuronal differentiation. Protein lysates were prepared from spinal cord (sc), brainstem (bs), cerebellum (cb), hippocampus (hc), and cortex (cc) at the stages indicated and constant amounts of protein subjected to Western blot analysis with an antiserum against KCC2 or actin, for normalization. Signals were detected using a chemoluminescence kit.

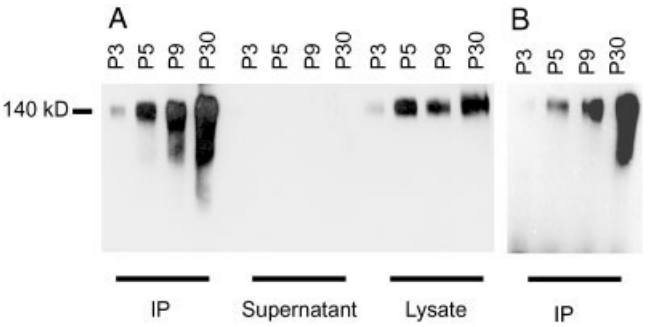

Fig. 5. Tyrosine phosphorylation of KCC2 during development. KCC2 was immunoprecipitated with a KCC2 antiserum from cortex preparations of P3, P5, P9, and P30 mice. The precipitate (IP) was subjected to Western analysis with the KCC2 antibody (**A**) or with an phosphotyrosine-antibody (**B**). Absence of KCC2 in the immunoprecipitation supernatants demonstrated that immunoprecipitation was quantitative (A). Increasing KCC2 levels in cortex lysates was paralleled by increasing levels of phosphorylated KCC2 (B).

Phosphorylation of KCC2 during development

As the transport activity of KCC2 might be regulated by posttranslational mechanisms, the maturation of postsynaptic inhibition does not necessarily correlate with the expression of the KCC2 protein. Indeed, it is known that cation-chloride cotransporters are regulated by phosphorylation/dephosphorylation events (Russell, 2000). For KCC2, it has been suggested that it is activated by endogenous protein tyrosine kinases (Kelsch et al., 2001). We immunoprecipitated KCC2 from cortex protein lysates of P3, P5, P9, and P30 mice with an antibody against KCC2 and subjected the resulting precipitates to Western blot analysis. The amount of precipitated protein detected with the KCC2 antibody increased from P3 to P30 (Fig. 5A). The absence of a band corresponding to KCC2 in Western blots from equal volumes of supernatants after precipitation compared to protein lysates before precipitation demonstrated that the majority of the KCC2 protein was indeed precipitated. When the blot was probed with a phosphotyrosine antibody, only a faint band corresponding to KCC2 could be observed at P3. The amount of phosphorylated KCC2 increased with the amount of KCC2 precipitated (Fig. 5B).

Changes of E_{GABA} during development

The time course of the GABA reversal potential (E_{GABA}) during mouse development was determined at P1, P3, P8,

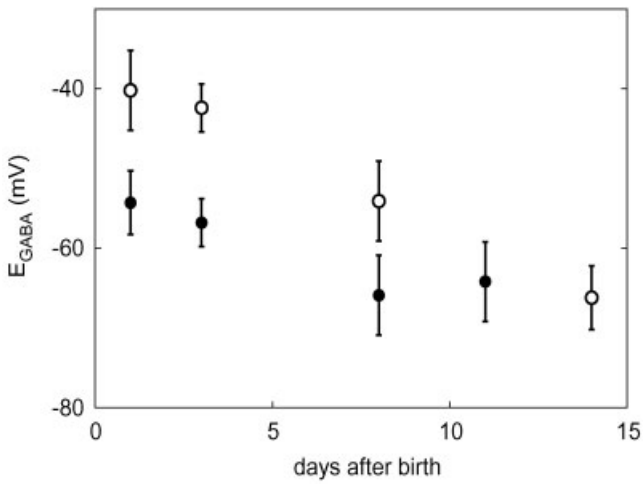

Fig. 6. E_{GABA} is shown for hippocampal pyramidal neurons (open symbols) at P1, P3, P8, and P14 ($n \geq 6$, error = SEM) and motoneurons (closed symbols) at P1, P3, P8, and P11 ($n \geq 5$, error = SEM). E_{GABA} is significantly higher for pyramidal neurons during the first days of development. At P8, motoneurons hyperpolarized in the presence of GABA, whereas hippocampal neurons still showed a slight depolarization. At P14 the pyramidal neurons also hyperpolarized in the presence of GABA.

and P14 for hippocampal pyramidal neurons (n = 6 from three animals for each time point) and at P1, P3, P8, and P11 for motoneurons (n = 5 from two animals for each time point) (Fig. 6). The resting potential did not change significantly during this time. The resting potentials of hippocampal pyramidal cells and spinal cord motoneurons did not differ significantly either. In the hippocampus the resting potential was -63.4 ± 2.1 mV (n = 6) at P1 compared to -64.8 ± 1.8 at P14 (n = 8). For motoneurons the resting potential was -65.1 ± 3.2 mV at P1 (n = 6) and -63.9 ± 2.3 mV at P11 (n = 7). During the first days of postnatal development E_{GABA} was significantly higher for pyramidal neurons than for motoneurons. At P8 motoneurons hyperpolarized in the presence of GABA, whereas hippocampal neurons still slightly depolarized. E_{GABA} did not decrease further in spinal cord motoneurons, as it was not significantly changed between P8 and P11. In hippocampal pyramidal neurons a GABA-induced hyperpolarization was only observed at P14.

DISCUSSION

Excitatory GABA and glycine responses are thought to play a central role in the ontogeny of emerging neuronal networks. In early postnatal hippocampal pyramidal neurons GABA-mediated responses are depolarizing (Ben-Ari et al., 1989). A gradual shift towards a hyperpolarizing response is established in the second postnatal week (Kakazu et al., 1999). The role of a furosemide-sensitive chloride transport mechanism for the maintenance of E_{GABA} in hippocampal pyramidal cells has been known for many years (Misgeld et al., 1986). When KCC2 was cloned in 1996, it was suggested that this protein might be the driving force for the developmental E_{GABA} reversal (Payne et al., 1996). For the developing rat hippocampus this hypothesis was further substantiated in an elegant in vitro study: The switch from excitatory to inhibitory GABA response, which normally occurs around P9, could be inhibited by cultivation of hippocampal slices in the presence of KCC2 antisense oligonucleotides (Rivera et al., 1999). KCC2 knockout mice died immediately after birth due to major motor deficits and respiratory failure (Hübner et al., 2001b), thus demonstrating that KCC2 is crucial in the spinal cord and the medulla around birth. In another mouse model a small amount (≈5%) of protein remained because of alternative splicing. The homozygous mice developed spasticity and had frequent generalized seizures, until they died in the third postnatal week (Woo et al., 2002).

Here we give a detailed survey of the expression of KCC2 in the developing mouse. Throughout development we found a strict neuronal expression of KCC2, as noted previously (Lu et al., 1999). KCC2 expression was detectable as early as E10.5, the time at which the first motoneurons differentiate in the neural tube (Fig. 1B). During further development, KCC2 expression was not restricted to neurons residing in the ventral horn of the spinal cord, but also included dorsal horn neurons and sensory nuclei. At birth, adult protein levels were established in the spinal cord and the brainstem. This time course of expression fits well with the phenotype of KCC2 knockout mice, which have an abnormal muscle tone and a severe movement disorder due to the defect of the inhibitory control of motoneurons at birth.

Neuronal differentiation describes the process that leads to the formation of mature neurons from neuronal precursor cells. The individual cell's fate is determined by the signals it receives from its immediate environment and by the profile of genes it expresses as a consequence of its developmental history. As a general rule, KCC2 expression started as soon as a particular immature neuron had reached its final position in the cerebellum or cortex. In the cerebellum, KCC2 transcripts were already detected at E12, a developmental stage where the formation of axon extensions of cerebellar neurons occurs (Hatten et al., 1997). In the basal older parts of the cortex, the differentiation starts around E15.5, when intense signals for KCC2 transcripts were detectable in the piriform cortex. Signals spread over the entire neocortex during further development and reached adult levels in the first postnatal week, as shown for the rat neocortex (Clayton et al., 1998). Adult protein levels were established in the second postnatal week (Fig. 4). A similar situation was found in the hippocampus, where expression of KCC2 started in the CA3 region around E15.5, where the first pyramidal cells reside. The signals subsequently spread to CA1 at E18.5. In the dentate gyrus, KCC2 transcripts were detectable not earlier than at the end of the first postnatal week, when the majority of its granule cells are generated. Adult hippocampal KCC2 protein levels occurred around P15 (Fig. 4).

In the rat hippocampus, the transition from GABA-induced Cl^--dependent depolarizations to hyperpolarizations occurs during the second week after birth (Ben-Ari et al., 1989; Zhang et al., 1990). However, a depolarizing GABA response does not necessarily entail a GABA-induced excitation of the neuron. GABA, although still depolarizing, can have an inhibitory effect on the neuron due to the increased conductance that can shunt excitatory synaptic currents (Gao and Ziskind-Conhaim, 1995) or by a voltage-dependent inactivation of Na^+ channels

(Zhang and Jackson, 1993). The decline of the GABA response of spinal cord motoneurons in rats was described to occur during the last week of gestation (Wu et al., 1992), which is ≈23 days in rats compared to ≈19 days in mice. In spinal cord motoneurons of mice E_{GABA} were also larger than in age-matched hippocampal preparations until ≈P14 (Fig. 6). Increasing hippocampal or spinal motoneuron KCC2 expression was paralleled by a decline of E_{GABA}. Finally, E_{GABA} of motoneurons and hippocampal neurons did not differ significantly any more. Although GABA-induced currents of independent measurements at a given time point were consistent, it has to be considered that hippocampal pyramidal neurons are very heterogeneous, as well as their response to GABA, in particular, at early developmental stages. Therefore, our recordings may not be representative for all hippocampal pyramidal neurons.

Our data raise the question of whether the GABA switch is the result of the expression level of KCC2 or whether an activation of the protein has to occur. The activity of members of the cation-chloride cotransporter family is regulated by phosphorylation or dephosphorylation events (Russell, 2000). The regulation of cation cotransporters by phosphorylation is very complex, as recently outlined for the Na^+-K^+-$2Cl^-$ cotransporter (Flatman, 2002). Protein–protein interactions either within the plasma membrane or with elements of the cytoskeleton seem to be important for transport. Conditions can be found where transport rate changes independently of cotransporter phosphorylation. KCC2 differs from KCC1 in a C-terminal tyrosine consensus site (Payne, 1997), but this site was not phosphorylated in the *Xenopus* oocyte expression system (Strange et al., 2000). Evidence that KCC2 activation may depend on tyrosine phosphorylation came from cultured hippocampal neurons (Kelsch et al., 2001): Neurons initially expressed an inactive KCC2 protein, which became activated during subsequent maturation. Activation of KCC2 in immature neurons was induced by IGF-1 or a Src kinase, whereas membrane-permeable protein tyrosine kinase inhibitors deactivated KCC2. It was concluded that activation of K^+Cl^- cotransporter function by endogenous protein tyrosine kinases mediates the developmental switch of GABAergic excitatory responses to hyperpolarizing inhibition. Immunoprecipitation of KCC2 from developing murine cortex demonstrated tyrosine phosphorylation of the protein at early stages of development, when the expression of KCC2 is rather low (Fig. 5). Thus, tyrosine-phosphorylation seems to be less important than the transcriptional upregulation of KCC2. Serine-threonine kinases like the stress-related SPAK and OSR1 kinases, which interact with cation chloride cotransporters (Piechotta et al., 2002), may also influence transport activity.

Other members of the K^+Cl^- cotransporter family can contribute to set neuronal $[Cl^-]_i$. NKCC1, which raises $[Cl^-]_i$, is highly expressed in some neurons early in development (Clayton et al., 1998), and was therefore postulated to set $[Cl^-]_i$ at a high level at an early stage. In the adult mouse brain neuronal NKCC1 expression is low in most neurons (Hübner et al., 2001a). Surprisingly, NKCC1-deficient mice have no major neurological deficit apart from an impairment of sensory perception (Sung et al., 2000). However, a neurological defect may be masked by the severe vestibular dysfunction of homozygous mutants resulting from impaired endolymph secretion that leads to deafness (Delpire et al., 1999; Flagella et al., 1999; Pace et al., 2000).

All components affecting synaptic inhibition by modulating $[Cl^-]_i$ and the $GABA_A$- or glycine-receptors are candidate genes for human neurological disorders. In fact, mutations in the γ2 or the α1 subunit of the $GABA_A$ receptor were demonstrated in epilepsy (Baulac et al., 2001; Wallace et al., 2001; Cossette et al., 2002). Mutations of the α1 glycine-receptor cause autosomal dominant hyperekplexia (startle disease) (Shiang et al., 1993), which is characterized by marked muscular hypertonia in infancy and a grossly exaggerated response to unexpected stimuli. As the electrophysiological effect of GABA and glycine depends on the intracellular chloride concentration, which is influenced by ion-transporting proteins like chloride channels and cation-chloride cotransporters, it is tempting to speculate that mutations of these genes cause severe neurological defects in humans. Indeed, mutations of the voltage-gated chloride channel ClC-2 might be associated with epilepsy (Haug et al., 2003), though no increased seizure susceptibility was found in a ClC-2 knockout mouse model (Bösl et al., 2001). KCC3 mutations were demonstrated in a severe form of polyneuropathy (Howard et al., 2002), but the underlying mechanisms are still unclear. A similar KCC2 expression pattern in humans with the early upregulation in the older parts, like spinal cord and brainstem, might explain why $GABA_A$ receptor agonists are useful in neonatal brainstem epilepsies but work for epilepsies involving the rostral parts of the CNS only later in life.

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