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Developmental localization of potassium chloride co-transporter 2 (KCC2) in granule cells of the early postnatal mouse cerebellum with special reference to the synapse formation

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ABSTRACT

In the adult central nervous system (CNS), γ -amino butyric acid (GABA) is the predominant inhibitory neurotransmitter, mediating the hyperpolarization of membrane potential and regulating the glutamatergic activity. In the immature CNS, on the other hand, GABA mediates depolarization and is involved in controlling morphogenesis. This developmental shift in GABA actions from depolarization to hyperpolarization occurs as a result of decreasing the intracellular chloride ion (Cl^-) concentration ($[\text{Cl}^-]_i$) which is regulated by the K^+ - Cl^- co-transporter 2 (KCC2). To clarify the time-course of changes in the GABA actions during development, we examined the developmental localization of the KCC2 in the granule cells of the postnatal mouse cerebellum using specific antibodies against KCC2. The granule cell precursors and migrating granule cells were devoid of immunoreactivity against KCC2 antibodies. At postnatal day 3 (P3), the KCC2-immunolabeling was negative in the internal granular layer, although synaptophysin-positive mossy fiber terminals were detected. At P5, we first detected the KCC2-immunolabeling at the somata of granule cells and their dendrites before granule cells received inhibitory input from Golgi cells. Almost all KCC2-positive dendrites (more than 98%) attached to and formed synapses with mossy fiber terminals. As development proceeded, the number of KCC2-positive granule cells increased, and all granule cells

became positive by P21. These results suggested that GABAergic transmission on granule cells might shift from excitation to inhibition after the synapse formation, and the excitatory synapse-formation and related factors might be the triggers for the expression and localization of the KCC2 in the granule cells. Furthermore, it was also suggested that formation of the GABAergic synapses and GABAergic transmission were not necessary for the KCC2-expression in the mouse cerebellar granule cells *in vivo*.

Key Words: NKCC1, GABA, synaptophysin, mossy fiber, synaptic glomerulus

Abbreviations

ABC: avidin-biotin-peroxidase complex

Ca²⁺: calcium ion

Cl⁻: chloride ion

[Cl⁻]_i: intracellular chloride ion concentration

CNS: central nervous system

FITC: fluoresceine isothiocyanate

GABA: γ -amino butyric acid

GAT: GABA transporter

K⁺: potassium ion

KCC: potassium chloride co-transporter

kD: kilo Dalton

KLH: keyhole limpet hemocyanin

Na⁺: sodium ion

NKCC: sodium potassium chloride co-transporter

NMDA: N-methyl D-aspartate

P: postnatal day

PB: phosphate buffer

VDCC: voltage dependent calcium channel

INTRODUCTION

In the adult central nervous system (CNS), γ -amino butyric acid (GABA) is the predominant neurotransmitter, mediating fast inhibitory synaptic transmission and regulating the excitatory activity of neurons (Olsen and Tobin, 1990, Macdonald and Olsen, 1994). During brain development, on the other hand, GABA is an excitatory transmitter, serves as a trophic factor and is involved in controlling morphogenesis, such as regulating cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death (Ben-Ari, 2002, McCarthy et al., 2002, Owens and Kriegstein, 2002, Represa and Ben-Ari, 2005).

These developmental changes in the actions of GABA occur as a result of a negative shift in the chloride ion (Cl^-) reversal potential, which is mainly regulated by two different chloride co-transporter families, $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ co-transporters (NKCCs) and $\text{K}^+ - \text{Cl}^-$ co-transporters (KCCs) (Ben-Ari, 2002, Owens and Kriegstein, 2002, Payne et al., 2003). In two NKCCs, only NKCC1 is detected in the mammalian CNS. The NKCC1 is dominantly expressed in the immature brain and acts to maintain a high intracellular Cl^- concentration ($[\text{Cl}^-]_i$). Under the high $[\text{Cl}^-]_i$, the activation of ionotropic GABA receptors mediates the depolarization of the membrane potential, and GABA acts as an excitatory transmitter. As development proceeds, $[\text{Cl}^-]_i$ is gradually decreased by the KCCs-expression, and GABA becomes an inhibitory transmitter. Among four isoforms of

KCCs, KCC1 and KCC2 are expressed in the CNS (Kanaka et al., 2001). The KCC1 is ubiquitously localized in the mammalian tissue (Gillen et al., 1996), and its expression-patterns do not developmentally change in both cerebral cortex (Wang et al., 2002) and cerebellum (Mikawa et al., 2002). Taken together, it is considered that KCC1 is the “housekeeping” isoform involved in cell volume regulation (Gillen et al., 1996, Williams et al., 1999). In contrast, the KCC2 is specifically expressed in neurons, and abundant in the mature CNS (Williams et al., 1999, Kanaka et al., 2001). Changes in the levels of KCC2 correlate with the modification of GABA actions. The transfection of KCC2 into hippocampal neurons converts the actions of GABA from excitatory to inhibitory. GABA is excitatory in the KCC2-knockout mice (Hubner et al., 2001, Ben-Ari, 2002, Payne et al., 2003). These results indicate that not KCC1 but KCC2 plays a key role in decreasing $[Cl^-]_i$, the molecular switch from NKCC1 to KCC2 drives the Cl^- influx in response to ionotropic GABA receptor activation and expression of the KCC2 might be the beginning of the GABAergic inhibition.

To clarify the time-course of changes in the GABA actions during development, we investigated the developmental localization of the KCC2 in the cerebellar granule cells by immunohistochemical staining. Although developmental expression of the KCC2 mRNA had previously been investigated using *in situ* hybridization (Mikawa et al., 2002),

little is known about (1) when granule cells start to express the KCC2 during development, and (2) where the KCC2-protein is localized in the granule cells.

We found that KCC2 was first observed at the granule cell dendrites, which formed synapses with synaptophysin-positive mossy fiber terminals in the internal granular layer, at postnatal day 5 (P5). The KCC2-positive granule cells increased in number during development and all granule cells became positive by P21. The results suggest that GABA might mediate depolarization of the granule cell membrane potential before synapse formation, and play important roles in development and maturation of the granule cells. After the synapse formation, GABA acts as an inhibitory neurotransmitter for the cerebellar granule cells. Furthermore, the results also suggest that the synapse-formation with mossy fiber terminals might be one of the triggers for the expression and localization of the KCC2 in the granule cells.

EXPERIMENTAL PROCEDURES

Animals

We examined mice from the C57Bl/6CrSlc mice of postnatal days 0 (P0), P3, P5, 7, 10, 12, 14, 21 and 90 (as an adult). At each age, at least five mice (three for light microscopic analysis, two for electron microscopic analysis) were sacrificed for

immunohistochemistry.

Establishment of the KCC2 antibody

A cystein residue was introduced at the amino terminal of the synthesized peptide, PVS SEG IKD FFS MKP EWE NLN, which are amino acid residues 1022-1042 of the mouse KCC2 (AF332064.1, GI:14193695). The peptide was conjugated with keyhole limpet hemocyanin (KLH). Rabbits and guinea pigs were immunized at two week-intervals by the subcutaneous injection of conjugates (500 μ g of peptide/rabbit, 150 μ g of peptide/guinea pig), which were emulsified in equal amounts of complete Freund's adjuvant. Two weeks after the last injection, IgG fractions were purified from the antiserum using a Protein G Sepharose column (Pharmacia Biotech, Sweden). The specific IgG fraction for synthetic peptide for KCC2 was affinity-purified with a CNBr-activated Sepharose 4B column (Pharmacia Biotech, Sweden). The specificity of the KCC2 antibody was checked by Western blot analysis, as described in a previous report (Takayama and Inoue, 2003).

Tissue preparation

Under deep ether anesthesia, the mice were fixed by transcardial perfusion with

4% paraformaldehyde in a phosphate buffer (PB, 0.1M pH 7.4). The brains were removed from the skulls and were immersed in the same fixative overnight at 4°C. For light microscopic analysis, the cerebella were cryoprotected with 30% sucrose in PB overnight, then cut into sagittal sections at a thickness of 20µm with a cryostat. The sections were mounted on gelatin-coated glass slides. For electron microscopic analysis, cerebella were cut into sagittal sections at a thickness of 100µm with a microslicer (Dosaka, Kyoto, Japan).

Immunohistochemistry for KCC2

Sections on the glass slides were treated as follows; with methanol containing 0.3% H₂O₂ for 30 minutes, PB for 10 minutes, 3% normal goat serum in PB for one hour, and a rabbit (1µg/ml) or guinea pig (1.5µg/ml) antibody against KCC2 overnight at room temperature. After rinsing three times with PB for 15 minutes, sections were visualized using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) or the reaction with Cy3TM-conjugated anti-rabbit IgG (diluted 1:400, Jackson ImmunoResearch, USA) for two hours at room temperature.

For the double staining of KCC2 and synaptophysin, sections on glass slides were reacted with a mixed solution of guinea pig KCC2 antibody (1.5µg/ml) and rabbit

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synaptophysin antibody (1:5, Zymed, Cat No. 08-1130, USA) overnight at room temperature, and visualized by the incubation with a mixed solution of Cy3TM-conjugated anti-guinea pig IgG (1:400) and fluoresceine isothiocyanate (FITC)-conjugated anti rabbit IgG. (1:100, Jackson ImmunoResearch, USA) for two hours at room temperature.

For the electron microscopic analysis, floating sections prepared with a microslicer were treated with 3% normal goat serum for one hour, and reacted with the rabbit KCC2 antibody (1µg/ml) overnight at room temperature. The sections were visualized using the ABC method and post-fixed with 1% glutaraldehyde in PB for 30 minutes at 4°C. After the fixation by 1% OsO₄ in PB for 2 hours at 4°C, the sections were stained with 2% uranyl acetate aqueous solution overnight at room temperature, and embedded in epoxy resin as previously reported (Takayama and Inoue, 2004b). Ultra thin sections at the culmen of the vermis were observed under an electron microscope, H-7100 (Hitachi Japan).

These experiments were permitted by the Animal Care and Use committee of Hokkaido University School of Medicine.

RESULTS

Immunohistochemical localization of KCC2 in the adult mouse cerebellar cortex

The specificity of the rabbit and guinea pig KCC2 antibodies was examined by immunoblot analysis and immunohistochemistry. Each immunoblot showed a major band around the predicted molecular weight (140kD), and the band was abolished by addition of the peptide for immunization into the primary antibody solution (Fig. 1A).

The immunohistochemical staining with rabbit KCC2-antibody showed that the immunolabeling was observed in the all three layers (Fig. 1B). In the molecular layer and Purkinje cell layer, dense immunolabeling was localized at the dendrites (black arrows) and cell bodies of stellate, basket and Purkinje cells (Fig. 1B). In addition, the neuropil region was moderately labeled. In the granular layer, the KCC2 was localized at the granule cell somata. In the synaptic glomeruli, granule cell dendrites were detected as ring-shaped profiles (arrowheads in Fig. 1B). This immunolabeling was completely abolished by the pre-incubation of the primary antibody with synthetic peptide (Fig. 1C). Confocal microscopic analysis in the granular layer revealed that all granule cells were positive for the KCC2 antibody, and their dendrites around mossy fiber terminals were more densely stained in the synaptic glomeruli (white arrows in Fig. 1D). The immunolabeling pattern with guinea pig antibody was identical to that with rabbit antibody (Fig. 1E), and the labeling was also abolished by the preincubation with synthetic peptide (Fig. 1F).

Electron microscopic analysis revealed that in the granular layer, the KCC2 was

localized within the dendrites (Gd) and cell bodies of granule cells in the granular layer (Fig. 1G, H). The immunolabeling was mainly localized near the cell membrane (black arrows in Fig. 1G), while the cytoplasm and organelles, including the mitochondria and endoplasmic reticulum, were negative (Fig. 1G). Both proximal (near the somata) and distal (in the synaptic glomeruli) part of the dendrites were labeled as well as cell bodies. Many KCC2-positive digits of dendrites often formed synapses with mossy fiber terminals in the synaptic glomeruli (Fig. 1H), while ascending axons and parallel fibers were negative.

These results indicated that the KCC2 was localized at the dendrites and cell bodies of granule cells, in particular the cell membrane. This was in agreement with the results by *in situ* hybridization histochemistry, and the similarity of these labeling patterns by immunohistochemistry in the previous reports suggests that both rabbit and guinea pig antibodies specifically bind to the KCC2 in the mouse brain (Payne et al., 1996, Williams et al., 1999, Kanaka et al., 2001, Mikawa et al., 2002).

Developmental changes in localization of the KCC2 in the cerebellar cortex

We examined the immunohistochemical localization of the KCC2 in the developing mouse cerebellar cortex using the ABC method. At P0 (Fig. 2A) and P3 (Fig.

2B), KCC2-immunolabeling was observed in the developing molecular layer and Purkinje cell layer, whereas the external granular layer and internal granular layer were negative, except for the Golgi cells (Go in Fig. 2A). At P5, the immunolabeling was observed near the white matter (arrows in Fig. 2C). In this stage, few granule cell somata and dendrites were labeled, but the majority of the granule cells were negative. The KCC2-positive granule cells (arrows) increased in number at P7 (Fig. 2D). At P10, KCC2-positive granule cells were distributed throughout the internal granular layer, and synaptic glomeruli were clearly discernible as ring-shaped profiles (arrowheads) in the internal granular layer (Fig. 2E). From P12 through P21, the KCC2-positive granule cells increased in number and the majority of the granule cells were positive by P21 (Fig. 2F-H). The KCC-immunolabeling pattern at P21 was almost the same as that in adults (Fig. 2I). Throughout development, the external granular layer was negative (Fig. 2A-G).

Electron microscopic localization of the KCC2 in the migrating granule cells

To detect the expression of the KCC2 in the migrating granule cells between KCC2-positive stellate, basket and Purkinje cells, we examined the electron microscopic localization of the KCC2 in the cerebellar cortex at P10, when the granule cells massively migrated from the external granular layer towards the internal granular layer.

At the bottom of the external granular layer, the KCC2 was negative (asterisks) in the granule cells (EGr), which were tangentially migrating (Fig. 3A). In the molecular layer, migrating granule cells (GC1 and GC2 in Fig. 3A, GC3 in Fig. 3B) were also negative, although dendrites and cell bodies of stellate, basket and Purkinje cells were clearly labeled. When granule cells went through the Purkinje cell layer, granule cells did not contain the KCC2 (GC4 in Fig. 3C). Beneath the Purkinje cell layer, granule cells, whose nuclei were quite similar to those of migrating granule cells in the molecular layer, were also negative (GC5 and 6 in Fig. 3D). We could not find any immunolabeling in the migrating granule cells, including the perikarya (asterisks), leading processes (lp), trailing processes (tp) (Rakic, 1971, Rakic and Komuro, 1995, Komuro and Yacubova, 2003) and parallel fibers (PF).

KCC2-localization and mossy fiber terminal development

Our results indicated that granule cells do not immediately express KCC2 when they enter the internal granular layer. In the internal granular layer, granule cells migrate slightly and finally begin their maturation, such as extending their dendrites and forming synapses (Rakic, 1971, Rakic and Komuro, 1995, Komuro and Yacubova, 2003). To reveal the spatial and temporal relationship between KCC2-localization and synapse formation

with mossy fibers, we performed the double staining of the KCC2 and synaptophysin as a marker of mossy fiber terminals in the early postnatal internal granular layer.

At P3, the KCC2-immunolabeling was not observed in the internal granular layer, but small synaptophysin-positive dots (green), which were immature mossy fiber terminals, were already observed (Fig. 4A). At P5, synaptophysin-positive dots, which were developing mossy fiber terminals, became bigger than that at P3 (Fig. 4B). The KCC2-immunolabeling (red) was detected in both somata (white arrows) and dendrites of a few granule cells in the deeper half of the internal granular layer, but the majority of the granule cells were KCC2-negative. In all five five-day-old mice, which we investigated, more than 98% of KCC2-positive dendrites attached to synaptophysin-positive dots, which were mossy fiber terminals (arrowheads). In addition, we also observed small synaptophysin-positive dots which did not attach to the KCC2-positive dendrites or cell bodies (small diamonds in Fig. 4B). At P7, the number of KCC2-positive granule cells and their dendrites increased (Fig. 4C). The synaptophysin-positive dots became much bigger than that at P5, and attached to and were often surrounded by the distal part of the KCC2-positive dendrites (arrowheads in Fig. 4C). At P10, KCC2-positive granule cells were detected in throughout the internal granular layer (Fig. 4C). The synaptic glomeruli consisting of mossy terminals (green) and granule cell dendrites (red) were clearly

discernible (arrowheads in Fig. 4D). Collectively, these results indicated that the KCC2 was first localized at the granule cell somata and dendrites, which attached to mossy fiber terminals at P5.

Next, we examined the electron microscopic analysis, to reveal that KCC2-positive dendrites formed synapses with mossy fiber terminals at P5. The KCC2-immunolabelling was localized near the cell membrane of granule cell bodies and dendrites (arrows in Fig. 5A-D). The dendrites attached to the immature mossy fiber terminals (Larramendi, 1969, Hamori and Somogyi, 1983, Jakab and Hamori, 1988, Altman and Bayer, 1997, Avoli, 2000). By the higher magnification analysis, KCC2-positive dendrites often formed synapses (asterisks) with mossy fiber terminals in the developing synaptic glomeruli before “*cup stage*” (Fig. 5B, C) and at “*cup stage*” (Fig. 5D) (Larramendi, 1969).

DISCUSSION

In the present study, we found that the KCC2 was first localized at the granule cell somata and dendrites, which formed synapses with synaptophysin-positive mossy fiber terminals at P5 as summarized in Fig. 6. The KCC2-positive granule cells increased in number during development, and all granule cells became positive by P21.

KCC2-expression during the granule cell development

The granule cell precursors proliferate in the top of the external granular layer beneath the pia matter. After final mitosis, immature granule cells cross the molecular layer, pass through the Purkinje cell layer and enter the internal granular layer (Uzman, 1960, Miale and Sidman, 1961, Mugnaini and Forstronen, 1967, Rakic, 1971, Komuro and Rakic, 1995, Altman and Bayer, 1997, Komuro and Yacubova, 2003). Within the internal granular layer, granule cells settle at the final destination, extend their dendrites and then start to form the excitatory synapses with mossy fibers before P3, and receive inhibitory input from Golgi cell axons after P7 (Larramendi, 1969, Hamori and Somogyi, 1983, Jakab and Hamori, 1988, Mugnaini, 2000, Takayama and Inoue, 2004b, Takayama, 2005a).

The present studies demonstrated that granule cells did not contain the KCC2 during proliferation in the upper half of the external granular layer and migration in the lower half of the external granular layer and molecular layer. Granule cells did not immediately express the KCC2 when they entered the internal granular layer. At P3, although immature synapses were formed between granule cells and small mossy fiber terminals (Larramendi, 1969, Takayama, 2005a), KCC2 was negative in the granule cells. We first detected KCC2-immunolabeling in the dendrites and cell bodies, which formed

synapses with mossy fibers at P5. At P5, we also often observed synaptophysin-positive mossy fiber terminals, which did not attach to KCC2-positive dendrites or somata. These results indicate that granule cells started to contain the KCC2 after they formed synapses with the mossy fiber terminals and before they received the inhibitory input from Golgi cell axons (Larramendi, 1969, Takayama and Inoue, 2004b, 2005).

Recent investigations demonstrated the mechanisms underlying the KCC2-expression and upregulation, such as GABA_A receptor activation (Ganguly et al., 2001), synaptic transmission-independent system (Ludwig et al., 2003, Titz et al., 2003, Wojcik et al., 2006), trophic factors (Kelsch et al., 2001, Aguado et al., 2003) and so on. In the present study, we found that the onset of the KCC2-localization in the granule cells was at P5 after forming excitatory synapse with mossy fibers and before receiving inhibitory input from the Golgi cells. If extrasynaptically released GABA (Takayama and Inoue, 2004a) could induce the expression, migrating granule cells might contain the KCC2 in the molecular layer and internal granular layer. Nevertheless, we could not detect the KCC2 in the migrating granule cells. If not glutamatergic but GABAergic synaptic transmission could be a trigger, the KCC2 would be detected after P7 when inhibitory synapses are formed (Larramendi, 1969, Takayama and Inoue, 2004b, 2005). Nevertheless, we detected the KCC2 at P5 before the formation of inhibitory synapses. These results indicated that

GABA_A receptor activation by GABAergic synaptic transmission or extrasynaptically released GABA were not necessary for the expression of KCC2 in the cerebellar granule cells *in vivo* as demonstrated by two groups (Ludwig et al., 2003, Titz et al., 2003), and suggested that the excitatory synapse formation might be one of the triggers. Several groups, however, demonstrated that the KCC2-upregulation was not inhibited by blocking action potentials (Ganguly et al., 2001, Ludwig et al., 2003, Titz et al., 2003). Therefore, other mechanisms which are not synaptic transmission but other factors related to the synapse-formation, such as cell to cell interaction and growth factor-releasing, might be involved in the expression (Ludwig et al., 2003).

Developmental Changes in GABA actions on the granule cell

The developmental shift in GABA actions on the membrane potential is determined by the $[Cl^-]_i$ (Ben-Ari, 2002, Owens and Kriegstein, 2002). The early expression of the NKCC1 maintains the high $[Cl^-]_i$, and the opening of the ionotropic GABA receptors depolarizes the membrane potential. GABA-induced depolarization mediates the calcium ion (Ca^{2+}) influx through the voltage dependent calcium channel (VDCC) and N-methyl D-aspartate (NMDA) type glutamate receptor-activation, and GABA is involved in controlling the morphogenesis (Connor et al., 1987, Yuste and Katz,

1991, Owens et al., 1996, Eilers et al., 2001). Late expression of the KCC2 decreases $[Cl^-]_i$, and GABA-mediated hyperpolarization negatively regulates the glutamatergic transmission.

This study demonstrated that the KCC2 started to become localized after granule cells formed synapses with mossy fibers. This indicated that before synapse formation, the KCC2 was negative in the granule cells, suggesting that GABA might be an excitatory transmitter on the granule cells. At P3, vesicular GABA transporters were already localized at the axon varicosities of GABAergic neurons in the molecular layer, Purkinje cell layer and internal granular layer (Takayama and Inoue, 2004a). Thus, GABA released from axon varicosities might act as an excitatory transmitter and play important roles in regulating cell proliferation, migration and maturation, such as extending their axons and dendrites.

After P5, few early-maturated granule cells, which formed synapses with mossy fibers, started to express the KCC2. Two days later, preliminary GABAergic synapses were formed, GABA transporter 1 (GAT-1) started to be localized at the axon terminals of GABAergic neurons, and mature type of GABA_A receptors containing $\alpha 1$ and $\alpha 6$ subunits were expressed on the granule cell dendrites (Takayama and Inoue, 2004c, b, Takayama, 2005a, b, Takayama and Inoue, 2005). It might take several days for the developmental shift from depolarization to hyperpolarization after the KCC2-expression. Taken together, GABA might become an inhibitory transmitter when GABAergic synapses were formed

between granule cell dendrites and Golgi cell axons. Until P21, GABA, released extrasynaptically, might control the maturation of late-developing granule cells, and GABA, released synaptically, might inhibit the glutamatergic activity of early-matured granule cells.

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FIGURE LEGENDS

Fig.1 Western blot analysis of the KCC-antibodies (A) and immunohistochemical localization of the KCC2 in the adult cerebellar cortex (B-H).

A) Western blot analysis with rabbit (Rb-ab) and guinea pig (GP-ab) KCC2 antibodies and the antibodies preincubated with the KCC2-peptide, which was used for immunization (Rb+pep and GP+pep). The position and molecular weights of standards (kD) are shown on the right.

B and C) Immunohistochemical localization of the KCC2 in the adult mouse cerebellar with rabbit antibody. The immunolabeling was detected at the dendrites (black arrows) and cell bodies of stellate (St), basket (Ba) and Purkinje cells (asterisks) in the molecular layer (Mo) and Purkinje cell layer (B). In the granular layer (Gr), granule cell somata and dendrites surrounding the mossy fiber terminals in the synaptic glomeruli (arrowheads) were clearly stained. The immunolabeling was completely abolished when the primary antibody was preincubated with the KCC2 peptide (C).

D) Confocal laser scanning microscopic imaging of the immunohistochemistry for the KCC2 in the granular layer. Cell bodies of granule cells and Golgi cells (Go) were stained and granule cell dendrites in the synaptic glomeruli were more densely labeled (white arrows).

E and F) Immunohistochemical localization of the KCC2 in the adult mouse cerebellar cortex with guinea pig antibody. The immunolabeling pattern was identical to that by rabbit antibody (E). The immunolabeling was also completely abolished when the primary antibody was preincubated with the KCC2 peptide (F).

G and H) Electron microscopic localization of the KCC2 in the granular layer.

The KCC2-immunolabelling was detected at the cell membrane (black arrows) and dendrites (Gd) of granule cells (G). In the synaptic glomeruli, the KCC2-immunolabeling was detected within the granule cell dendrites (Gd), which formed asymmetric synapses (arrowheads) with mossy fiber terminals (Mf).

Fig.2 Developmental localization of the KCC2 in the cerebellar cortex at P0 (A), P3 (B), P5 (C), P7 (D), P10 (E), P12 (F), P14 (G), P21 (H) and P90 (I).

In the internal granular layer (IGr), KCC-immunolabeling was detected at the cell bodies (arrows) and dendrites of granule cells at P5 (C). KCC2-positive granule cells (arrows) increased in number at P7 (D), and granule cell dendrites in synaptic glomeruli (arrowheads) were clearly discernible as ring-shaped profiles at P10 (E). No immunolabeling was detected within the external granular layer (EGr).

Abbreviations: asterisk: Purkinje cell body, Go: Golgi cell, Gr: granular layer.

Fig. 3 Electron microscopic localization of the KCC2 in the migrating granule cells at P10.

A) Bottom part of the external granular (EGr) and molecular layer

B) Middle part of the molecular layer

C) Purkinje cell layer

D) Internal granular layer beneath the Purkinje cells

No immunolabeling was detected in the migrating granule cells (GC1-6), including nuclei, cytoplasm (asterisks), trailing processes (tp) and leading processes (lp).

Abbreviations: Pd: KCC2-positive Purkinje cell dendrite, PF: parallel fiber bundle, Pc:

KCC2-positive Purkinje cell body

Fig. 4 Double labeling of KCC2 (red) and synaptophysin (green) in the internal granular layer (IGr) at P3 (A), P5 (B), P7 (C) and P10 (D).

A) At P3, perikarya (asterisks) and short dendrites of Purkinje cells were positive with KCC2 antibody, and the cell bodies (asterisks) were surrounded by numerous climbing fiber terminal dots in the Purkinje cell layer. In the internal granular layer (IGr), tiny synaptophysin-positive dots were observed, but the KCC2-immunolabeling was negative.

B) At P5, KCC2-immunolabeling was detected at few cell bodies (white arrows) and their

dendrites, which attached to synaptophysin-positive mossy fiber terminals (white arrowhead). Several small synaptophysin-positive terminals (small diamonds), which did not attach to KCC2-positive granule cell dendrites, were detected in the upper part of the internal granular layer.

C) At P7, synaptophysin-positive dots became bigger than those at P5, and attached to the distal part of the KCC2-positive granule cell dendrites (white arrowhead).

D) At P10, synaptophysin-positive dots were surrounded by KCC2-positive granule cell dendrites (white arrowhead) in the synaptic glomeruli.

Fig. 5 Electron microscopic localization of the KCC2 in the internal granular layer at P5.

A) KCC2-immunolabeling was localized at the cell membrane (black arrows) of the granule cell (GC) and its dendrites (Gd) which attached to mossy fiber terminals (Mf).

B and C) Higher magnification of the 5B and 5C in the Fig. 5A. KCC2-immunolabing (arrows) was detected within the dendrites (Gd), which formed synapses (asterisks) with mossy fiber terminals (Mf) containing numerous round synaptic vesicles.

D) Another higher magnification photo of developing synaptic glomerulus consisting of oval mossy fiber terminals (Mf) and granule cell dendrites (Gd). Mossy fiber terminals and KCC2-positive (arrows) granule cell dendrites (Gd) were formed synapses (asterisks).

Fig. 6 Schematic illustration of the developmental localization of the KCC2 in the granule cells of early postnatal mouse cerebellum.

The KCC2 was not localized in the granule cell somata (white ovals) and their leading processes (white triangles) during proliferation in the external granular layer (EGr) and migration in the molecular (Mo) and Purkinje cell (Pu) layers. Granule cells did not immediately contain the KCC2 when they formed synapses with mossy fiber terminals (Mf). The KCC2 might start to be localized in the cell bodies (black circle) and dendrites after excitatory synapses were formed. When inhibitory synapses were formed with Golgi cell axons (Go), the KCC was abundantly localized in the dendrites and cell bodies.

Abbreviations: WM: white matter

REFERENCES

- Aguado F, Carmona MA, Pozas E, Aguilo A, Martinez-Guijarro FJ, Alcantara S, Borrell V, Yuste R, Ibanez CF, Soriano E (BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K⁺/Cl⁻ co-transporter KCC2. *Development* 130:1267-1280.2003).
- Altman J, Bayer SA (1997) *Development of the cerebellar system in relation to its evolution, structure, and functions*. Boca Raton: CPC Press.
- Avoli M (2000) Epilepsy. In: *GABA in the nervous system: the view at fifty years*(Martin, D. L. and Olsen, R. W., eds), pp 293-316 Philadelphia: Lippincott Williams & Wilkins.
- Ben-Ari Y (Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci* 3:728-739.2002).
- Connor JA, Tseng HY, Hockberger PE (Depolarization- and transmitter-induced changes in intracellular Ca²⁺ of rat cerebellar granule cells in explant cultures. *J Neurosci* 7:1384-1400.1987).
- Eilers J, Plant TD, Marandi N, Konnerth A (GABA-mediated Ca²⁺ signalling in developing rat cerebellar Purkinje neurones. *J Physiol* 536:429-437.2001).
- Ganguly K, Schinder AF, Wong ST, Poo M (GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* 105:521-532.2001).
- Gillen CM, Brill S, Payne JA, Forbush B, 3rd (Molecular cloning and functional expression of the K-Cl cotransporter from rabbit, rat, and human. A new member of the cation-chloride cotransporter family. *J Biol Chem* 271:16237-16244.1996).
- Hamori J, Somogyi J (Differentiation of cerebellar mossy fiber synapses in the rat: a quantitative electron microscope study. *J Comp Neurol* 220:365-377.1983).
- Hsu SM, Raine L, Fanger H (Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29:577-580.1981).
- Hubner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ (Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron* 30:515-524.2001).
- Jakab RL, Hamori J (Quantitative morphology and synaptology of cerebellar glomeruli in the rat. *Anat Embryol* 179:81-88.1988).
- Kanaka C, Ohno K, Okabe A, Kuriyama K, Itoh T, Fukuda A, Sato K (The differential expression patterns of messenger RNAs encoding K-Cl cotransporters (KCC1,2) and Na-K-2Cl cotransporter (NKCC1) in the rat nervous system. *Neuroscience* 104:933-946.2001).
- Kelsch W, Hormuzdi S, Straube E, Lewen A, Monyer H, Misgeld U (Insulin-like growth factor 1 and a cytosolic tyrosine kinase activate chloride outward transport during maturation of hippocampal neurons. *J Neurosci* 21:8339-8347.2001).
- Komuro H, Rakic P (Dynamics of granule cell migration: a confocal microscopic study in acute cerebellar slice preparations. *J Neurosci* 15:1110-1120.1995).
- Komuro H, Yacubova E (Recent advances in cerebellar granule cell migration. *Cell Mol Life Sci* 60:1084-1098.2003).
- Larramendi LHM (1969) Analysis of synaptogenesis in the cerebellum of the mouse. In: *Neurobiology of cerebellar evolution and development*(Llinas, R., ed), pp 783-843 Chicago: Am Med Ass.

- Ludwig A, Li H, Saarma M, Kaila K, Rivera C (Developmental up-regulation of KCC2 in the absence of GABAergic and glutamatergic transmission. *Eur J Neurosci* 18:3199-3206.2003).
- Macdonald RL, Olsen RW (GABAA receptor channels. *Annu Rev Neurosci* 17:569-602.1994).
- McCarthy MM, Auger AP, Perrot-Sinal TS (Getting excited about GABA and sex differences in the brain. *Trends Neurosci* 25:307-312.2002).
- Miale IL, Sidman RL (An autoradiographic analysis of histogenesis in the mouse cerebellum. *Exp Neurol* 4:277-296.1961).
- Mikawa S, Wang C, Shu F, Wang T, Fukuda A, Sato K (Developmental changes in KCC1, KCC2 and NKCC1 mRNAs in the rat cerebellum. *Brain Res Dev Brain Res* 136:93-100.2002).
- Mugnaini E (2000) GABAergic inhibition in the cerebellar system. In: *GABA in the nervous system: the view at fifty years*(Martin, D. L. and Olsen, R. W., eds) Philadelphia: Lippincott Williams.
- Mugnaini E, Forstron PF (Ultrastructural studies on the cerebellar histogenesis. I. Differentiation of granule cells and development of glomeruli in the chick embryo. *Z Zellforsch Mikrosk Anat* 77:115-143.1967).
- Olsen RW, Tobin AJ (Molecular biology of GABAA receptors. *Faseb J* 4:1469-1480.1990).
- Owens DF, Boyce LH, Davis MB, Kriegstein AR (Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *J Neurosci* 16:6414-6423.1996).
- Owens DF, Kriegstein AR (Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci* 3:715-727.2002).
- Payne JA, Rivera C, Voipio J, Kaila K (Cation-chloride co-transporters in neuronal communication, development and trauma. *Trends Neurosci* 26:199-206.2003).
- Payne JA, Stevenson TJ, Donaldson LF (Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem* 271:16245-16252.1996).
- Rakic P (Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in *Macacus Rhesus*. *J Comp Neurol* 141:283-312.1971).
- Rakic P, Komuro H (The role of receptor/channel activity in neuronal cell migration. *J Neurobiol* 26:299-315.1995).
- Represa A, Ben-Ari Y (Trophic actions of GABA on neuronal development. *Trends Neurosci* 28:278-283.2005).
- Takayama C (Formation of GABAergic synapses in the cerebellum. *Cerebellum* 4:171-177.2005a).
- Takayama C (GABAergic signaling in the developing cerebellum. *Int Rev Neurobiol* 71:63-94.2005b).
- Takayama C, Inoue Y (Normal formation of the postsynaptic elements of GABAergic synapses in the reeler cerebellum. *Brain Res Dev Brain Res* 145:197-211.2003).
- Takayama C, Inoue Y (Extrasynaptic localization of GABA in the developing mouse cerebellum. *Neurosci Res* 50:447-458.2004a).
- Takayama C, Inoue Y (Morphological development and maturation of the GABAergic synapses in the mouse cerebellar granular layer. *Brain Res Dev Brain Res* 150:177-190.2004b).
- Takayama C, Inoue Y (Transient expression of GABA(A) receptor alpha2 and alpha3

- subunits in differentiating cerebellar neurons. *Brain Res Dev Brain Res* 148:169-177.2004c).
- Takayama C, Inoue Y (Developmental expression of GABA transporter-1 and 3 during formation of the GABAergic synapses in the mouse cerebellar cortex. *Brain Res Dev Brain Res* 158:41-49.2005).
- Titz S, Hans M, Kelsch W, Lewen A, Swandulla D, Misgeld U (Hyperpolarizing inhibition develops without trophic support by GABA in cultured rat midbrain neurons. *J Physiol* 550:719-730.2003).
- Uzman LL (The histogenesis of the mouse cerebellum as studied by its tritiated thymidine uptake. *J Comp Neurol* 114:137-159.1960).
- Wang C, Shimizu-Okabe C, Watanabe K, Okabe A, Matsuzaki H, Ogawa T, Mori N, Fukuda A, Sato K (Developmental changes in KCC1, KCC2, and NKCC1 mRNA expressions in the rat brain. *Brain Res Dev Brain Res* 139:59-66.2002).
- Williams JR, Sharp JW, Kumari VG, Wilson M, Payne JA (The neuron-specific K-Cl cotransporter, KCC2. Antibody development and initial characterization of the protein. *J Biol Chem* 274:12656-12664.1999).
- Wojcik SM, Katsurabayashi S, Guillemain I, Friauf E, Rosenmund C, Brose N, Rhee JS (A shared vesicular carrier allows synaptic corelease of GABA and glycine. *Neuron* 50:575-587.2006).
- Yuste R, Katz LC (Control of postsynaptic Ca²⁺ influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron* 6:333-344.1991).











