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Rapid Communication

**Developmental localization of potassium chloride co-transporter 2 (KCC2) in the
Purkinje cells of embryonic mouse cerebellum**

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Developmental shift in GABA actions from depolarization to hyperpolarization occurs as a result of decreasing the intracellular Cl^- concentration regulated by K^+ - Cl^- co-transporter 2 (KCC2). To clarify the time-course of the developmental shift on the Purkinje cells, we examined KCC2-localization in the embryonic mouse cerebellum. The KCC2 was first detected within the Purkinje cells in the Purkinje cell layer of the hemisphere at embryonic day 15 (E15) and the vermis at E17, but the ventricular and intermediate zones were negative. These results suggest that GABA might become inhibitory on the Purkinje cells after their settling in the Purkinje cell layer.

In the adult central nervous system (CNS), γ -amino butyric acid (GABA) mediates fast inhibitory synaptic transmission and regulates the excitatory activity of neurons (Olsen and Tobin, 1990; Macdonald and Olsen, 1994). In the immature CNS, on the other hand, GABA is an excitatory transmitter and is involved in controlling morphogenesis (Ben-Ari, 2002; McCarthy et al., 2002; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005). These developmental changes in the GABA actions occur as a result of a negative shift in the chloride ion (Cl^-) reversal potential, which is mainly regulated by two chloride co-transporters, $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ co-transporter 1 (NKCC1) and $\text{K}^+ - \text{Cl}^-$ co-transporter 2 (KCC2) (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003). 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 expression of the KCC2, and GABA becomes an inhibitory transmitter. Previous electrophysiological analysis directly demonstrated that KCC2-expression is crucial for the inhibitory transmission by GABA (Hubner et al., 2001; Okabe et al., 2003; Wang et al., 2005). Therefore, the molecular switch from NKCC1 to KCC2 drives the Cl^- influx in response to ionotropic GABA receptor activation, and might be the beginning of the GABAergic

inhibition.

In a previous study, we demonstrated that the cerebellar granule cells start to contain the KCC2 after forming synapses with mossy fibers, suggesting that GABA is excitatory during proliferation and migration, but is inhibitory after synapse formation (Takayama and Inoue, in press). Furthermore, it was suggested that synapse formation might be one of the triggers for the KCC2-expression. In the present study, we focused on the Purkinje cell, which is a principal and first-differentiating neuron in the cerebellar cortex. Although developmental expression of the KCC2 in the cerebellum had previously been investigated using *in situ* hybridization (Mikawa et al., 2002), little is known about when Purkinje cells start to express the KCC2 during development. To clarify the time-course of the changes in GABA actions during Purkinje cell development, we examined the developmental localization of the KCC2 in the embryonic mouse cerebellum.

We examined mice from the C57Bl/6CrSlc mice of embryonic day (E) 13 (E0=mating day), E15, E17 and postnatal day 0 (P0). At each age, at least five mice were sacrificed for immunohistochemistry. Embryonic mice were removed from their mothers under deep ether anesthesia. Mice were fixed by transcardial perfusion with 4% paraformaldehyde in a phosphate buffer (PB, 0.1M pH 7.4). The section-preparation and immunohistochemical staining were performed as described in a previous paper (Takayama

and Inoue, in press). For the double staining of KCC2 and synaptophysin or vesicular GABA transporter (VGAT), sections on glass slides were reacted with a mixed solution of guinea pig KCC2 antibody (1.5 μ g/ml) and rabbit synaptophysin antibody (1:5, Zymed, Cat No. 08-1130, USA) or rabbit VGAT antibody (1.5 μ g/ml) (Takayama and Inoue, 2004a) 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. These experiments were permitted by the Animal Care and Use committee of Hokkaido University School of Medicine.

At E13, the KCC2 was not localized in the cerebellum consisting of ventricular zone and intermediate zone (Fig. 1A). The KCC2-immunolabeling was first detected in the Purkinje cell layer beneath the external granular layer of the anterior-dorsal part of the hemisphere at E15 (Fig. 1B), but was still negative in the vermis. At E17, the KCC2 was distributed in the Purkinje cell layer of both the hemisphere and vermis. However, the intensity of KCC2-immunolabeling varied with the folia. The Purkinje cells in the anterior and posterior folia, lobules II, III, IX and X (Marani and Voogd, 1979), were clearly labeled (asterisks in Fig. 1C). In addition, clearly labeled Purkinje cell masses were also detected in other folia (asterisks in Fig. 1C). Throughout embryonic development, the

KCC2-immunolabeling was not observed in the ventricular zone, intermediate zone or external granular layer (Fig. 1A-C).

Next, the intracellular localization of the KCC2 in the anterior folia (lobules II and III) at E17 was examined by electron microscopy. The immunolabeling was associated with the cell membrane and cytoplasmic vesicular structures, which relate to the membrane recycling, within the immature dendrites (De) and perikarya (Pk and black arrows) of Purkinje cells. The axon hillock (AH) and axon (Ax), however, did not contain the KCC2 (Fig. 1D-F). Asymmetric synapses were often formed at the distal and proximal parts of the KCC2-immunopositive dendrites (arrowheads in Fig. 1E, F).

Furthermore, we performed the double staining of KCC2 and synaptophysin or VGAT in the anterior folia (lobules II and III), where Purkinje cells were clearly stained, to reveal the relationship between KCC2-expression and the formation of excitatory and inhibitory synapses. Synaptophysin-positive dots were detected within the Purkinje cell layer (white arrows in Fig. 2A). The dots often attached to the Purkinje cell somata and thick portion of their dendrites, where the KCC2-immunoreactivity exhibited granular appearance. At P0, the synaptophysin-positive dots remarkably increased in number, and many synaptophysin-positive dots (arrows in Fig. 2B) surrounded the Purkinje cell bodies and attached to their dendrites in the developing molecular layer beneath the external

granular layer. The VGAT-positive dots (arrows in Fig. 2C), on the other hand, were very few in the Purkinje cell layer at E17. The VGAT-positive dots increased in number at P0, and often attached to the bottom part of the Purkinje cell bodies (Fig. 2D). The VGAT-positive dots were considered to be the varicosities of axon collaterals from neighbor Purkinje cells, since they extended from the prospective white matter as observed in the adult cerebellum (Palay and Chan-Palay, 1974), and stellate and basket cells had not been differentiated before birth (Altman and Bayer, 1997; Takayama, 2005).

The Purkinje cell precursors proliferate in the ventricular zone and migrate towards the developing external granular layer (Yuasa et al., 1996; Altman and Bayer, 1997). After settling under the external granular layer, the Purkinje cells extend their dendrites and form the excitatory and inhibitory synapses (Larramendi, 1969; Altman and Bayer, 1997). The present studies demonstrated that Purkinje cells started to contain the KCC2 within the dendrites and somata after they settled in the Purkinje cell layer of the hemisphere at E15 and the vermis at E17. The expression of the KCC2 drives the Cl^- influx in response to ionotropic GABA receptors activation, and is thought to be the beginning of the GABAergic inhibition. The GABA-induced depolarization in the immature brain is involved in controlling the morphogenesis, whereas GABA-mediated hyperpolarization in the mature brain negatively regulates the glutamatergic transmission (Ben-Ari, 2002;

Owens and Kriegstein, 2002; Payne et al., 2003). Therefore, GABA might act as an excitatory transmitter for the Purkinje cell precursors which expressed GABA_A receptor containing the $\alpha 3$ subunit (Takayama and Inoue, 2004c), and play roles in regulating proliferation, migration and early maturation, including the initial formation of synapses. After Purkinje cells settled at their final position, GABA, which is released from the axon collaterals of neighbor Purkinje cells, might be inhibitory for the Purkinje cells which expressed GABA_A receptor containing the $\alpha 1$ subunit (Takayama and Inoue, 2004b), although it is excitatory for the developing granule cells. This difference in GABA actions between neuronal types in the same region had been demonstrated by electrophysiological analysis in the olfactory bulbs (Wang et al., 2005) and dentate gyrus (Okabe et al., 2003). In addition, GABA is not excitatory but inhibitory during drastic Purkinje cell maturation, including dendrite-extension, synapse-formation, and synapse-refinement. In a previous study, we demonstrated that granule cells also started to express the KCC2 after their settling at the final destination and forming the initial synapse (Takayama and Inoue, in press). Taken together, it is suggested that for the developing neurons, GABA is excitatory during proliferation and migration, but is inhibitory after forming the first synapses.

This study also demonstrated that densely stained Purkinje cells received synaptophysin-positive input, whereas only few synaptophysin-positive dots were detected

in the weakly stained Purkinje cell masses. In addition, the discontinuous localization of the clear KCC-immunolabeling in the Purkinje cell layer was quite similar to the patten of early climbing fiber innervations (Arsenio Nunes and Sotelo, 1985). These results suggest that the synapse formation, in particular with climbing fibers, might be closely related to the beginning of KCC2-expression, as demonstrated in the granule cells (Takayama and Inoue, in press).

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Figure legends

Figure 1 Immunohistochemical localization of the KCC2 in the mouse cerebellum at embryonic day (E) 13 (A), E15 (B) and E17 (C-F).

A-C) Light microscopic localization of the KCC2 in the cerebellum at E13 (A), E15 (B) and E17 (C). The KCC2-immunolabeling was first detected in the Purkinje cell layer (Pu) in the hemisphere at E15 (B), but the ventricular zone (VZ), intermediate zone (IZ) and external granular layer (EGr) were negative. At E17 (C), the Purkinje cells were not equally stained. Clearly (asterisks) and weakly labeled regions were detected in the vermis.

D-F) Electron microscopic localization of the KCC2 in the Purkinje cell layer of the anterior folium at E17. The KCC2-immunolabelling was localized within the dendrites (De) and perykarya (Pk and black arrows), but the axon hillock (AH) and axons (Ax) were negative. The KCC2-positive dendrites often formed asymmetric synapses (arrowheads in E and F).

Other abbreviations: Nu: deep cerebellar nucleus, WM: white matter

Figure 2 Double labeling of the KCC2 (red) and synaptophysin (green) (A, B) or VGAT (green) (C, D) in the cerebellum at E17 (A, C) and postnatal day (P) 0 (B, D)

A and B) External granular layer (EGr) was negative with synaptophysin and KCC2.

Synaptophysin-positive dots (white arrows) were observed in the Purkinje cell layer (Pu) at

E17 (A). At P0 (B), synaptophysin-positive dots (white arrows) remarkably increased in number and were detected in the molecular layer (Mo) and Purkinje cell layer (Pu).

Asterisks show the position of Purkinje cell bodies.

C and D) The VGAT-positive dots (white arrows), which extended from the deeper part of the Purkinje cell layer (Pu) towards the molecular layer, were few in the cerebellar cortex at

E17 (C). At P0 (D), the VGAT-positive dots (white arrows) increased in number and often attached to the KCC2-positive Purkinje cell somata (asterisks) and dendrites.

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