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Developmental localization of potassium chloride co-transporter 2 (KCC2), GABA and vesicular GABA transporter (VGAT) in the postnatal mouse somatosensory cortex

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ABSTRACT

Gamma-amino butyric acid (GABA) mediates the hyperpolarization of membrane potential, negatively regulating glutamatergic activity in the adult brain, whereas, mediates depolarization in the immature brain. This developmental shift in GABA actions is induced by the expression of potassium chloride co-transporter 2 (KCC2). In this study, we focused on the developing mouse somatosensory cortex, where the barrel structure in layer 4 alters by the whisker-lesion during the critical period, before postnatal day 4 (P4). First, to clarify the time-course of postnatal changes in GABA actions, we investigated the developmental localization of KCC2. Second, to reveal its spatial and temporal relationship with GABA-synapse formation, we examined the developmental localization of GABA and vesicular GABA transporter. KCC2 was localized within the pyramidal cells in layer 5 after P3, granule cells in layer 4 after P5 and neurons in layer 2 and 3 after P7, indicating that KCC2 was expressed in the chronological order of neuronal settling at the destination. The onset of KCC2 localization was almost concomitant with the formation of GABA synapses, suggesting that GABA was inhibitory after GABA synapse-formation. Furthermore, extrasynaptically released GABA might be involved in the maintenance of activity-dependent plasticity as an excitatory transmitter during the critical period.

Key Words: activity-dependent plasticity, barrel structure, corticogenesis, critical period, GABA synapse, KCC2, somatosensory cortex, VGAT

1. Introduction

In the adult central nervous system (CNS), γ-amino butyric acid (GABA) predominantly induces the hyperpolarization of membrane potential, and mediates fast inhibitory synaptic transmission, negatively 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; Ben-Ari et al., 2007).

This developmental change in GABA actions occurs as a result of a negative shift in the chloride ion (Cl^-) reversal potential, which is mainly regulated by two chloride co-transporters, sodium (Na^+)-potassium (K^+)-$2Cl^-$ co-transporter 1 (NKCC1) and K^+-Cl^- co-transporter 2 (KCC2) (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003). The molecular switch from NKCC1 to KCC2 drives Cl^- influx in response to ionotropic GABA receptor activation and the dominant expression of the KCC2 might be the beginning of GABAergic inhibition (Hubner et al., 2001; Lee et al., 2005; Rivera et al., 2005).

In previous studies, we investigated developmental changes in the localization of various molecules involved in GABAergic transmission and revealed GABA signaling in the developing cerebellum (Takayama and Inoue, 2004b; Takayama, 2005b). Dendrites, perikarya, axons and growth cones of immature GABAergic neurons in the developing cerebellar cortex are filled up with GABA. Subsequently, GABA is confined to the axon varicosities and terminals which form synapses. Concomitantly, GABA vesicles, labeled by VGAT-immunohistochemistry, become localized in axon varicosities and growth cones

(Takayama and Inoue, 2004c; Takayama and Inoue, 2004a), and GABAergic synapses are morphologically detected (Takayama, 2005a). These results indicate that GABA synapses are formed when GABA is accumulated in and VGAT is localized at axon varicosities. Furthermore, it was suggested that GABA could be extrasynaptically released from immature GABA neurons by non-vesicular mechanisms, diacrine, in the immature cerebellum (Takayama and Inoue, 2004b; Takayama, 2005b), although membrane-type GABA transporters, GATs, are not localized before synapse formation (Takayama and Inoue, 2005). In studies concerning KCC2 localization, we demonstrated that (1) granule cells express KCC2 in the chronological order of their differentiation, in particular the formation of synapses with mossy fiber terminals, (2) among five types of neurons in the cortex, Purkinje cells first express KCC2 after the settle at their final destination and form synapses before birth, suggesting that settling and synapse formation might be important triggers of the expression of KCC, and GABA is excitatory before the formation of GABA synapses (Takayama and Inoue, 2006; Takayama and Inoue, 2007).

Next, we focused on GABA signaling in the developing cerebral cortex, in particular the somatosensory cortex. The cerebral cortex exhibits distinct six layers. During corticogenesis, postmitotic excitatory neurons leave the ventricular zone, and generate the layers 2-6 in an “inside-out” sequence (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1972; Nadarajah and Parnavelas, 2002; Hevner et al., 2004). In contrast, GABAergic neurons are born in the subcortical area, lateral and medial ganglionic eminences, reach the cortex in tangentially migrating streams, and radially moved to the final position (Marin and Rubenstein, 2001). These corticogenesis is the final event in brain ontogeny. Neurons still continued to migrate to the superficial layers of the cerebral cortex at birth, and the majority of synapses are formed after birth. Furthermore, activity-dependent plasticity in the formation

of characteristic cytoarchitectonic patterns and functional columns is maintained after birth (Kaas et al., 1983; Hensch, 2004; Fox and Wong, 2005). In the rodent somatosensory cortex, for example, the barrel structure in layer 4 is sensitive to peripheral damage, such as the removal of whisker follicles, before postnatal day 4 (P4) (Durham and Woolsey, 1984).

In the present study, first, to clarify the time-course of changes in GABA actions during corticogenesis, we investigated the postnatal development of KCC2 localization in the mouse somatosensory cortex. Second, to reveal the time course of GABA synapse formation, we examined the developmental localization of GABA and vesicular GABA transporter (VGAT), which is a marker of GABA vesicles, in the same area. In addition, we compared the developmental shift in the GABA-release mechanism from non-vesicular extrasynaptic release, diacrine, to synaptic release, exocytosis, and the changes in GABA action. Third, to reveal the involvement of GABA in the activity dependent plasticity, we investigated differences in KCC2, GABA and VGAT localization in layer 4 during and after the critical period.

2. Materials and Methods

2.1 Animals

We examined C57Bl/6CrSlc mice of postnatal days 0 (P0), P3, P5, P7, P10, P12, P14 and P21. At each age, at least five mice were sacrificed for immunohistochemistry.

2.2 Antibody characterization

Table 1 shows the list of all antibodies used in the present study. Immunoblotting for the KCC2 antibody exhibited a major band around the predicted molecular weight (140kD). The band in the immunoblotting and the immunolabeling in the cerebellar sections were abolished by addition of 1μg/ml of the peptide for immunization into the primary antibody

solution (Takayama and Inoue, 2006). Dot blot analysis revealed that the GABA antibody bound GABA-conjugates, but did not react with other amino acid-conjugates. The preincubation of the primary antibody with 0.1μg/ml GABA completely abolished the immunolabeling in the cerebellar sections (Takayama and Inoue, 2004a). Immunoblot analysis for the VGAT antibody showed a single band at the predicted molecular weight, 51kd. The band in the immunoblotting and the immunolabeling in the cerebellar sections were abolished when the primary antibody was preincubated with the 1μg/ml of peptide, which was used for the immunization (Takayama and Inoue, 2004a).

2.3 Tissue preparation

Under deep ether anesthesia, mice were fixed by transcardial perfusion with 4% paraformaldehyde in phosphate buffer (PB, 0.1M pH 7.4) or mixed solution of 4% paraformaldehyde and 0.5% glutaraldehyde in PB. The brains were removed, immersed in the same fixative overnight, cryoprotected with 30% sucrose in PB for two days at 4°C, and then cut into coronal sections at a thickness of 20μm by a cryostat. The sections were mounted on gelatin-coated glass slides.

2.4 Immunohistochemistry for KCC2, VGAT, and GABA

Sections of brains fixed with 4% paraformaldehyde were treated as follows; with methanol containing 0.3% H_2O_2 for 30 minutes, PB for 10 minutes, 3% normal goat serum in PB for one hour, and KCC2 antibody (1μg/ml) or VGAT antibody (1μg/ml) overnight at room temperature. After rinsing three times with PB for 15 minutes, sections were visualized using the avidin-biotin-peroxidase complex (ABC) method (Histofine kit, Nichirei, Japan) (Hsu et al., 1981) as described in previous papers (Takayama and Inoue, 2004a; Takayama and Inoue, 2006).

Sections of brains fixed by mixed solution of 4% paraformaldehyde and 0.5%

glutaraldehyde were treated as follows; with methanol containing 0.3% H_2O_2 for 30 minuets, PB for 10 minutes, 0.2% glycine in PB for 30 minutes, 1% sodium borohydrate in PB for 30 minutes (Kosaka et al., 1986), PB for 15 minutes, 3% normal goat serum in PB for one hour, and GABA antibody (0.2μg/ml) overnight at room temperature. After rinsing three times with PB for 15 minutes, sections were visualized using the avidin-biotin-peroxidase complex (ABC) method (Histofine kit, Nichirei, Japan) (Hsu et al., 1981) as described in a previous paper (Takayama and Inoue, 2004a).

These experiments were approved by both Animal Care and Use Committees of Hokkaido University School of Medicine (No. 06044) and University of the Ryukyus (No. 4683) performed in conformance with the *Guide for the Care and Use of Laboratory Animals of Hokkaido University School of Medicine and University of the Ryukyus*. Every effort was made to minimize the number of animals used and their suffering.

3. Results

3.1 Nissl staining and immunohistochemistry for KCC2, GABA and VGAT in the mouse somatosensory cortex at P21

In the mouse somatosensory cortex, toluidine blue-staining exhibited the characteristic cytoarchitecture (Fig. 1A). Large pyramidal cells aligned in the layer 5. Layer 4 consisted of cell-sparse areas (i.e., patches) and cell dense walls (i.e. septum, black arrows in Fig. 1A) (Waite, 2004). In the adjacent section, cell-sparse areas were identical to the cytochrome oxidase-positive patches which receive thalamic afferents (data not shown). The borderline between layer 2 and 3 was not clear in the mouse cerebral cortex. KCC2 immunolabeling was seen in the neuropil region throughout all layers (Fig. 1B). GABA

immunolabeling was localized in the cell bodies and terminal dots of GABAergic neurons in all layers (Fig. 1C). Immunohistochemistry for VGAT was detected in all layers, and the labeling was usually surrounded the cell bodies of granule cells in layer 4 and pyramidal cells in layer 5 (Fig. 1D).

3.2 Immunohistochemical localization of KCC2, GABA and VGAT in the mouse somatosensory cortex at P0

In the newborn mouse somatosensory cortex, neurons continue to migrate superficially in the cortical plate (CP), which would give rise to layer 2/3 and 4.

KCC2 was localized at the dendrites and cell bodies of small neurons (arrows) in layer 5 and 6, whereas pyramidal cells in layer 5 and granule cells in the deep part of the cortical plate (CP) were negative (Fig. 2A). By GABA immunohistochemistry, numerous GABAergic neurons were labeled in all layers (Fig. 2B), but VGAT-positive dots were quite few or negative (Fig. 2C).

In the higher magnification view, weak KCC2 immunolabeling was detected in the dendrites and cell bodies of small multi-polar neurons in layer 5, but apical and basal dendrites, cell bodies and axons of pyramidal neurons were negative (Fig. 2D). By GABA immunohistochemistry, cell bodies and short dendrites of GABAergic neurons were stained, but axon varicosities and terminal-like dots were not detected. In the cortical plate, GABA-positive neurons were bipolar and spindle shaped (asterisks in Fig. 2E) with leading and trailing processes in the upper and lower sides, respectively. In contrast, GABA neurons had several dendrites (arrowheads in Fig 2.E) in layer 5, indicating that GABA neurons in the cortical plate were still radially migrating and those in layer 5 had already settled at their final position and had been differentiating. VGAT-positive dots were absent in the cortical plate

and were scarce in layer 5 (Fig. 2F).

These results suggested that KCC2 is first localized in non-pyramidal cells, probably GABAergic neurons, which had already finished migration in layer 5 and 6.

3.3 Developmental change in localization of the KCC2 in the mouse somatosensory cortex

Next, we examined developmental changes in the localization of KCC2 in somatosensory cortex. At P3, non-pyramidal neurons were stained in the layer 5 and 6 (Fig. 3A), and apical dendrites of pyramidal cells were weakly labeled. At P5, immunolabeling increased in intensity in all layers (Fig. 3B). In layer 4, patch-like-structures (asterisks) were clearly detected. In layer 5, the neuropil region became moderately positive. At P7, the neuropil region in all layers increased in intensity (Fig. 3C). At P10, the neuropil region was homogeneously stained in all layers and apical dendrites in layer 2 and 3 were not clearly discernible (Fig. 3D). The intensity of immunolabeling increased until P14 (Fig.3E, F). In summary, GABA neurons were localized in all layers throughout development (Fig. 4A, C, E, G, I, K). In contrast, GABA-positive fine dots in the neuropil and VGAT-positive dots were first detected in layer 5 and 6 at P3 (Fig. 4A, B). The dot-localizing area gradually expanded toward the upper layers until P10 (Fig. 4C-H), and the density and the intensity of the dots became homogeneous at P14 (Fig. 4I-L).

To reveal these developmental changes more precisely, we examined them in layer 5, 4 and 2/3 at higher magnification.

3.4 Developmental localization KCC2, GABA and VGAT in the layer 5

The principal neurons in layer 5 are pyramidal cells, mainly receiving intrinsic input from granule cells in layer 4 and sending axons to subcortical areas, such as the striatum and

several nuclei in the brain stem and spinal cord (Waite, 2004; Brecht, 2007).

At P3, KCC2 immunolabeling was observed in the dendrites and cell bodies of small multipolar neurons (arrows), as detected at P0 (Fig. 5A). Cell bodies and dendrites of pyramidal cells were weakly labeled. The KCC2-positive structure was still sparse, since the dendrites were immature. At P5, immunolabeling markedly increased in intensity (Fig. 5B). Moderate KCC2 immunolabeling was detected on the surface of cell bodies (asterisks) and dendrites (arrowheads) of pyramidal cells, and KCC2-positive fine granules were distributed in the neuropil. At P7, KCC2-positive fine dots homogeneously occupied the neuropil region and apical and basal dendrites were not clearly discernible, indicating that the majority of dendrites were KCC2-positive (Fig. 5C). KCC2-labeled fine dots in the neuropil gradually increased in intensity until P14, whereas perikarya and cytosol of the thick dendrites (white arrows) were negative (Fig. 5D-F).

Many GABAergic neurons were localized in layer 5 at P3 (Fig. 6A). By GABA immunohistochemistry, cell bodies, dendrites and short axons were clearly labeled. Several varicosities were detected, but the varicosities were still small. On the same day, several tiny dots were labeled by VGAT immunohistochemistry (Fig. 6B). At P5, GABA was also localized in the dendrites, cell bodies and axons (Fig. 6C). Immunolabeling in axons became faint and axon varicosities were more clearly discernible. VGAT-positive dots increased in number and became larger than those at P3, but the cell bodies of pyramidal cells were not completely surrounded (Fig. 6D). At P7, GABA-positive axon varicosities markedly increased in density and sometimes surrounded the pyramidal cells (asterisks in Fig. 6E). VGAT-positive dots also markedly increased in density and sometimes surrounded pyramidal neurons (asterisks in Fig. 6F). After P10 until P21, the density of GABA-positive axon varicosities and VGAT-positive dots increased greatly, and dots around the pyramidal neurons

became much dense, and these dots completely occupied the neuropil and attached to the surface of neurons in layer 5 (Fig. 6G-L). Concomitantly, GABA immunolabeling disappeared from the dendrites and axons themselves (Fig. 6E, G, I, K).

KCC2 was localized in the pyramidal cells after P3, and markedly increased in expression after P5. GABAergic neurons were already localized at P0. GABA- and VGAT-positive axon varicosities appeared at P3, and markedly increased after P5.

3.5 Developmental changes in localization of KCC2, GABA and VGAT in layer 4

Layer 4 of the somatosensory cortex is filled in a characteristic cytoarchitectonic pattern, barrel structures, consisting of “patches”, where granule cells, the principal neurons, extend their dendrites and receive sensory input from whisker follicles by way of the ventral posteromedial nucleus of the thalamus, and the “septum”, receiving afferents from the posteromedial nucleus of the thalamus. The axons of granule cells have intrinsic connections with other layers in the same column and layer 4 in adjacent columns (Waite, 2004; Brecht, 2007).

At P3, apical dendrites of pyramidal cells (arrowheads) were moderately stained, and small non-granular cells (arrows) were weakly labeled, as detected in layer 5 and 6 on P0 (Fig. 7A). In contrast, granule cells were negative. At P5, patches were observed at lower magnification (Fig. 3B). On the higher magnification view, KCC2 immunolabeling was localized at the neuropil within patches where cell bodes of granule cells are densely packed, whereas it was weak or negative between patches (Fig. 7B). At P7, KCC2-positive patches were also clearly discernible (Fig. 3C). The fine KCC2-positive granules occupied the neuropil region within patches (Fig. 7C). In addition, immunolabeling between patches slightly increased in intensity, but was still weak. At P10, immunolabeling markedly increased

in intensity and fine granules homogeneously occupied the neuropil (Fig. 7D). After P10, the neuropil region was homogeneously and densely labeled, and apical dendrites of pyramidal cells and patches were not clearly discernible, but the cytosol of cell bodies and apical dendrites was negative (white arrows in Fig. 7E, 7F).

At P3, GABA was localized in the cell bodies, dendrites and axons of GABAergic neurons (Fig. 8A). GABA-positive axon varicosities and VGAT-positive dots were detected, but they were few and sparse compared to those in layer 5 (Fig. 8A, B). At P5, both dots increased in density (Fig. 8C) and became as numerous as those in layer 5 at P3 (Fig. 6A), but GABA was also detected in axons. From P7 to P10, the density of GABA- and VGAT-positive dots markedly increased (Fig. 8E-H). The dots sometimes surrounded granule cell bodies. Concomitantly, GABA disappeared from the axons and was confined to perikarya and axon varicosities. At P12 and P14, the dots increased in density, occupied the neuropil region and surrounded granule cell bodies (Fig. 8I-L).

KCC2 was negative in layer 4 neurons at P3, and was localized in patches after P5 and the septum after P7. KCC2 immunolabeling became dense in patches after P7, and homogeneous after P10. GABA- and VGAT-positive axon varicosities were detected after P5, and markedly localized after P7.

3.6 Developmental change in localization of KCC2, GABA and VGAT in layer 2 and 3

Layer 2/3 mainly consist of small granule cells and small pyramidal cells, receiving cortico-cortical association fibers from the ipsilateral hemisphere and commissural fibers from the contralateral hemisphere to the areas, such as the motor cortex, and secondary sensory cortex and sending axons to the same areas. In rodents, the borderline between layer 2 and 3 is not clear (Waite, 2004; Brecht, 2007).

Apical dendrites of pyramidal cells in layer 5 (arrows) were moderately stained with KCC2 antibody at P3 (Fig. 9A), and their immunolabeling increased in intensity at P5 (Fig. 9B), but neurons in layer 2 and 3 were negative. At P7, small neurons became weakly positive in the lower half (Fig. 9C). At P10, the majority of neurons in layer 2 and 3 contained KCC2, but there was a deep to superficial intensity gradient in KCC2 immunolabeling (Fig. 3D, 9D). After P12, KCC2-positive fine granules homogeneously occupied the neuropil region, increased in intensity and apical dendrites were not clearly discernible, but the cytoplasm in cell bodies and the thick part of apical dendrites were not labeled (Fig. 9D-F).

At P3 and P5, GABA was localized within the short dendrites and axons in the developing layer 2 and 3, but GABA-positive axon varicosities and VGAT-positive dots were absent at P3 (Fig. 10A), and scarce in the deep part at P5 (Fig. 10B). At P7, axon varicosities were clearly detected by GABA and VGAT immunohistochemistry in all parts of layer 2 and 3 after P7 (Fig. 10E, F), but there was a deep to superficial density gradient by P10 (Fig. 10D-H). GABA and VGAT-positive dots gradually increased in density, were homogeneously localized and surrounded neurons in the deep part after P12 (Fig. 10 I, J) and superficial part after P12 (Fig. 10K, L). During development, GABA in the dendrites and axons disappeared by P12. In summary, KCC2 was localized in the deep part after P7, and all layers after P10, and markedly localized after P12.

3.7 Developmental change in localization of KCC2, GABA and VGAT in other layers

In the layer 1, not only GABA but also KCC2 and VGAT were highly expressed at birth (Fig. 2), and continued to be localized throughout postnatal development (Figs. 3, 4). In the layer 6, the time-course of the changes in expression of three molecules seemed to be similar to that in layer 5. However it was too complicated to reveal the precise changes in

their localization in the proper neurons in layer 6, since various heterogenic cells were intermingled during corticogenesis. In the subplate, GABA, KCC2, and VGAT were also localized at birth (Fig. 2), but the subplate had disappeared by the end of the first postnatal week (Figs. 3, 4).

4. Discussion

4.1 Developmental changes in GABA actions in the mouse somatosensory cortex

Previous electro-physiological analyses demonstrated that GABA is excitatory in the superficial layers of the cerebral cortex, but is inhibitory in the deep layers during the first postnatal week (Yamada et al., 2004). GABA action developmentally shifts from depolarization to hyperpolarization during the second postnatal week (Sutor and Luhmann, 1995; Yamada et al., 2004). In the present study, to precisely elucidate the developmental change in GABA action, we examined the localization of KCC2 in the developing mouse somatosensory cortex. The result is schematically illustrated in Figure 11. First, non-pyramidal small neurons expressed KCC2 in layer 5 and 6 at P0. Subsequently, KCC2 was localized in pyramidal cells in layer 5 after P3, granule cells in layer 4 after P5, neurons in layer 2 and 3 after P7, and was markedly distributed two days after the onset of the localization in each layer. These results support previous studies demonstrating the developmental increase in expression of KCC2 protein (Lu et al., 1999; Balakrishnan et al., 2003; Vale et al., 2005) and indicate that KCC2 was expressed in the chronological order of neuronal settling at their final destination in the cerebral cortical layers (Miller, 1988) as demonstrated in the cerebellar cortex (Takayama and Inoue, 2006; Takayama and Inoue, 2007). Marked expression of KCC2 in neurons induces the decrease of $[Cl^-]_i$, resulting in the shift of GABA actions from excitation to inhibition (Hubner et al., 2001; Lee et al., 2005;

Rivera et al., 2005). Furthermore, previous histochemical and electrophysiological investigations demonstrated that functional $GABA_A$ receptors are expressed in all layers of cortex during postnatal development (Laurie et al., 1992; LoTurco et al., 1995; Yamada et al., 2004), indicating GABA is functional in the developing cortex. Taken together with these previous and presnt data, the functional roles of GABAergic neurons are change during the early postnatal days as follows: (1) GABA acts as an excitatory transmitter to all neurons except for those in layer 1 cells and several small multipolar neurons in layer 5 and 6 at birth. (2) The shift of GABA actions first occurs in the deep layer, and GABAergic inhibition gradually extends toward the superficial part. (3) Around P7, GABA might be inhibitory within patches but excitatory in the septum of the barrel field in layer 4. (4) GABA acts as an excitatory transmitter to neurons in the superficial part, whereas is inhibitory in the deep part by P7. (5) After P10, GABA becomes an inhibitory neurotransmitter for all neurons in the cerebral cortex.

4.2 Developmental formation of GABA synapses and their relationship with the shift in GABA actions

In the present study, we demonstrated that GABA was homogeneously localized in GABAergic neurons, including dendrites, cell bodies and axons, in the immature cerebral cortex, whereas it was accumulated in axon varicosities after maturation. VGAT became localized to axon varicosities, when GABA accumulated in varicosities. These developmental changes in the localization of GABA and VGAT were also detected in the cerebellar cortex (Takayama and Inoue, 2004c; Takayama, 2005a), suggesting that GABA synapses are formed when GABA and VGAT are confined to axon varicosities. The developmental formation of GABA synapses in the mouse somatosensory cortex is summarized in the Table 2, and this

result is in agreement with a previous electron microscopic study (De Felipe et al., 1997). The time course of GABA synapse formation in each layer was almost identical to KCC2 localization, suggesting that GABA could be released as an excitatory transmitter by a non-vesicular system, diacrine, before synapse formation, whereas it is synaptically released as an inhibitory transmitter after synapse formation.

The mechanism underlying non-vesicular release, diacrine, is still unknown. Previously, it was speculated that diacrine might be driven by the reverse action of the membrane type GABA transporters (GATs) (Taylor and Gordon-Weeks, 1991; Gao and van den Pol, 2000; Varju et al., 2001). GATs, however, were not detected before synapse formation in the developing cerebellar cortex (Takayama, 2005b; Takayama and Inoue, 2005). No deep to superficial gradient in expression, as detected in the present study, was detected, although previous studies reported that GATs are localized in the immature cerebral cortex (Conti et al., 1999; Conti et al., 2004). Furthermore, developmental shift in GATs localization from axons to varicosities was not detected. These results suggested that novel mechanisms might be involved in non-vesicular GABA release, diacrine, during maturation in the CNS.

4.3 Relationship between plasticity and GABA action

The somatosensory cortex is one of the best areas to investigate activity-dependent plasticity. Rearrangement of characteristic structure, barrel formation in layer 4, is morphologically detected after damage to the sensory input from rodent whiskers during the critical period (Kaas et al., 1983; Durham and Woolsey, 1984). In mice, the critical period ends by P4 or P5, and no changes are detected after the critical period (Durham and Woolsey, 1984). Our present study demonstrated that the onset of KCC2 localization in granule cells of layer 4 was P5, and no KCC2 immunolabeling was detected before P3, indicating that GABA

may be excitatory during critical period. Furthermore, GABA was homogeneously localized in GABAergic neurons, and GABA- and VGAT-positive axon varicosities were scarce during the critical period, indicating GABA signaling is the immature type. These results suggested that extrasynaptically released GABA might act as an excitatory transmitter during the critical period.

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Figure legends

Figure 1 Nissl staining (A) and immunohistochemistry for the KCC2 (B), GABA (C) and VGAT (D) in the mouse somatosensory cortex at P21

Abbreviations and Symbols

1: layer 1, 2/3: layer 2 and 3, 4: layer 4, 5: layer 5, 6: layer 6

Asterisk: patch in the barrel structure, arrow: septum between patches

Figure 2 Immunohistochemical localization of KCC2 (A, D), GABA (B, E) and VGAT (C, F) at lower magnification (A-C) and higher magnification (D-F) in the mouse somatosensory cortex at P0

KCC2 immunolabeling was detected in layer 1 and small multipolar neurons (arrows) in layer 5 (5), and layer 6 (6), whereas it was negative in the cortical plate (CP) (A and D). GABAergic neurons are bipolar in the cortical plate (asterisks), whereas they were multipolar in layer 5 (arrowheads) (B and E). VGAT-positive dots were scarce in the layer 5 (C and F).

Figure 3 Developmental change in localization of KCC2 in the mouse somatosensory cortex at P3 (A), P5 (B), P7 (C), P10 (D), P12 (E) and P14 (F)

KCC2 was localized in small neurons (arrows) in layer 5 (5) and layer 6 (6) and pyramidal neurons at P3 (A), and patches (asterisks) in layer 4 (4) at P5 (B). The KCC2-positive area expanded toward layer 2 and 3 (2/3).

Figure 4 Developmental change in localization of GABA (A, C, E, G, I, K) and VGAT (B, D, F, H, J, L) in the mouse somatosensory cortex at P3 (A, B), P5 (C, D), P7 (E, F), P10 (G, H), P12 (I, J) and P14 (K, L)

Figure 5 Developmental change in localization of KCC2 in layer 5 at P3 (A), P5 (B), P7 (C), P10 (D), P12 (E) and P14 (F)

KCC2 was localized in small neurons (arrows) and pyramidal neurons (asterisks) at P3. Dendrites (arrowheads) and cell bodies (asterisks) of pyramidal cells were clearly labeled at P5 (B). Immunolabeling increased in intensity during development, whereas it was negative in the cytoplasm in the dendritic shaft (white arrows) and cell bodies (asterisks) at P12 (E) and P14 (F).

Figure 6 Developmental change in localization of GABA (A, C, E, G, I, K) and VGAT (B, D, F, H, J, L) in layer 5 at P3 (A, B), P5 (C, D), P7 (E, F), P10 (G, H), P12 (I, J) and P14 (K, L)

GABA- and VGAT-positive dots were detected after P3 (A, B), markedly increased in density after P5 (C, D) and clearly surrounded pyramidal cell bodies (asterisks) after P10 (E-L).

Figure 7 Developmental change in localization of KCC2 in layer 4 at P3 (A), P5 (B), P7 (C), P10 (D), P12 (E) and P14 (F)

KCC2 was localized in the small neurons (arrows) and apical dendrites of pyramidal cells (arrowheads) at P3 (A). Patches (asterisks) were clearly detected at P5 (B) and P7 (C), whereas neuropil was homogeneously stained after P10 (D-F). The cytoplasm in the dendritic shaft (white arrows) was negative at P12 (E) and P14 (F).

Figure 8 Developmental change in localization of GABA (A, C, E, G, I, K) and VGAT (B, D, F, H, J, L) in the layer 4 at P3 (A, B), P5 (C, D), P7 (E, F), P10 (G, H), P12 (I, J) and P14 (K, L)

GABA- and VGAT positive dots were scarce at P3 (A, B), increased in density after P5 (G, H)

and clearly surrounded the granule cell bodies (asterisks) after P10 (E-L).

Figure 9 Developmental change in localization of KCC2 in layer 2 and 3 at P3 (A), P5 (B), P7 (C), P10 (D), P12 (E) and P14 (F)

KCC2 was localized in the apical dendrites of pyramidal cells (arrows) at P3 (A) and P5 (B). After P7, as immunolabeling increased in layer 2 and 3, dendrites were not clearly discernible after P12 (E, F). The cytoplasm in the dendritic shaft (white arrows) was negative at P12 (E) and P14 (F).

Figure 10 Developmental change in localization of GABA (A, C, E, G, I, K) and VGAT (B, D, F, H, J, L) in layer 4 at P3 (A, B), P5 (C, D), P7 (E, F), P10 (G, H), P12 (I, J) and P14 (K, L)

GABA- and VGAT-positive dots were negative at P3 (A, B), scarce at P5 (C, D), gradually increased in density after P7 (E, F), and surrounded the cell bodies (asterisks) after P12 (I-L).

Figure 11 Schematic illustration of the developmental localization of KCC2 in the somatosensory cortex.