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Developmental expression of GABA transporter-1 and 3 during formation of the GABAergic synapses in the mouse cerebellar cortex

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Abstract

In the brain, \(\gamma\)-amino butyric acid (GABA), released extrasynaptically and synaptically from GABAergic neurons, plays important roles in morphogenesis, expression of higher functions and so on. In the GABAergic transmission system, plasma membrane GABA transporters (GATs) mediate GABA-uptake from the synaptic cleft in the mature brain, and are thought to mediate diacrine of cytosolic GABA in the immature brain. In the present study, we focused on two GATs (GAT-1 and GAT-3) in the mouse cerebellar cortex, which are widely localized in neural and glial cells. Firstly, we examined the localization of GATs in the dendrites and cell bodies of developing GABAergic neurons, where GABA is extrasynaptically distributed, to clarify the GABA-diacrine before synaptogenesis. Secondly, we examined the developmental changes in the localization of GATs to reveal the development of the GABA-uptake system. Neither transporter was detected within the dendrites and cell bodies of GABAergic neurons, including Purkinje, stellate, basket and Golgi cells, in the immature cerebellar cortex. GAT-1 was observed within the Golgi cell axon terminals after postnatal day 5 (P5) and presynaptic axons of stellate and basket cells after P7. GAT-3 was localized within the astrocyte processes sealing the GABAergic synapses in the Purkinje cell and granular layers after P10. These results indicated that GABA-diacrine did not work in the mouse cerebellar cortex. The onset of
GAT-1-expression was prior to that of GAT-3. GAT-1 started to be localized within the GABAergic axon terminals during synapse formation. GAT-3 started to be localized within astrocyte processes when they sealed the synapses.

**Classification Terms**

*Theme A:* Development and Regeneration

*Topic:* Neurotransmitter systems and channels

*Key Words:* vesicular GABA transporter, diacrine, exocytosis, GABAergic synapse, astrocyte.
Introduction

In the adult central nervous system (CNS), γ-amino butyric acid (GABA) is a predominant neurotransmitter, mediating fast inhibitory synaptic transmission, and regulating the excitatory activity of neurons [22,25]. Recent studies have revealed that GABA serves as a trophic factor during brain development, inducing morphogenesis, and regulating cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death [4,23,26,41].

In the cerebellar cortex, four of the five types of neurons, Purkinje, stellate, basket and Golgi cells, release GABA as a neurotransmitter [13,21,27]. The stellate cell axons make many GABAergic synaptic contacts with the dendritic shafts of Purkinje cells in the molecular layer. The basket cell axons form GABAergic synapses with Purkinje cell bodies and the initial segment of the Purkinje cell axons in the Purkinje cell layer. Golgi cell axons form inhibitory synapses with granule cell dendrites at the peripheral part of the synaptic glomeruli in the granular layer. GABAergic inputs regulate the neuronal activity of Purkinje and granule cells, which organize the major stream of neural circuitry in the cerebellar cortex. During formation of the GABAergic network in the cortex, mice open their eyes and start moving around [16,39,42]. Eye opening and increasing motor activity imply extensive development of motor control, coordination and learning. Furthermore,
elimination of GABAergic input from the Golgi cells in the cerebellar granular layer causes overexcitation of granule cells resulting in severe ataxia during the acute phase [43]. These results suggest that GABAergic input regulates glutamatergic excitation and might play a crucial role in expression of cerebellar functions such as motor skill learning [13,20,21].

We are morphologically investigating the development of the GABAergic transmission system in the cerebellar cortex. We previously demonstrated the extrasynaptic localization of GABA in developing cerebellar GABAergic neurons, including Purkinje, stellate, basket and Golgi cells [33-35]. Before formation of GABAergic synapse, GABA is localized in the cytoplasm of the dendrites and cell bodies of GABAergic neurons, and is concentrated in the vesicles within the axon varicosities and growth cones. This suggested that GABA could be extrasynaptically released in the developing cerebellum in two ways: (1) exocytosis of GABAergic vesicles from axon varicosities and growth cones and (2) extracellular GABA diacrine from dendrites and cell bodies. Physiological and biochemical studies have demonstrated that the non-vesicular form of GABA is released via the plasma membrane by the reverse transporter action of plasma membrane GABA transporters (GATs) [2,3,11,37,38,41]. In the present study, we examined the localization of GATs in the dendrites and cell bodies of developing cerebellar GABAergic neurons, where GABA is extrasynaptically localized, to reveal the GABA-diacrine system during cerebellar
In previous studies, we also demonstrated the developmental change in expression and localization of the various synaptic elements, including GABA and vesicular GABA transporter (VGAT), in the presynaptic axons and GABA\textsubscript{A} receptors in the postsynaptic membrane during formation of GABAergic synapses [32-36]. In the present study, we examined the developmental change in expression and localization of GATs to clarify the development of the GABA-uptake system in the mouse cerebellar GABAergic synapse.

GATs mediate the GABA-uptake from the synaptic cleft by exchanging Na\textsuperscript{+} and Cl\textsuperscript{-} ions in the mature brain [5,10,17]. Molecular cloning has isolated four GATs: GAT-1, GAT-2, GAT-3 and BGT-1. Mouse GAT2, GAT3 and GAT4 (no hyphen) are species homologs of rat BGT-1, GAT-2 and GAT-3, respectively. Among them, we focused on GAT-1 and GAT-3 (mouse homologues of GAT1 and GAT4, respectively), which are localized widely in neural and glial cells [7,8,14,24,28,29]. Furthermore, we used not the rat [44], but the mouse cerebellum as a comparison with the previous studies demonstrating the extrasynaptic localization of GABA in cerebellar GABAergic neurons [33,35].

We found that GABA might be extrasynaptically released not by diacrine, but by only exocytosis from GABAergic axons in the developing mouse cerebellar cortex. During formation of GABAergic synapses, GABA might be re-uptaken firstly into the presynaptic
terminals via GAT-1 and subsequently into both presynaptic terminals via GAT-1 and astrocyte processes via GAT-3.

2. Materials and Methods

2.1 Animals

We examined mice from the C57Bl/6CrSlc strain at postnatal days 5 (P5), 7, 10, 14, 21 and 90 (as adults). At each postnatal stage, at least five mice were sacrificed for immunohistochemistry.

2.2 Establishment of a guinea pig VGAT antibody

Guinea pigs were immunized by subcutaneous injection of the VGAT peptide (CGDEGAEAPVEGDIHYQRGGA) [30] conjugates with keyhole limpet hemocyanin (KLH). The specific IgG fraction of the synthetic peptide for VGAT was affinity-purified, and the specificity of the antibody was checked as previously described [33]. The immunohistochemistry was identical to that detected by the rabbit antibody [33] and the results in previous studies [6,31,32].

2.3 Tissue preparation

Under deep ether anesthesia, mice were fixed by transcardial perfusion with 4% paraformaldehyde in a phosphate buffer (PB, 0.1M pH 7.4). For light microscopic analysis,
cerebella were cryoprotected with 30% sucrose in PB overnight, then cut into sagittal sections at a thickness of 20\(\mu\)m with a cryostat. The sections were mounted on gelatin-coated glass slides. For electron microscopic analysis, cerebella at P5, P10 and P90 were cut into sagittal sections at a thickness of 100\(\mu\)m with a microslicer (Dosaka, Kyoto, Japan).

2.4 Immunohistochemistry for GAT-1 and GAT-3

Sections on the glass slides were treated as follows; with methanol containing 0.3% \(\text{H}_2\text{O}_2\) for 30 minutes, PB for 10 minutes, 3% normal goat serum in PB for one hour, and an antibody against GAT-1 (diluted 1:1000, Sigma G0157) or GAT-3 (diluted 1:4000, Sigma G8407) overnight at room temperature. After rinsing three times with PB for 15 minutes, sections were visualized by the reaction with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:100, Jackson ImmunoResearch, USA) for two hours at room temperature. After rinsing with PB for 30 minutes, the immunohistochemical staining was observed under a confocal laser-scanning microscope (MRC-1024, BIOLAD).

For the double staining with the VGAT or calbindin antibody, sections, stained as above, were reacted with the guinea pig VGAT (1.5\(\mu\)g/ml) or mouse calbindin antibody (diluted 1:2000, Sigma C9848) overnight, rinsed with PB three times for 15 minutes, and visualized by the reaction with Cy3\textsuperscript{TM}-conjugated anti-guinea pig IgG (diluted 1:200,
Jackson ImmunoResearch, USA) or Cy3™-conjugated anti-mouse IgG (diluted 1:200, Jackson ImmunoResearch, USA), respectively. The stained sections were rinsed with PB for 30 minutes and observed under a confocal laser scanning microscope (MRC-1024, BIOLAD).

For the electron microscopic analysis, floating sections prepared by a microslicer were treated with 3% normal goat serum for 30 minutes and the GAT-1 or GAT-3 antibody overnight at room temperature, and visualized by the ABC method (Histofine SAB-P0(R) Kit, Nichirei Co. Japan) after rinsing three times with PB for 10 minutes. The sections were post-fixed with 1% glutaraldehyde in PB for 30 minutes and 1% OsO₄ in PB for 2 hours at 4°C, stained with 2% uranil acetate aquarius solution overnight, and embedded in epoxy resin as previously reported [33]. 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.

3. Results

3.1 Immunohistochemical localization of GAT-1 and GAT-3 in the adult mouse cerebellar
GAT-1-immunolabeling was observed throughout the cerebellar cortex (Fig. 1A).

GAT-1-positive dots and short lines were scattered in the molecular layer, and surrounded Purkinje cell bodies in the Purkinje cell layer. In the granular layer, the GAT-1-immunolabeling often exhibited ring-shaped profiles. Next, we performed double staining by using a guinea pig antibody against VGAT, which we newly raised, or a mouse calbindin antibody. The GAT-1-immunolabeling was localized at VGAT-positive dots in the molecular layer, basket formation and pinceau in the Purkinje cell layer and ring-shaped profiles at the synaptic glomeruli in the granular layer (Fig. 1B). In contrast, GAT-1-immunolabeling was not observed within the dendrites or cell bodies of Purkinje cells stained with the calbindin antibody (Fig. 1C). GAT-1-positive dots often attached to the dendritic shafts and cell bodies of Purkinje cells. Beneath the Purkinje cell bodies, GAT-1-immunolabeling surrounded the Purkinje cell axons. By electron microscopic analysis, GAT-1-immunolabeling was localized within the presynaptic terminals containing flattened vesicles in the molecular (Fig. 1D), Purkinje cell (Fig. 1E) and granular layers (Fig. 1F-H). In addition, weak immunolabeling was also detected within the processes of astrocytes (Fig. 1D-F). These results indicated that GAT-1 was abundantly localized within presynaptic axon terminals originated from GABAergic neurons, including stellate, basket,
Purkinje and Golgi cells, but was negative in their dendrites and cell bodies.

GAT-3-immunolabeling was detected in the neuropil of the Purkinje cell and granular layers, but was negative in the molecular layer (Fig. 1I). By double staining with the VGAT antibody, GAT-3-immunolabeling was not localized at, but surrounded, the VGAT-positive rings in the granular layer (Fig. 1J). With electron microscopic analysis, the immunolabeling was localized within the astrocyte processes surrounding the synaptic glomeruli (Fig. 1K). By light microscopic observation, the GAT-3-immunolabeling was colocalized with the VGAT-immunostaining at the pinceau (Fig. 1J). Electron microscopic analysis, however, revealed that the distribution of GAT-3-immunolabeling was differ from that of VGAT. GAT-3 was localized at the processes of astrocytes (Fig. 4F), while VGAT was localized at the axon terminals of basket cells [6,31,32]. These results indicated that GAT-3 was localized at the astrocyte processes in the Purkinje cell and granular layers.

These results, as a whole, were identical to previous reports by in situ hybridization histochemistry and immunohistochemistry in the mouse and rat cerebella [8,14,24,28,29].

3.2 Developmental expression of GAT-1 in the mouse cerebellar cortex

We examined the localization of GAT-1 in the developing mouse cerebellar cortex
in the culmen (lobules IV+V) of the vermis.

In the molecular and Purkinje cell layers, GAT-1-immunolabeling (FITC) was detected as dots and short line at P7 (Fig. 2A). During development, the immunolabeling expanded towards the pial surface (Fig. 2A-D). The upper part of the molecular layer, however, continued to be negative by the end of the third postnatal week (Fig. 2A-C). Throughout development, the GAT-1-positive dots and lines were not localized within, but rather attached to, the Purkinje cell dendrites and cell bodies stained with the calbindin antibody (Cy3<sup>TM</sup>) (Fig. 2A-D). Double staining of GAT-1(FITC) and VGAT (Cy3<sup>TM</sup>) demonstrated that GAT-1-positive dots and lines were localized at VGAT-positive dots (Fig. 2E-H). In the upper part of the molecular layer, however, GAT-1-immunolabeling was not detected at the VGAT-positive dots by the end of the third postnatal week (Fig. 2E-G).

In the granular layer, the GAT-1-immunolabing (FITC) was observed as fine dots at P5 near the white matter (Fig. 2I). The immunolabeling was detected in all part of the granular layer at P7 (Fig. 2J), and exhibited ring-shaped profiles after P10 (Fig. 2K-M). At P5, VGAT-positive (Cy3<sup>TM</sup>), but GAT-1-negative dots, were often detected in the upper part of the granular layer (Fig. 2I). After P10, the GAT-1-immunolabeling was completely colocalized with the VGAT-positive dots and rings in the granular layer (Fig. 2I-M).

Electron microscopic analysis revealed that the GAT-1-immunolabeling was
localized within the axons, varicosities and terminals, which contained flattened vesicles, although dendrites and cell bodies were also negative during cerebellar development (Fig. 3A-H). At P5, GAT-1-immunolabeling was observed within the axons and synaptic terminals in the granular layer (Fig. 3A-C). At P10 and P21, immunolabeling was detected in all three layers of the cortex. In the molecular, GAT-1-immunolabeling was detected within the axon terminals forming symmetric synapses with Purkinje cell dendrites (Fig. 3D, F). In the Purkinje cell layers, axon terminals forming synapse with Purkinje cell bodies were positive (Fig. 3G). In the granular layer, presynaptic terminals forming synapses with granule cell dendrites were labeled in the synaptic glomeruli (Fig. 3E, H). Since light microscopic analysis demonstrated that the GAT-1-immunolabeling was colocalized with VGAT-immunolabeling in all three layers of the cortex, GAT-1-positive profiles, which contained flattened vesicles, were considered to be GABAergic axon terminals originated from stellate cells in the molecular layer, basket cells in the Purkinje cell layer and Golgi cells in the granular layer.

These results indicated that throughout development, GAT-1 was densely localized in axon varicosities and terminals of GABAergic neurons, but was negative in their dendrites and cell bodies. During development, VGAT is expressed in the GABAergic axons before the onset of GAT-1-expression. GAT-1 was localized within the Golgi cell
axon terminals after P5 and presynaptic axons of stellate and basket cells after P7.

3.3 Developmental expression of GAT-3 in the mouse cerebellar cortex

Lastly, we examined the localization of GAT-3 in the developing mouse cerebellar cortex. At P10, weak GAT-3-immunolabeling was localized around the VGAT-positive rings at the deep part of the granular layer (Fig. 4A). Electron microscopic analysis revealed that the immunolabeling was localized within the astrocyte processes, which often surrounded the synaptic glomeruli, consisting of mossy fiber terminals, granule cell dendrites and Golgi cell axons (Fig. 4B, C). However, the cell bodies, dendrites, and axons of cerebellar neurons, including Golgi cells, were negative. After P14 and P21, the immunolabeling increased in intensity and the GAT-3-immunolabeling was localized in the neuropil of all part of the granular layer, but was not colocalized with VGAT-immunolabeling (Fig. 4D, E). Electron microscopic analysis revealed that GAT-3 was also detected within the astrocyte processes in the Purkinje cells (Fig. 4F) and granular layers (Fig. 4G), but was not localized within the axons, dendrites or cell bodies.

4. Discussion

In the present study, we investigated the developmental changes in expression and
localization of GAT-1 and GAT-3 in the mouse cerebellar cortex. GAT-1 was detected within the presynaptic axons of Golgi cells in the granular layer after P5, and those of stellate and basket cells in the molecular and Purkinje cell layers after P7. GAT-3 was observed within the astrocyte processes in the Purkinje and granular layers after P10. Neither transporter was detected within the dendrites and cell bodies of GABAergic neurons in the immature cerebellum.

4.1 GABA-releasing mechanism in the developing cerebellar cortex

GABA appears in the CNS long before the onset of synaptogenesis [9,19,40]. During development, GABA mediates excitatory transmission, and plays a role in brain morphogenesis [4,23,26,41]. In previous studies, we demonstrated the extrasynaptic localization of GABA in developing cerebellar GABAergic neurons [33,35]. This result suggests that during development, GABA could be secreted in two ways [34]: (1) exocytosis of GABAergic vesicles from axon varicosities and growth cones and (2) diacrine via the plasma membrane of dendrites and cell bodies. Physiological and cell biological analysis demonstrated that the diacrine is mediated by the reverse action of GATs [2,3,11,37,38,41]. In the present study, however, we could not find GATs in the dendrites or cell bodies in the immature mouse cerebellar GABAergic neurons, including
Purkinje, stellate, basket and Golgi cells, although GABA was localized throughout
GABAergic neurons. Furthermore, the onset of GAT-1- and GAT-3- expression was closely
related to the periods of synapse formation and astocyte-sealing, respectively. These results
suggested that GABA could be released only by exocytosis from axon varicosities and
growth cones, and that GATs are not involved in the diacrine secretion in the mouse
cerebellar cortex.

4.2 Development of the GABA-uptake system

During development of the cerebellum, GABA and VGAT have been already
localized in the GABAergic neurons in the cortex at P3 before the onset of GABAergic
synaptogenesis [33,35]. GABAergic synapses appear in the molecular and Purkinje cell
layers at P5, and are often observed at P7. In the granular layer, GABAergic synapses
appear in the deep part near the prospective white matter at P5, and are often detected
between Golgi cell axons and granule cell dendrites in all layers at P10 [32,35]. In all three
layers, the number of GABAergic synapses massively increases during the second and third
postnatal weeks [1,15,18,35]. During the same period of synaptogenesis, GAT-1 started to
be localized within the axon terminals of GABAergic synapses. Astrocytes, including
Bergmann glia, seal the synapses several days after the initial formation of GABAergic
synapses [1,12,15,18]. During the same period, GAT-3 starts to be expressed and localized within the astrocyte processes in the Purkinje and granular layers.

These results indicated that development of the GABA-uptake system can be classified into three stages, as shown in Figure 5. Before formation of GABAergic synapses, GABA might be released by the fusion of GABAergic vesicles with the membrane at the axon varicosities and growth cones. During this period, GABA-uptake systems are not established and GABA may disappear by diffusion in the extracellular space (Fig. 5A). Subsequently, GAT-1 is distributed within the axons varicosities and terminals. During this period, preliminary GABAergic synapses are being formed. GABA is synaptically released, and reuptaken by GAT-1 into presynaptic axons (Fig. 5B). After synapses were sealed by astrocyte processes, GABA is uptaken and reuptaken from synaptic clefts by both GAT-1 and GAT-3 (Fig. 5C).
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Figure Legends

**Figure 1** Immunohistochemical localization of GAT-1 and GAT-3 in the adult mouse cerebellar cortex

(A) Light microscopic localization of GAT-1. GAT-1-immunolabeling (FITC) was detected in the molecular (Mo), Purkinje cell and granular (Gr) layers. The immunostaining surrounded the Purkinje cell bodies (asterisks) and axons (white arrows), and exhibited ring shaped profiles (white arrowheads) in the granular layer.

(B) Double staining of GAT-1 (FITC) and VGAT (Cy3™). The GAT-1-immunolabeling was colocalized with the VGAT-immunolabeling in the molecular layer (Mo), around the Purkinje cell bodies (asterisks), at the *pinceau* (white arrows) and in the synaptic glomeruli (white arrowheads).

(C) Double staining of GAT-1 (FITC) and calbindin (Cy3™). The GAT-1-immunolabeling was not localized within, but attached to, the dendrites, and surrounded cell bodies (asterisks) and axons (white arrow) of the Purkinje cells.

(D-H) Electron microscopic localization of GAT-1 in the molecular (D), Purkinje cell (E) and granular (F-H) layers. The GAT-1-immunolabeling was localized within the axon terminals of stellate (St in D), basket (Bt in E) and Golgi cells (Gt in F), which contained flatten vesicles (v) and often formed symmetric synapses (black arrowheads) with Purkinje
cell dendrites, cell bodies (Pc in E) and granule cell dendrites (Gd). G and H show the Golgi cell terminals in F at higher magnification. Weak signals (black arrows) were also detected within the astrocyte processes (D-F).

(I) Immunohistochemical localization of GAT-3 (FITC). GAT-3-immunolabeling was detected in the neuropil region in the Purkinje cell (asterisks) and granular (Gr) layers, but was negative in the molecular layer (Mo).

(J) Double staining of GAT-3 (FITC) and VGAT (Cy3\textsuperscript{TM}). In the granular layer (Gr), the GAT-3-immunolabeling was not colocalized with, but surrounded, the VGAT-positive rings (white arrowheads).

(K) Electron microscopic localization of GAT-3 in the cerebellar granular layer. GAT-3-immunolabeling (black arrows) was detected within the processes of astrocytes around the synaptic glomeruli and granule cell bodies (Gc). The mossy fiber terminal (Mf) often form synapses (black asterisks) with granule cell dendrites (Gd).

Abbreviations: nf: neurofilament, Ec: endothelial cell of the blood vessel.

Bars in the electron micrographs= 1\mu m

\textbf{Figure 2} Developmental expression of GAT-1 in the cerebellar cortex.

(A-D) Double staining of GAT-1 (FITC) and calbindin (Cy3\textsuperscript{TM}) in the molecular and
Purkinje cell layers at P7 (A), P10 (B), P14 (C) and P21 (D). At P7, the GAT-1-immunolabeling was observed around and between Purkinje cell bodies (asterisks). At P7 (A), P10 (B) and P14 (C), dense immunostaining was detected mainly in the lower part of the molecular layer, while the upper layer was negative. During development, GAT-1-immunolabeling was not observed within, but attached to, the dendrites and cell bodies of Purkinje cells.

(E-H) Double staining of GAT-1 (FITC) and VGAT (Cy3<sup>TM</sup>) in the molecular (Mo) and Purkinje cell layers at P7 (E), P10 (F), P14 (G) and P21 (H). GAT-1-immunolabeling was localized at VGAT-positive dots. During development, the upper part of the molecular layer was negative with the GAT-1 antibody, although VGAT-positive dots were detected (E-G).

(I-M) Double staining of GAT-1 (FITC) and VGAT (Cy3<sup>TM</sup>) in the granular layer (Gr) at P5 (I), P7 (J), P10 (K), P14 (L) and P21 (M). GAT-1-immunolabeling was observed at the lower part of the granular layer at P5 (I) and localized at the VGAT-positive dots. At the upper part of the granular layer (Gr), however, only VGAT-positive dots were detected (I). After P10, GAT-1-immunolabeling exhibited ring-shaped profiles at the VGAT-positive rings (white arrowheads) (K-M).

**Figure 3** Electron microscopic localization of GAT-1 in the cerebellar cortex at P5 (A-C),
At P5, GAT-1-immunolabeling was localized within the axons and their varicosities, which contained flatten vesicles (v) (A-C). At P10 (D, E) and P21 (F, H), the GAT-1-immunolabeling was detected in the varicosities and terminals of stellate cells (St), Golgi cells (Gt), and basket cells (Bt), which contained flattened vesicles (v). The labeled terminals and varicosities often formed symmetric synapses (arrowheads) with granule cell dendrites (Gd in D, F), Purkinje cells dendrites (Pd in G) and Purkinje cell bodies (Pc in E, H).

Abbreviations: Mf: Mossy fiber terminal, nf: neurofilament, Mf:

Bars in the electron micrographs: 0.5μm

**Figure4** Immunohistochemical localization of GAT-3 in the developing cerebellar cortex.

(A) Double staining of GAT-3 (FITC) and VGAT (Cy3™) in the Purkinje cell (asterisks) and granular layers (Gr) at P10. Weak GAT-3-immunolabeling (white arrows) was detected at the deep part of the granular layer near the synaptic glomeruli.

(B and C) Electron microscopic localization of GAT-3 in the cerebellar granular layer. Immunolabeling was detected within the astrocyte processes (black arrows) around the granule cell bodies (Gc) and the synaptic glomeruli consisting of mossy fiber terminal (Mf),
granule cell dendrites (Gd), and Golgi cell axon terminals (Gt). In the synaptic glomeruli, asymmetric synapses (black asterisks) were formed between granule cell dendrites (Gd) and mossy fiber terminal (Mf).

(D and E) Double staining of GAT-3 (FITC) and VGAT (Cy3TM) in the Purkinje cell (asterisks) and granular layers (Gr) at P14 (D) and P21 (E). GAT-3-immunolabeling was detected in the neuropil and surrounded the VGAT-positive rings (white arrowheads).

(F and G) Electron microscopic localization of GAT-3 in the Purkinje cell (F) and granular layers (G) at P21. In the Purkinje cell layer, the immunolabeling was detected within the processes of astrocytes (black arrows), but was negative in the basket cell axons (Ba) and terminals (Bt), which contained flatten vesicles and neurofilaments (nf) and formed symmetric synapses (black arrowhead) with Purkinje cell body (Pc). In the granular layer, GAT-3-positive astrocyte processes surrounded the granule cell bodies (Gc) and synaptic glomeruli (black arrows in G). In the synaptic glomeruli, asymmetric synapses (black asterisks) were formed between granule cell dendrites (Gd) and mossy fiber terminal (Mf).

Bars in the electron micrographs: 0.5μm

**Figure 5** Schematic illustrations of the developmental changes in the GABA-uptake system.
A) Before synapse formation, GABAergic vesicles accumulate to the axon varicosities. GABA, released from the varicosities by exocytosis, is diffused in the extracellular space and activates GABA receptors in the neighboring neurons.

B) During formation of preliminary GABAergic synapses, GABA is released from the varicosities and terminals by exocytosis, activates the GABA receptors (GABAR) at the postsynapse and is reuptaken into the presynapse by GAT-1.

C) After mature GABAergic synapses were formed, GABA is released from the varicosities and terminals by exocytosis, and activates the GABA receptors (GABAR) at the postsynapse. Released GABA is reuptaken into presynapse by GAT-1 and uptaken into astocytes by GAT-3.
References


