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Research Report

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, γ-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.

Theme: Development and regeneration
Topic: Neurotransmitter systems and channels

Keywords: Vesicular GABA transporter; Diacrine; Exocytosis; GABAergic synapse; Astrocyte

1. 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

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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 synapses, 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) cytosolic 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 development.

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 $GABA_A$ 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^+ and Cl^- 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 reuptaken 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, 0.1 M 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 μm with a cryostat. The sections were mounted on gelatin-coated glass slides. For electron microscopic analysis, cerebella at P5, P10, P21 and P90 were cut into sagittal sections at a thickness of 100 μm 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% H_2O_2 for 30 min, PB for 10 min, 3% normal goat serum in PB for 1 h 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 min, sections were visualized by the reaction with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:100, Jackson ImmunoResearch, USA) for 2 h at room temperature. After rinsing with PB for 30 min, 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 μg/ml) or mouse calbindin antibody