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Normal formation of the postsynaptic elements of GABAergic synapses in the reeler cerebellum

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Abstract

Synaptic transmission mediated by γ -amino butyric acid (GABA) plays an important role in inhibition of glutamatergic excitatory transmission and expression of higher brain functions, such as memory, learning and anxiety. To elucidate mechanisms underlying formation of the postsynaptic elements for GABAergic transmission, we employed the reeler mutant mice in this study. In the reeler cerebellum, abnormal cytoarchitecture and an aberrant environment affect the formation of neural networks and maturation of neurons. We examined the expression and localization of GABA_A receptor α subunits in the reeler cerebellum and determined whether various abnormalities in the reeler mice affected formation of the postsynaptic elements. *In situ* hybridization analysis revealed that the specific expression of α subunit mRNAs in each neuronal type was preserved. Abnormal expression of α subunits was not detected, although GABAergic networks were altered and neuronal maturation was severely disturbed. Immunohistochemistry for the $\alpha 1$ and $\alpha 6$ subunits, which were expressed abundantly in the reeler cerebellum, revealed that both subunit-proteins accumulated at positions adjacent to GABAergic terminals. These results, taken together, suggested that expression of the GABA_A receptor subunits in postsynaptic neurons might be genetically determined, but trafficking and accumulation of the subunit proteins at the GABAergic synapse may be induced by GABAergic innervation.

Classification Terms

Theme D: Neurotransmitters, modulators, transporters, and receptors

Topic: GABA receptors

Keywords: GABA_A receptor, glutamic acid decarboxylase, Purkinje cell, synaptic glomerulus, neuronal maturation

1. Introduction

In the central nervous system (CNS), γ -amino butyric acid (GABA) is a predominant inhibitory neurotransmitter which regulates glutamatergic activity [31, 37, 39, 44, 56]. Recent studies revealed that GABAergic synaptic transmission also controls experience-dependent plasticity in the visual cortex [20], induces long-term potentiation, which is the electrophysiological basis of memory and learning [4, 13, 34, 35, 37, 46], modulates anxiety [41, 48] and generates circadian rhythms [62, 65].

For efficient GABAergic synaptic transmission, it is crucial that GABA_A receptors are targeted to and clustered at an appropriate synaptic site opposite the GABA-releasing site [3, 16, 39, 64]. In the cerebellum, granule cells express the GABA_A receptor $\alpha 1$ and $\alpha 6$ subunit mRNAs and the subunit proteins are targeted to the GABAergic synaptic site in the synaptic glomeruli [28, 47, 69]. Purkinje cells, on the other hand, express only the $\alpha 1$ subunit but not remaining five α subunits and its protein is trafficked to the dendritic shafts and cell bodies opposite the GABAergic terminals. To elucidate the mechanism underlying formation of the postsynaptic elements for GABAergic transmission, in particular expression and targeting of the GABA_A receptors, we employed the reeler mutant mouse. The reeler mouse is a consequence of the reelin gene mutation [10, 21, 43], and suffers from reeling ataxic gait, tremor action, and dystonic posture [12, 18]. In the reeler CNS, there are widespread abnormalities characterized by malposition of neurons [8, 18, 33]. The cytoarchitecture of the reeler cerebellum is quite characteristic [8, 17, 19, 22, 33, 51, 60]. In the outer surface of the cerebellum, molecular and granular layers are present, but the cortical structure is poorly developed. Under the cortical structure, many Purkinje, stellate and Golgi cells are intermingled, and a central cerebellar mass is formed. Malposition of neurons affects the neural circuitry and neuronal maturation [8, 19, 22, 32, 33, 51, 54, 58, 59, 60, 67]. In the cortical surface of the reeler cerebellum, the neural network is mostly preserved, and Purkinje cells normally extend their dendrites towards the pial surface. In the central cerebellar mass, on the other hand, neuronal development is severely disturbed in terms of the synaptic

architecture and dendritic arborization. Purkinje cells directly form synapses with mossy fibers and Golgi cell axons, whereas parallel fibers and axons from stellate and basket cells do not innervate the Purkinje cells. Moreover, multiple innervation from climbing fibers remains in the adult reeler cerebellum. Dendrites of Purkinje cells are poorly developed and extend almost randomly.

In the present study, we examined the expression and localization of the GABA_A receptor α subunits in the reeler cerebellum to clarify the influences of various abnormalities on postsynaptic differentiation. (1) Did abnormalities of neuronal networks affect the expression and accumulation of GABA_A receptor subunits? (2) Did neuronal maturation disturbances affect developmental changes in expression of the GABA_A receptor subunits? Finally, we attempted to elucidate the mechanisms underlying assembly of functional GABAergic synapses.

We found that the specific expression of the α subunit mRNAs in each neuronal type was preserved under the aberrant environment, and that subunit proteins accumulated at sites opposite the GABAergic terminals. Expression of ectopic subunits was not detected, although GABAergic networks were changed and neuronal maturation was severely disturbed in the central cerebellar mass of the reeler cerebellum. Taken together, expression of the GABA_A receptor subunits in postsynaptic neurons may be genetically determined, and GABAergic innervation may subsequently induce trafficking and accumulation of the subunit proteins at the GABAergic synapses.

2. Materials and Methods

2. 1. Animals

Reeler strain (B6C3Fe-a/a-rl) was obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in our laboratory. We examined one-month-old homozygous reeler mutant mice and behaviorally unaffected littermate control mice.

2. 2 in situ hybridization

The 45-mer oligonucleotide probes used for the detection of GABA_A receptor α subunits were as follows:

α 1: complementary to nucleotide residue of 1385-1429 of the α 1 subunit [66];

3'- GAC TAA AAG AAC CTA TTG ATG GGG TGT GGG GGC TTT TAG CTG AGG -5'

α 2: complementary to nucleotide residue of 1216-1260 of the α 2 [66];

3'- TTC AGC TGG CTT GTT CTC TGG CTT CTT GTT CGG TTC TGG CGT CGT -5'

α 3: complementary to nucleotide residue of 1450-1494 of the α 3 [66];

3'- CAG ATA AGT AGC CTT GGG TGA AGC AAT CGC TGT TGG AGT TGA AGA-5'

α 6: complementary to nucleotide residue 1276-1320 of the α 6 [23];

3'- CAG ATG GTA CTT GGA GTC AGA ATG CAC AAC AAT CTC CGC TTC CAG -5'

Under deep ether anesthesia, cerebella were removed from skulls of normal, reeler mutant and littermate mice, and immediately frozen in powdered dry ice. Sagittal and horizontal sections at a thickness of 20 μ m were prepared in a cryostat and mounted on glass slides pre-coated with 4% 3-aminopropyltriethoxysilane in acetone. After fixation with 4% paraformaldehyde in 0.1M phosphate buffer (PB pH 7.4) and pre-hybridization, hybridization was performed at 42°C for 10 hours in a hybridization buffer containing 50% formamide, 0.1M Tris-HCl (pH.7.5), 4 \times SSC (1 \times SSC; 150mM NaCl and 15mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2% sarkosyl, 250 μ g/ml of salmon sperm DNA, 10% dextran sulfate, 0.1M dithiothreitol, and 10⁴ dpm/ μ l of ³⁵S-labeled oligonucleotides. The glass slides were rinsed in 2 \times SSC containing 0.1% sarkosyl for 40 minutes at room temperature and in 0.1 \times SSC containing 0.1% sarkosyl for 80 minutes at 55°C. The sections were exposed to Hyperfilm- β -max (Amersham, Sweden) for three weeks, dipped in nuclear track emulsion (NTB2, Kodak) and exposed for two months.

2. 3. Antibodies against GABA_A receptor α 1 and α 6 subunits

To produce subunit-specific antibodies against the GABA_A receptor α 1 and α 6 subunits, two peptides corresponding to the intracellular loop between the third and fourth transmembrane regions of both subunit proteins were synthesized; PTA TSY TPN LAR GDP

GLA (amino acid residues 342-359 of the $\alpha 1$ subunit) [66], and KAE RQA QTA ATP PVA KSK AS (amino acid residues 324-343 of the $\alpha 6$ subunit) [23]. A cystein residue was introduced at the carboxyl terminal of each synthetic peptide in order to conjugate the peptides with keyhole limpet hemocyanin (KLH). Guinea pigs were immunized at two week-intervals by intraperitoneal injection of peptide/KLH conjugates, which were emulsified in equal amounts of complete Freund's adjuvant. One week after the last injection, an IgG fraction was purified from the antiserum using a Protein G Sepharose column (Pharmacia Biotech, Sweden). The peptide-specific IgG was affinity-purified with a CNBr-activated Sepharose 4B column (Pharmacia Biotech, Sweden).

2. 4. Brain membrane preparation and immunoblot analysis

Under ether anesthesia, cerebella were removed from two-month-old mice skulls and homogenized with a glass-Teflon device in ice-cold Tris buffered saline (10mM, pH7.4) containing 0.32M sucrose, 1mM ethylene glycol tetra acetic acid and 1mM phenyl methyl sulfonyl fluoride. After centrifugation for 10 minutes at 1000g, the supernatant was used as a tissue sample. A tissue sample (50 μ g/lane) solubilized with sodium lauryl sulfate (SDS) sampling buffer, including 2-mercapto ethanol was separated by 10% SDS-polyacrylamide gel electrophoresis, and electro-transferred onto nitrocellulose membranes. After incubation with 5% skim milk in Tris buffered saline (50mM, pH 7.4), the membrane was reacted with antiserum against the $\alpha 1$ or $\alpha 6$ subunit at a concentration of 1 μ g/ml overnight, incubated with alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch laboratory, USA) at a dilution of 1:5000 for two hours, and stained in a substrate solution containing 0.0165% bromochloroindolyl phosphate and 0.033% nitro blue tetrazolium. For the control experiment, the primary antibody was preincubated with the respective peptide (1 μ g/ml) and immunoblot analysis was performed as above.

2. 5. Immunohistochemistry for the GABA_A receptor $\alpha 1$ and $\alpha 6$ subunits

Under deep ether anesthesia, reeler mutant and littermate control mice were fixed by transcardial perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4).

Brains were removed from skulls and immersed in the same fixative overnight. After cryoprotection by immersion with 30% sucrose in PB overnight, cerebella were cut sagittally at a thickness of 20 μ m in a cryostat. Sections mounted on gelatin-coated glass slides were treated as follows; with methanol containing 0.3% H₂O₂ for one hour at room temperature, pepsin solution (1 μ g/ml 0.2N HCl) for ten minutes at 37°C, 3% normal goat serum in PB for one hour at room temperature, and an antibody against the GABA_A receptor α 1 (0.4 μ g/ml) or α 6 subunit (0.2 μ g/ml) overnight at room temperature. Immunoperoxidase staining was performed by the ABC method. For immunofluorescence staining, sections treated as above were visualized by incubation with Cy3TM-conjugated anti-guinea pig IgG diluted 1:200 in PB (Jackson ImmunoResearch, USA) for two hours, and observed under a laser-scanning microscope. For double immunofluorescence staining, sections treated with methanol, pepsin solution and normal goat serum, were incubated in a mixed antibody solution containing glutamic acid decarboxylase (GAD) 65 (diluted 1:2000, Chemicon International Inc. AB5082, USA), GAD67 (diluted 1:4000, Chemicon International Inc. AB108, USA) and α 1 (0.4 μ g/ml) or α 6 (0.2 μ g/ml) antisera overnight at room temperature, and visualized by reaction with a mixed solution containing Cy3TM-conjugated anti-guinea pig IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Jackson ImmunoResearch, USA).

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

3. Results

3. 1. Expression of the GABA_A receptor α subunit mRNAs in the reeler cerebellum

We investigated whether the alteration of networks and disturbance in neuronal maturation affects the expression of the GABA_A receptor α subunits in the reeler cerebellum. To test this, we examined the distribution of GABA_A receptor α subunit mRNAs in the reeler cerebellum by *in situ* hybridization.

First, we examined the distinct distribution of the GABA_A receptor α subunit

mRNAs in the cerebella of one-month-old behaviorally unaffected littermate control mice using the oligonucleotide probes specific to the $\alpha 1$ (Fig. 1A), $\alpha 2$ (Fig. 1B), $\alpha 3$ (Fig. 1C) and $\alpha 6$ (Fig. 1D) subunits. The $\alpha 1$ subunit mRNA was widely detected in the cerebellum, including the molecular layer, Purkinje cell layer, granular layer, and cerebellar nuclei (Fig. 1A). The $\alpha 2$, on the other hand, was weakly and sparsely expressed in the Purkinje cell layer, granular layer, white matter and deep cerebellar nuclei (Fig. 1B). The $\alpha 3$ -signals were faintly detected only in the deep cerebellar nuclei (Fig. 1C). Expression of the $\alpha 6$ subunit was restricted to the granular layer (Fig. 1D). Using bright field observation, we determined which α subunits were expressed in each neuronal cell type in the cerebellar cortex (Fig. 1E-H) and deep cerebellar nuclei (Fig. 1I-L). Stellate (arrows), basket and Purkinje cells (asterisks) expressed the $\alpha 1$ subunit (Fig. 1E) and granule cells expressed both the $\alpha 1$ and $\alpha 6$ subunits (Fig. 1E, H). On the other hand, the $\alpha 2$ subunit was expressed in Bergmann cells (arrowheads, Fig. 1F). Large neurons in the cerebellar nuclei expressed the $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunits (arrowheads, Fig. 1I-L). Identical expression-patterns were obtained with additional non-overlapping oligonucleotide probes and the signals were completely abolished in the presence of a 20-fold excess amount of each unlabeled oligonucleotide (data not shown). These results were also in agreement with previous reports [28, 47, 69].

Next, we examined the distribution of the α subunit mRNAs in the reeler cerebellum. Signals for the $\alpha 1$ subunit mRNA were detected widely in the reeler cerebellum, including the central cerebellar mass (asterisks) and nuclei (Fig. 2B). The $\alpha 2$ mRNA was sparsely localized in the reeler cerebellum, and the signals were very weak (Fig. 2C). The $\alpha 3$ -signals were faintly detected only in the cerebellar nuclei (Fig. 2D). Signals for the $\alpha 6$ subunit were highly expressed in the granular layer, while no significant signals were detected under the white matter (Fig. 2E).

These results demonstrated that specific expression of the α subunit in each neuronal type was preserved under the aberrant environment of the reeler cerebellum.

3. 2. Immunohistochemical localization of the GABA_A receptor $\alpha 1$ and $\alpha 6$ subunits in the reeler cerebellar cortex

We investigated whether the network alterations and disturbances in neuronal maturation affected the trafficking and accumulation of the subunit-proteins at synaptic sites. To address this, we examined the immunohistochemical localization of the $\alpha 1$ and $\alpha 6$ subunits, which were expressed highly in the reeler cerebellum.

First, we newly established subunit-specific antibodies against the GABA_A receptor $\alpha 1$ and $\alpha 6$ subunits. An immunoblot analysis of each antibody showed a single band at the predicted molecular weight, 51kd and 58kd for the $\alpha 1$ and $\alpha 6$ subunits, respectively (Fig. 3A). Each band was abolished after the primary antibody was preincubated with its respective peptide (Fig. 3A). Immunostaining with the $\alpha 1$ - and $\alpha 6$ -antibodies showed characteristic staining in the control cerebellum (Fig. 3B-E). $\alpha 1$ -immunoreactivity was detected widely in the cerebellar cortex and nuclei (Fig. 3B, D), whereas $\alpha 6$ -immunoreactivity was restricted to the granular layer (Fig. 3C, E). These staining patterns were consistent with the distribution of the $\alpha 1$ and $\alpha 6$ subunit mRNAs by *in situ* hybridization (Fig. 1). Preincubation of each antibody with the respective peptide completely abolished the staining (Fig. 3F, G). These results indicated that each antibody specifically bound to the $\alpha 1$ and $\alpha 6$ subunits.

Second, we observed immunohistochemical localization of the GABA_A receptor $\alpha 1$ and $\alpha 6$ subunits in the cerebellar cortex of the littermate mice by immunofluorescence staining. Moreover, we examined the spatial relationship between the localization of the GABA_A receptors and GABAergic terminals by a double labeling of the $\alpha 1$ or $\alpha 6$ subunit and glutamic acid decarboxylase (GAD), which is a synthetic enzyme for GABA and is localized at GABAergic terminals [24, 42, 52, 57]. In the control cerebellar cortex, $\alpha 1$ -immunohistochemistry exhibited fine dots in the molecular and Purkinje cell layers, and ring-shaped profiles in the granular layer (arrowheads, Fig. 4A). Using double staining, $\alpha 1$ -positive dots (Fig. 4D), which were scattered in the molecular layer and surrounded Purkinje cell bodies (asterisks), were localized adjacent to the GAD-positive dots and patches

(Fig. 4C). In the granular layer, $\alpha 1$ -positive rings consisting of finer dots (Fig. 4D) were detected at the identical position to the GAD- positive rings (Fig. 4E). In contrast, $\alpha 6$ -immunoreactivity was detected only in the granular layer as ring-shaped profiles (arrowheads, Fig. 4F). Using double staining, $\alpha 6$ -positive rings (Fig. 4G) were also detected at the identical position of the GAD-positive rings (Fig. 4H). These results demonstrated that the GABA_A receptor $\alpha 1$ subunit accumulated at majority of the GABAergic synapses, while the $\alpha 6$ subunit was confined to GABAergic synapses in the synaptic glomeruli.

Third, we observed localization of the $\alpha 1$ and $\alpha 6$ subunits in the reeler cerebellum at a lower magnification by the immunoperoxidase method. $\alpha 1$ -immunoreactivity was observed widely in the vermis (Fig. 5C) and hemisphere (Fig. 5D), including the molecular layer, granular layer and central cerebellar mass (asterisks) beneath the granular layer and under the white matter, and deep cerebellar nuclei (Fig. 5C, D). In contrast, $\alpha 6$ - immunoreactivity was localized in the granular layer, whereas was absent in the central part (Fig. 5E, F).

Fourth, we examined localization of the $\alpha 1$ and $\alpha 6$ subunits in the reeler cerebellum by immunofluorescence staining. In the cortical surface, $\alpha 1$ -immunohistochemistry was quite similar to that in the cerebellar cortex of the control mice (Fig. 6A, B). The $\alpha 1$ -positive dots were localized in the molecular layer and surrounded the Purkinje cell bodies (asterisks), and $\alpha 1$ -positive ring-shaped profiles (arrowheads) were observed in the granular layer (Fig. 6A, B). In addition, many $\alpha 1$ -positive dots were also detected on the dendrites (arrows) and cell bodies (asterisk) of ectopic Purkinje cells in the granular layer (Fig. 6B). In the central cerebellar mass beneath the granular layer, $\alpha 1$ -positive dots were scattered in the neuropil region and on the somata (asterisks) of Purkinje cells, and ring-shaped profiles (arrowheads) were often detected near the granular layer (Fig. 6C, D). In the white matter, $\alpha 1$ -positive dots were detected on the Purkinje cell dendrites (arrows) extending towards the cerebellar surface (Fig. 6E). Under the white matter, $\alpha 1$ -positive dots were also detected between and on Purkinje cell bodies, whereas the ring-shaped profiles were absent (Fig. 6E, F). On the other hand, majority of the $\alpha 6$ -immunoreactivity exhibited ring-shaped profiles (arrowheads, Fig.

7A-D). The $\alpha 6$ -positive rings (arrowheads) were detected in the granular layer and on the surface of central mass beneath the granular layer of both the vermis and hemisphere (Fig. 7A-D). In the deep part of the central cerebellar mass beneath the granular layer, the $\alpha 6$ -immunoreactivity became weaker and exhibited fragmentation (arrows, Fig. 7B, C). No immunoreactivity was detected under the white matter and in the cerebellar nuclei. These results demonstrated that both $\alpha 1$ and $\alpha 6$ subunit proteins normally accumulated, and no obvious retention of the GABA_A receptors containing $\alpha 1$ and $\alpha 6$ subunits was detected in cell bodies or axons of the reeler cerebellum.

Lastly, we examined the spatial relationship between localization of the GABA_A receptors and GABAergic terminals in the reeler cerebellum by double labeling. In the Purkinje cell and granular layers, GAD-positive dots and large patches, which surrounded Purkinje cell bodies (asterisks), are considered to show a *pinceau* of basket cell axon terminals (Fig. 8A, B) [45]. Ring-shaped profiles stained by GAD-immunohistochemistry show the terminals of Golgi cells in the cerebellar glomeruli. In the white matter and central cerebellar mass, GAD-positive dots detected in the neuropil and on the Purkinje cell bodies (asterisks) (Fig. 8C-F) are thought to include heterologous synapses with Golgi axon terminals and collaterals of Purkinje cell axons [8, 33, 51, 58, 60, 67]. $\alpha 1$ -immunoreactive dots and ring-shaped profiles were completely attached to the GAD-positive dots or patches and rings, respectively, in all regions, including the cortical surface (Fig. 8A), granular layer (Fig. 8B), white matter (Fig. 8C), and central cerebellar mass (Fig. 8D, E). Most of the ring-shaped profiles labeled by $\alpha 6$ -immunohistochemistry, on the other hand, were merged with the GAD-positive rings in the granular layer and the central mass beneath the granular layer (Fig. 8F, G). In the middle of the central cerebellar mass beneath the granular layer, $\alpha 6$ -immunoreactivity exhibiting fragmentation was attached to the GAD-positive rings (arrows, Fig. 8G). These results demonstrate that GABA_A receptors containing the $\alpha 1$ and $\alpha 6$ subunits were targeted normally to synaptic sites and accumulated adjacent to the GABAergic terminals in the reeler cerebellum.

4. Discussion

To establish efficient GABAergic synaptic transmission, it is crucial that both pre- and post- synaptic elements mature [3, 16, 39, 64]. Maturation of postsynaptic elements requires that GABA_A receptor subunit-genes are expressed, the subunit-proteins are trafficked and the receptors are clustered at appropriate places adjacent to the GABA-releasing sites. Within the aberrant environment of the reeler cerebellum, each neuron expressed the same GABA_A receptor α subunits as in the normal cerebellum. Both $\alpha 1$ and $\alpha 6$ subunit proteins were trafficked and clustered opposite the GABAergic terminals.

4. 1. Regulatory mechanisms underlying the expression of the GABA_A receptor α subunits

We examined expression of the GABA_A receptor α subunits under aberrant conditions of the reeler cerebellum, and investigated the following two issues. First, we investigated the relationship between presynaptic neurons and α subunits in the postsynaptic neurons. In the normal cerebellum, Purkinje cells receive GABAergic input from stellate and basket cells at dendritic shafts and cell bodies [45], GABAergic transmission at Purkinje cell synapses is mediated by GABA_A receptors containing only the $\alpha 1$ subunit but not the remaining five α subunits [28, 47, 69]. In contrast, Golgi cell axons form synapses with granule cell dendrites within the synaptic glomeruli of the granular layer, and their GABAergic transmission is mediated by GABA_A receptors containing both $\alpha 1$ and $\alpha 6$ subunits. In the central cerebellar mass of the reeler cerebellum, however, Purkinje cells directly form synapses with Golgi cell axons [8, 33, 51, 58, 60, 67]. If presynaptic neurons determine the type of receptor subunits in the postsynaptic neurons, GABAergic innervation from Golgi cells would induce Purkinje cells to express $\alpha 6$ subunit in the central cerebellar mass. Nevertheless, Purkinje cells in the central cerebellar mass did not express the $\alpha 6$ subunit. Therefore, Golgi cell innervation does not induce expression of the $\alpha 6$ subunit in Purkinje cells. These results suggest that postsynaptic self-autonomous manners, but not

presynaptic neurons, determine the types of subunits.

Second, we investigated the relationship between neuronal maturation and developmental changes in expression of the subunits. In the case of malpositioned Purkinje cells in the reeler cerebellum, neuronal maturation was assumed to be arrested due to lack of excitatory input from parallel fibers and inhibitory input from stellate and basket cells, presence of climbing fibers' multiple innervation, and undeveloped dendritic arborization [8, 17, 19, 22, 33, 51, 59, 60]. Moreover, maturation of other neurons may be disturbed in the central cerebellar mass. Early in cerebellar development, $\alpha 2$ and $\alpha 3$ subunits are abundantly expressed [2, 3, 4, 5, 29, 53, 63]. Upon maturation, expression of the $\alpha 2$ and $\alpha 3$ subunits decrease dramatically and the $\alpha 1$ and $\alpha 6$ subunits start to be expressed in the cerebellum [29]. If neuronal maturation initiates changes in subunit composition of the GABA_A receptors, higher expression of the $\alpha 2$ and $\alpha 3$ subunits should continue in the reeler cerebellum. Nevertheless, expression of the $\alpha 2$ and $\alpha 3$ subunits was as low as that in the normal cerebellum and furthermore, ectopic Purkinje cells expressed the $\alpha 1$ subunit at high level [14] as well as other molecules [22, 30, 61, 70]. These results suggest that developmental changes in subunit composition are independent of neuronal maturation, such as settling in the normal neuronal positions, maturation of excitatory networks, and formation of inhibitory synapses with basket and stellate cells. Therefore, as reported previously using culture granule cells [3, 6, 7, 11, 15, 36, 49, 55], $\alpha 1$ -expression may be initiated by GABAergic innervation from Golgi cells and neighboring Purkinje cells in the central cerebellar mass. In addition, the suppression of $\alpha 2$ and $\alpha 3$ subunit-expression might be related to the GABAergic innervation.

4. 2. Regulatory mechanism underlying the targeting and accumulation of the GABA_A receptor α subunit proteins at the GABAergic synapse

We examined the immunohistochemical localization of the GABA_A receptor $\alpha 1$ and $\alpha 6$ subunits and their spatial relationship with the GABAergic terminals in the reeler cerebellum, and investigated the specificity of subunit localization. In the normal cerebellum,

stellate and basket cell axon terminals form synapses not with spines, but with shafts of the proximal dendrites and cell bodies of Purkinje cells [45]. In the central cerebellar mass of the reeler cerebellum, on the other hand, GABAergic terminals, which are thought to originate from Golgi cells or neighboring Purkinje cells, form synapses with dendritic spines [8, 33, 51, 58, 59, 60, 67]. If the destination of subunit proteins is determined genetically, the $\alpha 1$ subunit might be localized independently of GABAergic terminals. Nevertheless, the $\alpha 1$ subunit was localized opposite majority of the GABAergic terminals in the reeler cerebellum. Moreover, the $\alpha 6$ subunit was detected only within the synaptic glomeruli, where Golgi cell axons were innervated. These results supported that the previous report [9] and suggested that GABAergic synapse-formation might induce targeting of the receptor subunits to the synaptic site, and prevent retention in the cell bodies and axons *in vivo*.

4. 3. Cytoarchitecture of the reeler cerebellum revealed by GABA_A receptor channel immunohistochemistry

In the present study, we revealed the distribution of granule cells in the reeler mice by *in situ* hybridization and immunohistochemistry for the $\alpha 6$ subunit, which is specifically expressed in granule cells [28, 68]. In the reeler cerebellum, $\alpha 6$ -mRNA and protein were localized in the granular layer and surface of the central cerebellar mass beneath the granular layer. During normal cerebellar development, granule cells are born in the external granular (germinal) layer beneath the pia matter, cross the molecular layer and settle within the internal granular layer [1, 25, 26, 27, 38, 40, 50]. Our results suggest that granule cells migrate into the surface of the central cerebellar mass beneath the granular layer in the reeler cerebellum, and complete synaptic glomeruli are formed there.

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Figure Legends

Figure 1 Distinct expression of the GABA_A receptor α subunits in the control cerebellum.

(A-D) Dark field micrographs showing the distribution of the $\alpha 1$ (A), $\alpha 2$ (B), $\alpha 3$ (C) and $\alpha 6$ (D) subunit mRNAs.

(E-H) Bright field micrographs showing the distribution of the $\alpha 1$ (E), $\alpha 2$ (F), $\alpha 3$ (G) and $\alpha 6$ (H) subunit mRNAs in the cerebellar cortex. The $\alpha 1$ -signals were detected in the stellate cells (arrows), Purkinje cells (asterisks), and granular layer (E). The $\alpha 2$ -signals (arrowheads) were accumulated between Purkinje cell bodies (asterisks) (F).

(I-L) Bright field micrographs showing the distribution of the $\alpha 1$ (I), $\alpha 2$ (J), $\alpha 3$ (K) and $\alpha 6$ (L) subunit mRNAs in the cerebellar nucleus. The $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -signals were localized at the large neurons (arrowheads).

Abbreviations: IC: inferior colliculus, Mo: molecular layer, Pu: Purkinje cell layer, Gr: granular layer, Nu: cerebellar nucleus, WM: white matter

Figure 2 Distinct expression of the GABA_A receptor α subunit in the reeler cerebellum.

(A) Sagittal section of the reeler hemisphere stained with Toluidine blue.

(B-D) Darkfield micrographs showing the distinct expression of the GABA_A receptor $\alpha 1$ (B), $\alpha 2$ (C), $\alpha 3$ (D), and $\alpha 6$ (E) subunit mRNAs in sagittal sections of the reeler cerebellum.

Abbreviations and symbols: Mo: molecular layer, Gr: granular layer, WM: white matter, asterisks: central cerebellar mass beneath the granular layer, CM: central cerebella mass under the white matter, Nu: cerebellar nucleus

Figure 3 Immunohistochemistry for the $\alpha 1$ and $\alpha 6$ subunits in the control cerebellum.

(A) Immunoblot analysis with antibodies against the $\alpha 1$ subunit ($\alpha 1$), antibodies against the $\alpha 1$ preincubated with the $\alpha 1$ -peptide, which was used for immunization ($\alpha 1$ +pep), antibodies against the $\alpha 6$ ($\alpha 6$), and antibodies against the $\alpha 6$ preincubated with the $\alpha 6$ -peptide ($\alpha 6$ +pep). The position and molecular weights of standards (kd) are shown on the right.

(B and C) Sagittal sections of the control mouse cerebellum stained with antibodies against the $\alpha 1$ (B) and $\alpha 6$ (C) subunits.

(D-G) Higher magnification micrographs of the immunohistochemistry for the $\alpha 1$ (D) and $\alpha 6$

(E) subunits in the cortex of culmen. Each immunoreactivity was diminished after the primary antibodies was preincubated with the respective peptides (F. G).

Abbreviations: Mo: molecular layer, Gr: granular layer, WM: white matter, Nu: cerebellar nucleus

Figure 4 Immunohistochemical localization of the GABA_A receptor α 1 and α 6 subunits in the control cerebellar cortex.

(A) Immunohistochemistry for the α 1 subunit in the cerebellar cortex.

(B and C) Double staining of the α 1 (B) and GAD (C) in the molecular (Mo) and Purkinje cell (Pu) layers.

(D and E) Double staining of the α 1(D) and GAD (E) in the granular layer.

(F) Immunohistochemical localization of the α 6 subunit in the cerebellar cortex.

(G and H) Double staining of the α 6 (G) and GAD (H) in the granular layer.

Abbreviations and symbols: Mo: molecular layer, Pu: Purkinje cell layer, asterisk: position of Purkinje cell body, thick white arrows: *pinceau* of basket cell terminal, Gr: granular layer, arrowhead and a-m: ring-shaped profile in the synaptic glomerulus

Figure 5 Immunohistochemistry for the α 1 and α 6 subunits in the reeler cerebellum.

(A and B) Sagittal sections of vermis (A) and hemisphere (B) stained with Toluidine Blue.

(C and D) Immunohistochemistry for the α 1 subunit in the vermis (C) and hemisphere (D).

(E and F) Immunohistochemistry for the α 6 subunit in the vermis (E) and hemisphere (F).

Abbreviations and symbols: Mo: molecular layer, Gr: granular layer, WM: white matter, asterisks: central cerebellar mass beneath the granular layer, CM: central cerebella mass under the white matter, Nu: cerebellar nucleus

Figure 6 Immunohistochemical localization of the GABA_A receptor α 1 subunit in the reeler cerebellum observed by an immunofluorescence staining.

(A) Cerebellar surface consisting of three layers.

(B) Granular layer.

(C and D) Central cerebellar mass (CM) beneath the granular layer (Gr).

(E) White matter (WM) and central cerebellar mass (CM).

(F and G) Deep part of the central cerebellar mass (CM).

(G, H) Sagittal section of the reeler vermis (G) and hemisphere (H) stained with Toluidine Blue showing the place of each micrograph in Figure 6 and Figure 8A-E.

Abbreviations and symbols: Mo: molecular layer, asterisk: position of Purkinje cell body, Gr: granular layer, arrowheads: ring-shaped profiles in the synaptic glomerulus, CM: central cerebella mass, arrows: $\alpha 1$ -positive dots on the Purkinje cell dendrites, WM: white matter.

Figure 7 Immunohistochemical localization of the $\alpha 6$ subunit in the cerebellum.

(A) Cerebellar surface consisting of three layers.

(B-D and F) Granular layer (Gr) and central cerebellar mass (CM) beneath the granular layer.

(H and I) Sagittal sections of the reeler vermis (H) and hemisphere (I) stained with Toluidine Blue showing the place of each micrograph in Figure 7 and Figure 8F, G.

Abbreviations and symbols: Mo: molecular layer, Gr: granular layer, arrowheads: ring-shaped profiles in the synaptic glomerulus, CM: central cerebella mass, arrows: $\alpha 6$ -positive fragment

Figure 8 Double labeling of GAD and the $\alpha 1$ (A-E) or $\alpha 6$ (F, G) in the reeler cerebellum.

(A) Cerebellar surface consisting of three layers (Gr).

(B) Granular layer.

(C) White matter.

(D and E) Deep part of the central cerebellar mass.

(F and G) Granular (Gr) layer and central cerebellar mass (CM) beneath the granular layer.

Abbreviations and symbols: Mo: molecular layer, asterisk: position of Purkinje cell body, Bt: *pinceau* of basket cell terminal, Gr: granular layer, arrowheads: ring-shaped profiles in the synaptic glomerulus, CM: central cerebella mass, arrows: $\alpha 1$ -positive dots on the Purkinje cell dendrites (C), and $\alpha 6$ -positive fragment (G).















