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Author(s)	Takayama, Chitoshi; Inoue, Yoshiro
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Research Report

Extrasynaptic localization of GABA in the developing mouse cerebellum

Chitoshi Takayama* and Yoshiro Inoue

Department of Molecular Neuroanatomy, Hokkaido University School of Medicine, Sapporo
060-8638, Japan

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To whom correspondence and proofs should be sent:

Chitoshi Takayama

Department of Molecular Neuroanatomy, Hokkaido University School of Medicine,
Kita-15 Nishi-7, Kita-Ku, Sapporo 060-8638, Japan

Phone +81-11-706-5028

Fax. +81-11-706-7863

E-mail takachan@med.hokudai.ac.jp

Abstract

In the adult brain, γ -amino butyric acid (GABA) is synaptically released and mediates inhibitory transmission. Recent studies have revealed that GABA is a trophic factor for brain development. To reveal the distribution of GABA and its secretion mechanisms during brain development, we investigated the immunohistochemical localization of two molecules, GABA and vesicular GABA transporter (VGAT), which is a GABAergic vesicle protein, in the developing mouse cerebellum by means of newly developed antibodies. Furthermore, we tested the relationship between developmental changes in distribution of above two molecules in the presynapses and ontogeny of GABAergic synapses. GABAergic synapses were detected by immunohistochemistry for the GABA_A receptor α 1 subunit, which is an essential subunit for inhibitory synaptic transmission in the mature cerebellar cortex. Until postnatal day 7 (P7), GABA was localized throughout the GABAergic neurons, and VGAT accumulated at axon varicosities and growth cones, where the α 1 subunit did not accumulate. After P10, both GABA and VGAT became confined to the terminal sites where the α 1 subunit was localized. These results suggested that GABA was extrasynaptically released from axon varicosities and growth cones by vesicular secretion 'exocytosis' and from all parts of GABAergic neurons during the cerebellar development by non-vesicular secretion 'diacrine'.

Key Words: Vesicular GABA transporter, GABA_A receptor, Purkinje cell, Golgi cell, GABAergic synapse, diacrine, exocytosis, calbindin

1. Introduction

In the adult central nervous system (CNS), γ -amino butyric acid (GABA) is a predominant neurotransmitter which is synaptically released, mediates fast inhibitory synaptic transmission, and regulates excitatory activity of neurons (Olsen and Tobin, 1990; Macdonald and Olsen, 1994). Recent studies have revealed that GABA serves as an excitatory transmitter during brain development, and induces trophic responses, such as regulation of cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death (Varju et al., 2001; Ben-Ari, 2002; McCarthy et al., 2002; Owens and Kriegstein, 2002).

In previous studies, we demonstrated that granule and Purkinje cells expressed the GABA_A receptor $\alpha 2$ and $\alpha 3$ subunits, respectively, after final mitosis until finishing maturation (Takayama and Inoue, 2004c). While GABAergic synapses were formed in the cerebellar cortex, the $\alpha 2$ and $\alpha 3$ subunits disappeared from the granule and Purkinje cells, the $\alpha 1$ and $\alpha 6$ subunits started to be expressed and the subunit proteins were confined to synaptic sites (Takayama and Inoue, 2004b). In the granular layer, glutamic acid decarboxylase (GAD), which is a synthetic enzyme of GABA, was localized throughout the Golgi cells during the first postnatal week and became confined to the synaptic glomeruli during synaptogenesis (Takayama and Inoue, 2004b). These results as a whole suggested that during cerebellar development, GABA might be extrasynaptically secreted from GABAergic neurons, and play roles in cerebellar morphogenesis by activating GABA_A receptors, whose compositions differ from those in the mature cerebellum (Takayama and Inoue, 2004a).

In the present study, we focused on two points. The first was which neurons secrete GABA for differentiating and maturing neurons in the developing cerebellar cortex. Previous studies using GAD-immunohistochemistry did not directly reveal the localization of

GABA (McLaughlin et al., 1975; Takayama and Inoue, 2004b). In the granular layer, distribution of GAD was almost the same as that of GABA (Oertel et al., 1981; Ottersen and Storm-Mathisen, 1984; Ribak and Roberts, 1990; Takayama, 1994). In the molecular and Purkinje cell layers, however, GABA is absent in the dendrites and cell bodies of Purkinje cells, although GAD is localized throughout the Purkinje cells. Therefore, we investigated the developmental changes in localization of GABA itself in the cerebellum. The second was how GABA is released from the GABAergic neurons during development. The mechanism of GABA-secretion is classified into two types (Jaffe and Vaello, 1988; Taylor et al., 1990; Taylor and Gordon-Weeks, 1991; Attwell et al., 1993; Gao and van den Pol, 2000). One is exocytosis of GABAergic vesicles, which is the main process of synaptic transmission in the mature cerebellum. The other is non-vesicular secretion which is called 'diacrine' by the reverse action of GABA transporters (GATs). Distribution of vesicular secretion 'exocytosis' in the immature cerebellum was examined by immunohistochemistry for the vesicular GABA transporter (VGAT), which is localized in the membrane of GABAergic vesicles, and loads synthesized GABA into the vesicles (McIntire et al., 1997; Fon and Edwards, 2001). The distribution of diacrine was identified as the localization of GABA, where VGAT was not accumulated. Here, we investigated the distribution of GABA and VGAT in the developing cerebellum, and revealed the developmental changes in localization of GABA and its secretion mechanisms. Furthermore, we tested the relationship between developmental changes in the distribution of the above two molecules in the presynapses and ontogeny of GABAergic synapses. The GABAergic synapses were detected by immunohistochemistry for the GABA_A receptor $\alpha 1$ subunit since the $\alpha 1$ subunit is considered to be an essential subunit for inhibitory synaptic transmission in the matured cerebellar cortex. The $\alpha 1$ subunit is the most dominant α subunit in all types of mature cerebellar neurons, and appears when and

where GABAergic synapses are formed (Laurie et al., 1992; Merlo et al., 2000; Takayama and Inoue, 2003; Takayama and Inoue, 2004b).

We found that in the immature cerebellum, GABA was localized throughout the GABAergic neurons including dendrites, cell bodies, axons, axon varicosities and growth cones. VGAT was confined to the axon varicosities and growth cones where GABA receptor $\alpha 1$ subunit was not yet localized. These results suggested that GABA might be extrasynaptically released via the plasma membrane from all parts of GABAergic neurons by diacrine and from axon varicosities and growth cones by exocytosis during cerebellar development. In the molecular layer, Purkinje cells secreted GABA by diacrine, and stellate and basket cells release it by exocytosis. In the granular layer, Golgi cells secrete GABA by both mechanisms.

2. Materials and Methods

2.1. Establishment of antibodies against GABA and VGAT

GABA was conjugated to bovine serum albumin (BSA) by glutaraldehyde as described in a previous paper (Takayama, 1994). A 21-mer peptide, CGDEGAEAPVEGDIHYQRGGA, which included amino acid residues 44-64 of the vesicular GABA transporter (VGAT) (Sagne et al., 1997), was conjugated with keyhole limpet hemocyanin (KLH).

Rabbits were immunized at two week-intervals by subcutaneous injection of conjugates (100 μ g of GABA or 1 μ g of VGAT peptide), which were emulsified in equal amounts of complete Freund's adjuvant. Two weeks after the last injection, IgG fractions were purified from the antiserum using a Protein G Sepharose column (Pharmacia Biotech, Sweden). The specific IgG fraction for GABA or synthetic peptide for VGAT was

affinity-purified with a CNBr-activated Sepharose 4B column (Pharmacia Biotech, Sweden).

The specificity of the GABA antibody was verified by dot blot analysis. Various conjugated amino acids (0.1µg amino acid/spot), including GABA, β-alanine (β-Ala), glycine (Gly), glutamic acid (Glu), and aspartic acid (Asp), were blotted onto nitrocellulose membranes. After incubation with 5% skim milk in Tris buffered saline (TBS, 50mM, pH 7.4), the membrane was reacted with antiserum against GABA at a concentration of 0.5µg/ml TBS overnight, incubated with an 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. The specificity of the VGAT antibody was checked by Western blot analysis, as described in a previous report (Takayama and Inoue, 2003). A tissue sample (50µg/lane) solubilized with a 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 TBS, the membrane was reacted with the VGAT antibody (1µg/ml TBS) and visualized as above.

2.2. Animals

We examined mice from the C57Bl/6CrSlc strain at postnatal days 3, 5, 7, 10, 14, 21 and 90 (as adults). At each postnatal stage, five mice were sacrificed for immunohistochemistry.

2.3. Tissue preparation

Under deep ether anesthesia, mice were fixed by transcardial perfusion with 4% paraformaldehyde with 0.5% glutaraldehyde in a phosphate buffer (PB, 0.1M pH 7.4) for GABA-immunohistochemistry or 4% paraformaldehyde in PB for VGAT-immunohistochemistry. For light microscopic analysis, cerebella were cryoprotected

with 30% sucrose in PB overnight, and were cut into sagittally at a thickness of 20 μ m by a cryostat. The sections were mounted on gelatin-coated glass slides. For electron microscopic analysis, cerebella at postnatal day 5 (P5) were cut into sagittal sections at a thickness of 100 μ m by a microslicer (Dosaka, Kyoto, Japan).

2.4. Immunohistochemistry for GABA

Sections on the glass slides were treated as follows; with methanol containing 0.3% H₂O₂ for 30 minutes, 0.2% Gly in PB for 30 minutes, 1% sodium borohydrate in PB for 30 minutes (Kosaka et al., 1986), 3% normal goat serum in PB for one hour, and GABA antibody (0.2 μ g/ml) overnight at room temperature. Sections were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:250, Jackson ImmunoResearch, USA). For electron microscopic analysis, free floating sections, prepared by a microslicer, were treated with Gly, sodium borohydrate, normal goat serum and GABA antibody, visualized by peroxidase staining by ABC method (Histofine SAB-P0(R) Kit, Nichirei Co. Japan), post-fixed with 1% glutaraldehyde in PB and 1% OsO₄ in PB for 30 minutes and 2 hours, respectively, at 4°C. After staining with 2% uranyl acetate aqueous solution overnight, the sections were embedded in epoxy resin in the usual manner. Ultra thin sections at the culmen were observed under an electron microscope.

2.5. Immunohistochemistry for VGAT

Sections on the glass slides were treated as follows; with methanol containing 0.3% H₂O₂ for 30 minutes, 3% normal goat serum in PB for one hour, and VGAT antibody (1 μ g/ml) overnight at room temperature. Sections were visualized by incubation with FITC-conjugated anti-rabbit IgG. For the double staining with the calbindin antibody, sections treated as above, were reacted with the calbindin antibody (diluted 1:2000, Sigma), and visualized by the reaction with Cy3TM-conjugated anti-mouse IgG (Jackson ImmunoResearch,

USA). For the double staining with the GABA_A receptor α 1 subunit antibody, sections mounted on gelatin-coated glass slides were treated as follows; with methanol containing 0.3% H₂O₂ for 30 minutes 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 a mixed antibody solution containing VGAT antibody (1 μ g/ml) and the α 1 subunit antibody (0.4 μ g/ml) (Takayama and Inoue, 2003). The sections were visualized by incubation with a mixed solution containing FITC-conjugated anti-rabbit IgG and Cy3TM-conjugated anti-guinea pig IgG at room temperature. For electron microscopic analysis, free floating sections, prepared by a microslicer, were reacted with the VGAT antibody and were stained by the ABC method. Ultra-thin sections were prepared as above.

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

3. Results

3.1. Immunohistochemical localization of GABA and VGAT in the mature cerebellar cortex

We newly established antibodies against two molecules, GABA and VGAT, which are major elements in the presynapse for GABAergic transmission.

Dot blot analysis revealed that the GABA antibody bound GABA-conjugates, but did not react with other conjugates, including β -alanine, aspartic acid, glutamic acid and glycine (Fig.1A). In the mature cerebellar cortex, GABA-immunolabeling was detected in the cell bodies and axon terminals of stellate, basket and Golgi cells (Fig.1B). In addition, *pinceau* of basket cell axon terminals (Palay and Chan-Palay, 1974) also labeled by GABA-immunohistochemistry (arrows in Fig. 1B). Cell bodies and dendrites of Purkinje cells,

however, were negative. This immunolabeling pattern was identical to that demonstrated in previous studies (Ottersen and Storm-Mathisen, 1984; Seguela et al., 1984). Preincubation of the primary antibody with GABA completely abolished the staining (Fig.1C).

Immunoblot analysis for the VGAT antibody showed a single band at the predicted molecular weight, 51kd. The band was abolished when the primary antibody was preincubated with the peptide, which was used for the immunization (Fig.1D). In the mature cerebellar cortex, the VGAT-immunolabeling exhibited fine dots in the molecular and Purkinje cell layers, and ring-shaped profiles in the granular layer, whereas cell bodies of stellate, basket, Purkinje and Golgi cells were negative (Fig.1E). In addition, *pinceau* of basket cell axon terminals (Palay and Chan-Palay, 1974) also labeled by VGAT-immunohistochemistry (arrows in Fig. 1E). This staining pattern was identical to that in previous report (Chaudhry et al., 1998; Takamori et al., 2000). Preincubation of the primary antibody with the synthetic peptide completely abolished the staining (Fig.1F).

These results indicated that each antibody specifically binds to GABA or VGAT.

3.2. Developmental changes in immunohistochemical localization of GABA and VGAT

To detect the distribution of GABA in the cytoplasm and vesicles in the immature CNS, we used immunohistochemistry for GABA and VGAT in the developing cerebellum. Furthermore, we performed double staining with calbindin, which is a marker protein of Purkinje cells (Yamakuni et al., 1984), or the GABA_A receptor α 1 subunit, which is an essential component of the GABA_A receptors at the GABAergic synapses in mature cerebellar neurons (Laurie et al., 1992; Merlo et al., 2000; Takayama and Inoue, 2003).

3.2.1. First postnatal week

During the first postnatal week, GABA-immunolabeing was moderately detected in

the dendrites and cell bodies of Purkinje cells (Fig. 2A-C). In the molecular layer, GABA-positive dots were observed between Purkinje cell dendrites at P7 (Fig.2C). In the internal granular layer, dense immunolabeling was detected in the cell bodies, dendrites, axon plexi, and axon varicosities of Golgi cells. GABA-positive mesh and the plexi of Golgi axons developed markedly during the first postnatal week (Fig. 2A-C).

Throughout development, VGAT-immunolabeling exhibited fine dots in the molecular, Purkinje cell and internal granular layers, but was negative in the dendrites and cell bodies of stellate, basket, Purkinje and Golgi cells (Fig.2D-F). The density of the dots increased during the development in throughout the cortex. By double staining with calbindin, VGAT-positive dots were localized on and between cell bodies and dendrites of Purkinje cells (arrowheads) in the molecular and Purkinje cell layers, and on the axon collaterals of Purkinje cells (arrows) in the upper part of the internal granular layer stained with calbindin antibody (Fig. 2G). In the internal granular layer, however, majority of the dots were not localized on the main axons (arrows) of Purkinje cells (Fig.2H). This indicated that VGAT is distributed not on the Purkinje cell axons but Golgi cell axons. By double staining with the GABA_A receptor $\alpha 1$ subunit, $\alpha 1$ -immunolabeling was not detected in the cerebellar cortex at P3 (Fig. 2I). At P7, many VGAT-positive dots were merged with $\alpha 1$ -positive dots in the molecular and Purkinje cell layers (Fig. 2J), but almost no $\alpha 1$ -immunolabeling was detected in the internal granular layer (Fig. 2K).

Furthermore, we examined the electron microscopic localization of GABA and VGAT in the cerebellar cortex at P5. GABA-immunolabeling was diffusely and widely distributed within the GABAergic neurons (Fig. 3). In the molecular layer (Fig. 3A, B), GABA was localized within dendritic shafts and spines of Purkinje cells, and stellate cell axons. In the granular layer (Fig. 3C-F), dendrites, axons and terminal like structures of Golgi

cells were densely labeled. In addition, GABA was localized within growth cones (GC) which contained large vacuoles and a smooth endoplasmic reticulum (De Cerro and Snider, 1968; Kawana et al., 1971) (Fig. 3D). These results in the granular layer are in agreement with previous reports of GAD-immunohistochemistry (McLaughlin et al., 1975; Takayama and Inoue, 2004b). VGAT-immunolabeling was detected around synaptic vesicles (Fig. 4). In both molecular (Fig. 4A-C) and internal granular (Fig. 4D-G) layers, VGAT-positive vesicles were detected at the axon varicosities between VGAT-negative axons (Fig. 4C, F) and growth cones which included large vacuoles and a smooth endoplasmic reticulum (De Cerro and Snider, 1968; Kawana et al., 1971) (Fig. 4A, B, D, E). Furthermore, majority of the profiles, which contained VGAT-positive vesicles, were not yet formed obvious symmetric synapses.

These results indicated that GABA was localized throughout the cytosol of GABAergic neurons during the first postnatal week. In contrast, GABAergic vesicles were confined to the axon varicosities and growth cones, which had not yet formed synapses.

3.2.2. Second and third postnatal week

In the molecular layer at P10, Purkinje dendrites and cell bodies were still moderately GABA-positive, and many fine dots were observed between Purkinje dendrites (Fig.5A). At P14 (Fig. 5B) and P21 (Fig. 5C), immunolabeling disappeared from dendrites and cell bodies of Purkinje cells, while axon terminals and cell bodies of stellate cells in the molecular layer and axon terminals of basket cells, including *pinceau* (Palay and Chan-Palay, 1974) around the Purkinje cell bodies became clearly discernible.

During the second postnatal week, the density of VGAT-positive dots also gradually increased in both the molecular and Purkinje layers (Fig. 5D-F). The VGAT-positive dots were almost the same as those of GABA-positive dots at P14 (Fig. 5E) and P21 (Fig. 5F). By

double staining with the $\alpha 1$ subunit, more than half of the VGAT dots merged with the $\alpha 1$ -positive dots at P10 (Fig. 5G), and most of the dots were stained with both antibodies at P21 (Fig. 5H) in the molecular and Purkinje layers.

In the granular layer at P10, GABA-immunolabeling was detected in the mesh and plexi of Golgi cell axons (Fig. 6A). During the second and third postnatal week, GABA-immunolabeling became confined to the terminals at the glomeruli (arrowheads), which exhibited ring shaped profiles (Fig. 6B-C). During the same period, VGAT-immunolabeling exhibited ring shaped profiles consisting of fine dots (Fig. 6D-F). By double staining with calbindin, VGAT-positive dots were localized on the collateral axons of Purkinje cells and their terminals around Purkinje cell bodies at P10 (Fig. 6G) and P21 (Fig. 6H). At the synaptic glomeruli, $\alpha 1$ -positive rings merged to VGAT- positive rings at P10 (Fig. 6I) and P21 (Fig. 6J).

These results indicated that during the second and third postnatal week, GABA disappeared from the cell bodies and dendrites of Purkinje cells and dendrites and axons of Golgi cells, and became confined to the terminals. GABAergic vesicles also became confined to the GABAergic synapses.

4. Discussion

4.1. Developmental Changes in the GABA secretion mechanism

In the present study, we found developmental changes in intracellular localization of GABA in GABAergic neurons. The results are summarized in Figure 7. In the immature cerebellum, cytosolic GABA is localized throughout cell bodies, dendrites, axons, axon varicosities, and growth cones of all GABAergic neurons (Fig. 7A, C). The vesicular GABA, detected by immunohistochemistry for VGAT, was localized at the axon varicosities and

growth cones where GABAergic synapse were not yet formed. These results indicated that in the immature cerebellum, GABA was distributed in the cytosol of all part of GABAergic neurons and the vesicles at the axon varicosities and growth cones. Physiological and biochemical studies have demonstrated that non-vesicular forms of GABA are also secreted via the plasma membrane by reverse transporter actions of GATs (Jaffe and Vaello, 1988; Taylor et al., 1990; Taylor and Gordon-Weeks, 1991; Attwell et al., 1993; Gao and van den Pol, 2000). Therefore, in the developing brain, cytosolic GABA might be extrasynaptically released from dendrites, axons and cell bodies via the plasma membrane by GATs (Evans et al., 1996; Yan and Ribak, 1998), diacrine, while GABA in the vesicles might be also extrasynaptically released from axon varicosities and growth cones by exocytosis (Fig. 7A, C). During the development, GABA disappeared from the cell bodies and dendrites of Purkinje cells and dendrites and axons of Golgi cells, and became confined to the terminals as reported in previous reports (McLaughlin et al., 1975; Takayama and Inoue, 2004b). In the adult cerebellum, GABA is exclusively transported into the synaptic vesicles by VGAT at the axon terminals (Fon and Edwards, 2001), released synaptically, and may mediate inhibitory transmission in the adult cerebellum (Fig. 7B, D). The same results by immunohistochemistry were obtained in the developing cerebral cortex (data not shown). Therefore, GABA secretion mechanisms might developmentally change in a similar manner in all brain regions.

4.2. Origin of GABA for cerebellar development

In the developing CNS, GABA appears long before the onset of synaptogenesis (Lauder et al., 1986; Van Eden et al., 1989; Fairen et al., 1998). This study also demonstrated that GABA is localized in cerebellar neurons before synapse formation. These results suggested that GABA might be extrasynaptically released from GABAergic neurons *in situ*

(Table 1). In the molecular layer, GABA might be secreted from Purkinje cell dendrites by diacrine, and axon varicosities and growth cones of stellate and basket cells by exocytosis. In contrast, Golgi cells mainly supply GABA in the granular layer by both types of secretion. GABA, which is extrasynaptically released in the developing cerebellum, might play various roles in cerebellar morphogenesis.

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Figure legends

Figure 1 Immunohistochemistry for GABA and VGAT in the mature cerebellum

A) Dot blot analysis with the antibody against GABA. The antibody only bound the GABA-conjugates (GABA), but did not react with other amino acid conjugates, such as β -alanin (β -Ala), aspartic acid (Asp), glutamic acid (Glu), and glycine (Gly).

B and C) Immunohistochemical localization of GABA in the mature cerebellar cortex. In the molecular (Mo) and Purkinje cell layers, immunolabeling was detected in the cell bodies of stellate (St) and basket (Ba) cells (B). Axon terminals of stellate cells exhibited fine dots in the molecular layer (Mo), and terminal portions of basket cell axons, including *pinceau* (arrows) (Palay and Chan-Palay, 1974), surrounded the Purkinje cell bodies (asterisks) (B). Dendrites and cell bodies (asterisks) of Purkinje cells, however, were negative (B). In the granular layer (Gr), Golgi cell bodies (Go) and axon terminals in the synaptic glomeruli (arrowheads) were clearly stained (B). The immunolabeling was completely abolished when the primary antibody was preincubated with GABA (C).

D) Western blot analysis with the antibody against VGAT (a-VGAT) and the antibody against VGAT preincubated with the VGAT-peptide, which was used for immunization (a-VGAT+pep). The position and molecular weights of standards (kd) are shown on the right.

E and F) Immunohistochemical localization of VGAT in the mature cerebellar cortex. The VGAT- immunolabeling exhibited fine dots in the molecular (Mo) and Purkinje cell (asterisks) layers and ring shaped profiles (arrowheads) in the granular layer (Gr) (E). In addition, *pinceau* (arrows) of basket axon terminals were heavily stained beneath Purkinje cell bodies (asterisks) (E). The immunolabeling was completely abolished when the primary antibody was preincubated with the VGAT peptide (F).

Figure2 Developmental changes in the distribution of GABA and VGAT in the cerebellar cortex at P3 (A, D, I), P5 (B, E, G, H), and P7 (C, F, J, K).

A-C) Immunohistochemical localization of GABA in the cerebellar cortex at P3 (A), P5 (B), and P7 (C). The GABA-immunolabeling is localized at dendrites and cell bodies (asterisks) of Purkinje cells in the molecular (Mo) and Purkinje cell layers, and cell bodies (Go) and axon plexi (arrows) of Golgi cells in the internal granular layer (Gr).

D-F) Immunohistochemical localization of VGAT in the cerebellar cortex at P3 (D), P5 (E), and P7 (F). The VGAT-immunolabeling exhibited fine dots in the molecular (Mo), Purkinje cell and internal granular layers (Gr). Asterisks show the position of Purkinje cell bodies.

G and H) Double staining of calbindin (red) and VGAT (green) at P5. VGAT-positive dots (arrowheads) were detected on and between Purkinje cell dendrites and cell bodies (asterisks). In addition, VGAT- positive dots were localized on the axon collaterals (arrows) of Purkinje cells (G). In the deeper part of the granular layer (Gr), VGAT-positive dots are almost negative on the calbindin positive Purkinje cell axons (arrows in H).

I-K) Double staining of VGAT (green) and the GABA_A receptor $\alpha 1$ subunit (red) at P3 (I) and P7 (J, K). At P3 (I), only VGAT-positive dots were detected around Purkinje cell bodies (asterisks) and in the granular layer (Gr). At P7 (J, K), many $\alpha 1$ -positive dots were merged with VGAT-positive dots (arrowheads) in the molecular (Mo) and Purkinje cell (asterisks) layers (J), whereas they were negative in the internal granular layer (Gr) (K).

Figure3 Electron microscopic localization of GABA in the cerebellar cortex at P5.

A and B) GABA immunohistochemistry in the molecular layer. GABA-immunolabeling was localized within the Purkinje cell dendrites (arrows in A, Pu in B), and the terminal like structure of stellate cell axons (St). Between parallel fiber bundles (Pf), asymmetric synapses

(asterisks) were formed between GABA-positive dendrites (Pu) and parallel fiber varicosities containing spherical vesicles (B).

C-F) GABA immunohistochemistry in the internal granular layer. GABA-immunolabeling was detected in the axons (arrows) and growth cones (GC) containing vacuoles (v) (De Cerro and Snider, 1968; Kawana et al., 1971). At the developing synaptic glomeruli, GABA was localized in the terminal like structures (Ga) and dendrites (Gd) of Golgi cells, but granule cells (Gr) were negative. GABA-positive profiles often included synaptic vesicles (arrowheads). Bar 1 μ m.

Figure 4 Electron microscopic localization of VGAT in the cerebellar cortex at P5.

A-C) VGAT immunohistochemistry in the molecular layer.

D-G) VGAT immunohistochemistry in the internal granular layer.

In both layers, VGAT-immunolabeling was observed around vesicles (arrowheads) in the axon varicosities (Va) between immuno-negative axons (Ax) and growth cones (GC) which contained vacuoles (v).

Bar 1 μ m.

Figure5 Developmental changes in the distribution of GABA and VGAT in the molecular (Mo) and Purkinje cell layers of the cerebellar cortex at P10 (A, D, G), P14 (B, E), and P21 (C, F, H).

A-C) Immunohistochemical localization of GABA at P10 (A), P14 (B), and P21 (C).

At P10 (A), GABA-immunolabeling is localized at dendrites and cell bodies of Purkinje (asterisks), stellate (st) and basket (Ba) cells in the molecular (Mo) and Purkinje cell layers.

At P14 (B) and P21 (C), Purkinje cell dendrites and cell bodies were negative.

D-F) Immunohistochemical localization of VGAT at P10 (D), P14 (E), and P21 (F).

VGAT-immunolabeling exhibited fine dots in the molecular (Mo) and Purkinje cell layers.

Asterisks show the position of Purkinje cell bodies.

G and H) Double staining of VGAT (green) and the GABA_A receptor α 1 subunit (red) in the molecular (Mo) and Purkinje cell layers at P10 (G) and P21 (H). The majority of α 1-positive dots merged with VGAT-positive dots (arrowheads).

Asterisks show the position of Purkinje cell bodies.

Figure6 Developmental changes in the distribution of GABA and VGAT in the cerebellar granular layer at P10 (A, D, G, I), P14 (B, E), and P21 (C, F, H, J).

A-C) Immunohistochemical localization of GABA at P10 (A), P14 (B), and P21 (C).

GABA-immunolabeling is localized at cell bodies of Golgi cells (Go). Plexi (arrows) and terminals (arrowheads) of Golgi cell axons were also labeled in the granular layer (Gr) at P10 (A), whereas GABA became accumulated to terminals (arrowheads) of Golgi cells at P14 (B) and P21 (C). Asterisks show the position of Purkinje cell bodies.

D-F) Immunohistochemical localization of VGAT at P10 (D), P14 (E), and P21 (F).

VGAT-immunolabeling showed ring shaped profiles (arrowheads) in the granular layer (Gr).

Asterisks show the position of Purkinje cell bodies.

G and H) Double staining of calbindin (red) and VGAT (green) at P10 (G) and P21 (H).

VGAT-positive dots were detected on the axon collaterals of Purkinje cells (arrows). Asterisks show the position of Purkinje cell bodies.

I and J) Double staining of VGAT (green) and the GABA_A receptor α 1 subunit (red) at P10 (I)

and P21 (J) in the granular layer. Arrowheads show the synaptic glomeruli where α 1 and VGAT merged. The intensity of α 1-immunolabeling increased markedly during the second

and third postnatal week.

Figure7 Schematic illustrations of developmental changes in the distribution of GABA and its secretion mechanisms in the cerebellar neurons

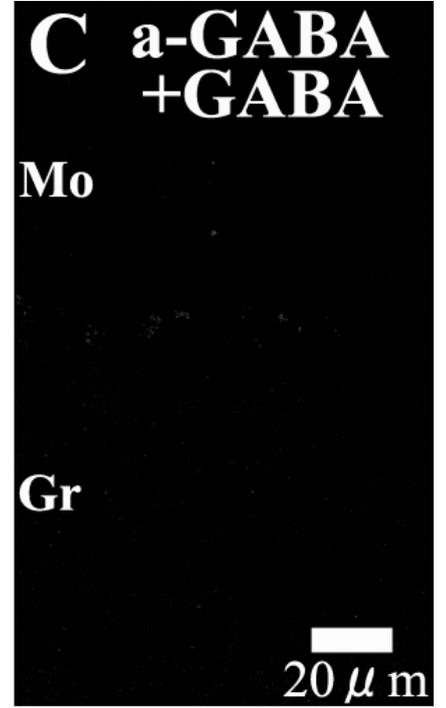
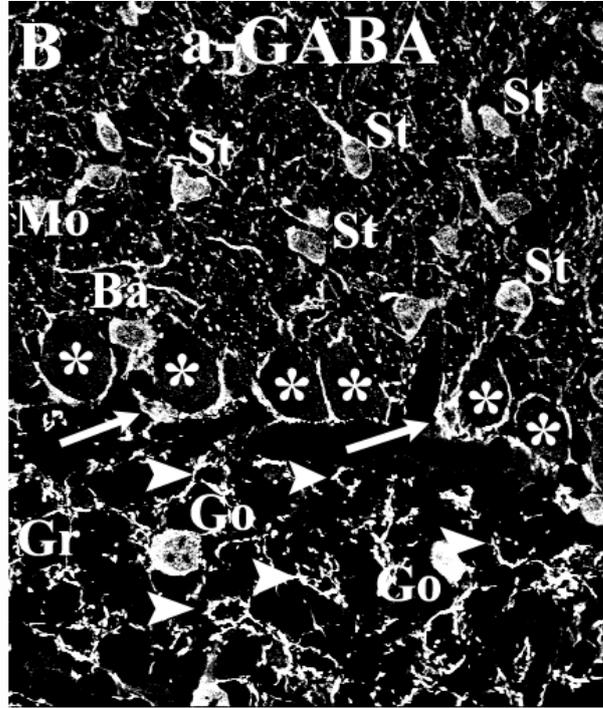
A and B) In the immature Purkinje cells (A), GABA was localized throughout the neurons (gray painting). GABA in dendrites (large triangle) and cell bodies (large circle) could be released by non-vesicular (NV) mechanisms, and GABA in the vesicles at varicosities (small circles) and terminals/growth cones (square) of collateral axons could be released by exocytosis (V). In the mature Purkinje cells (B), GABA is localized at terminals (square) of axon collaterals in the Purkinje cell layers and released by exocytosis (V). Axons themselves were negative (straight and curved dotted lines) (A, B).

C and D) In the immature Golgi cells (C) as well as other GABAergic interneurons, GABA is localized throughout the neurons (gray painting). GABA might be secreted from dendrites (long square), cell bodies (large circle), and axons (straight line) by non-vesicular secretion (NV), and from axon varicosities (small circles) and terminals/growth cones (small square) by exocytosis (V). In the adult Golgi cells (D), GABA is localized in cell bodies (large circle) and axon terminals (square) at synaptic glomeruli, and is released from axon terminals (square) by exocytosis (V). Present results could not determine the non-vesicular secretion from the cell bodies was not (NV?).

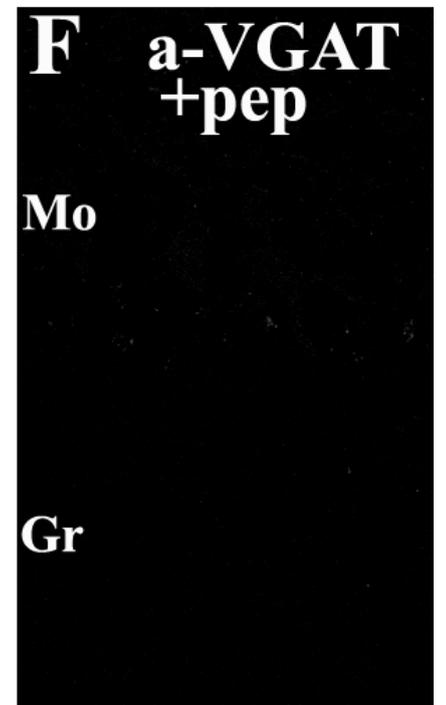
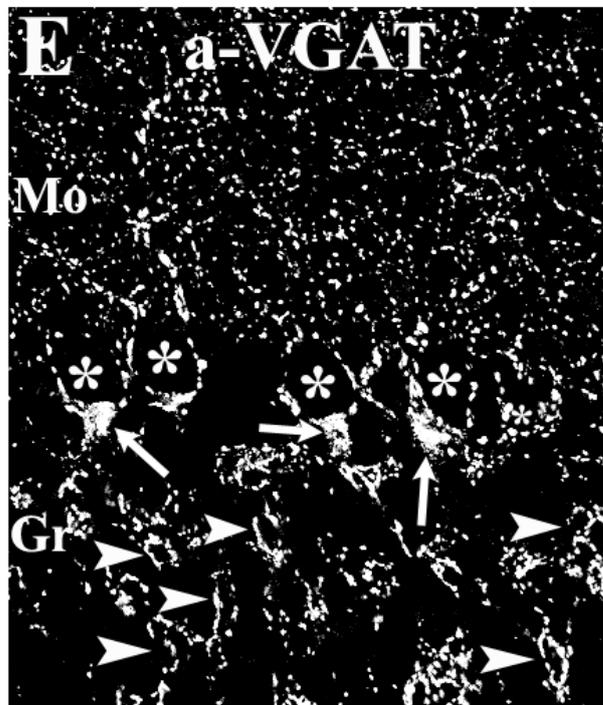
Table 1 GABA secretion mechanisms in the immature cerebellum

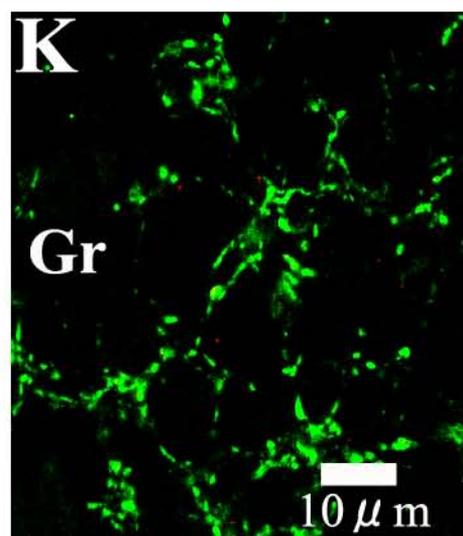
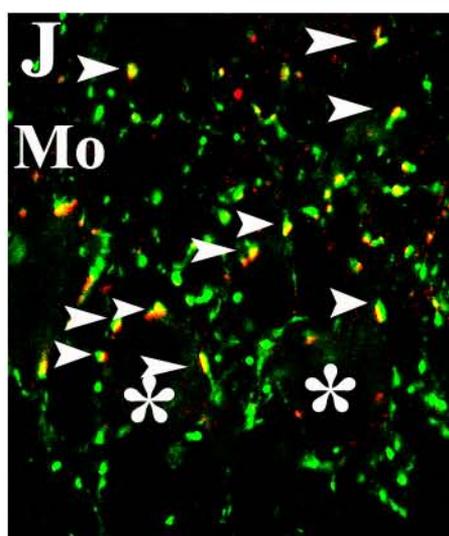
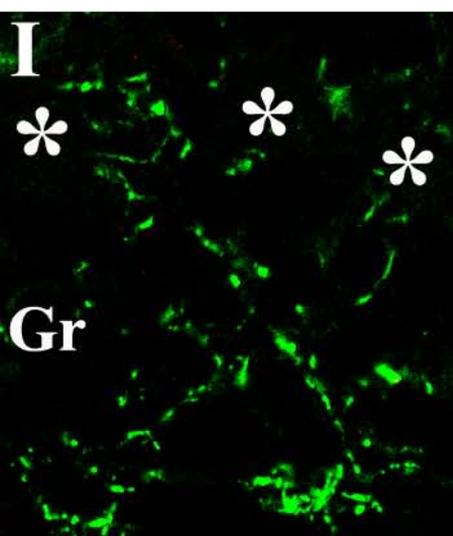
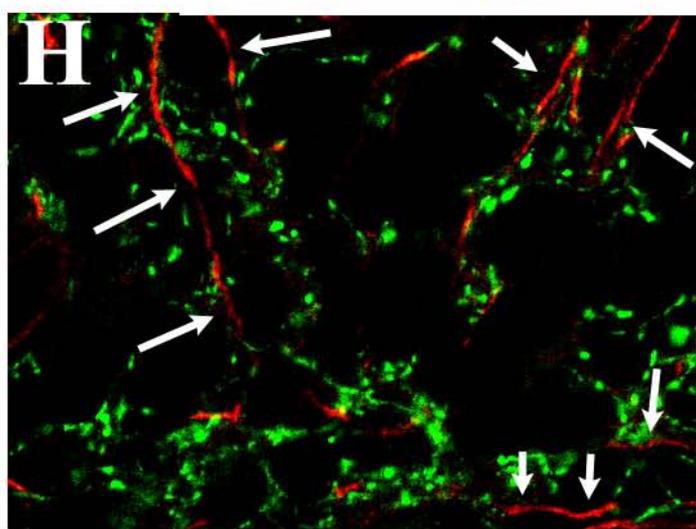
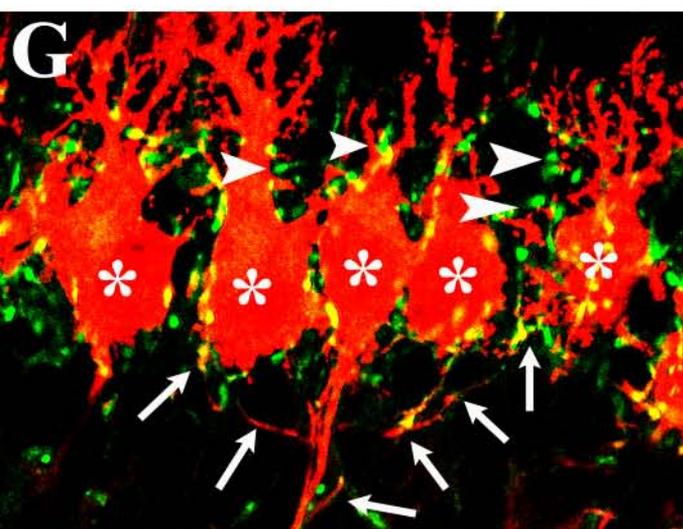
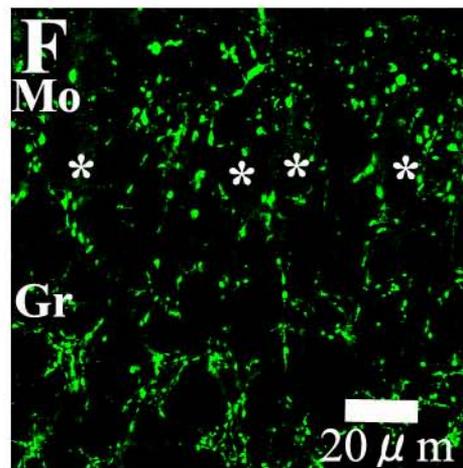
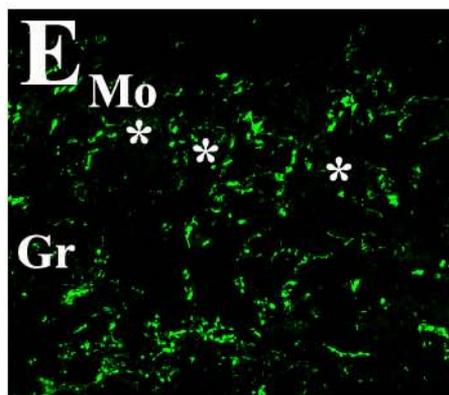
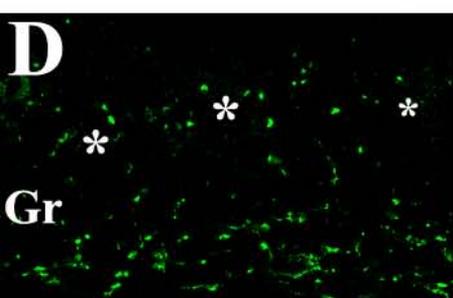
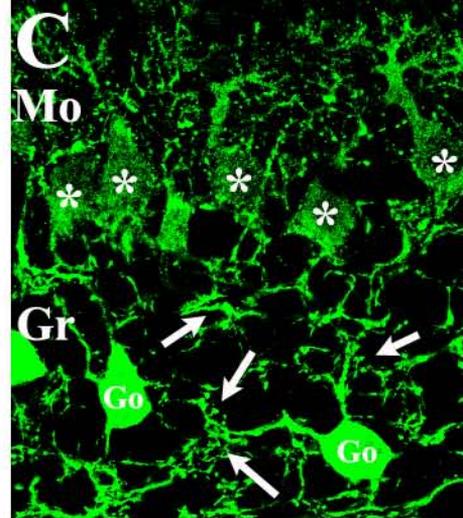
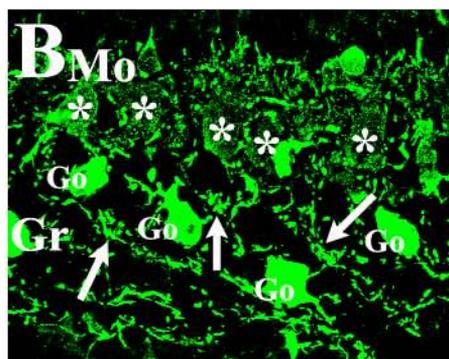
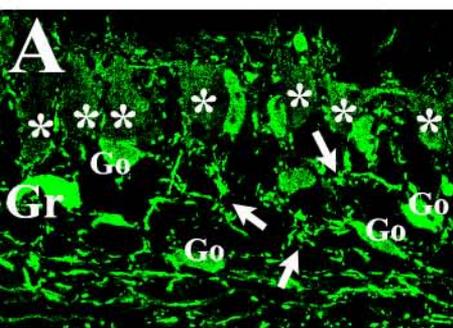
	Diacyrine by GABA transporters	Exocytosis of GABAergic vesicles
Molecular and Purkinje cell layers	Dendrite of Purkinje cells (and stellate and basket cells)	Axon varicosities of stellate and basket cells
Granular layer	Dendrites and axons of Golgi cells	Axon varicosities of Golgi cells Purkinje cell axon collaterals

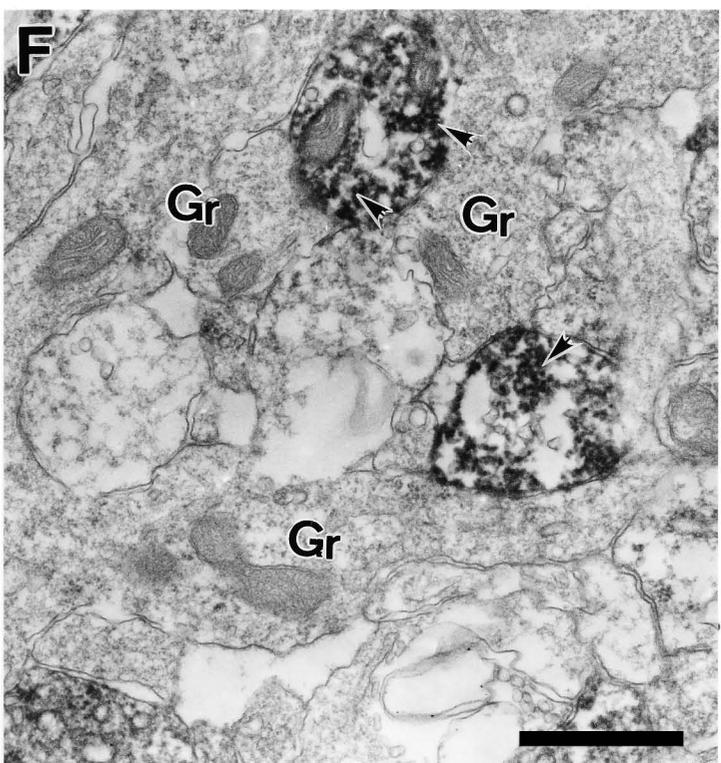
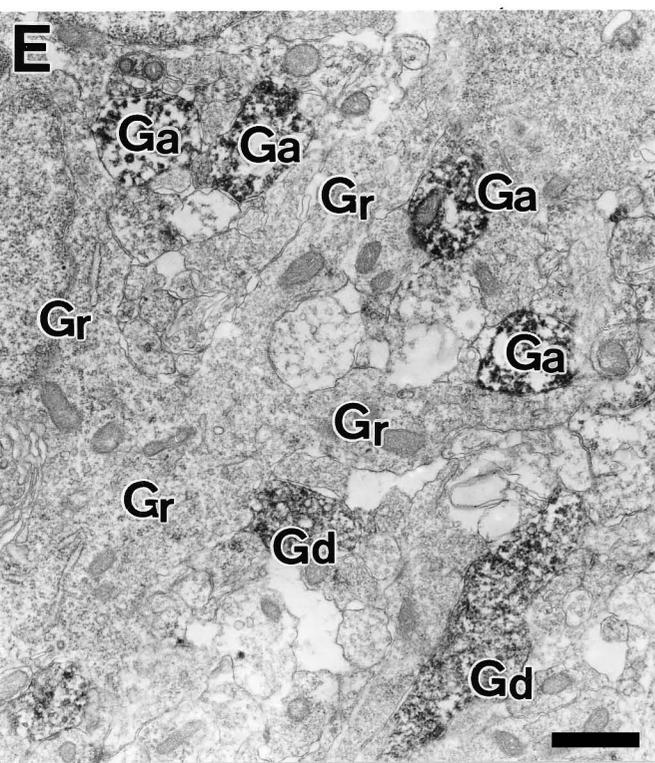
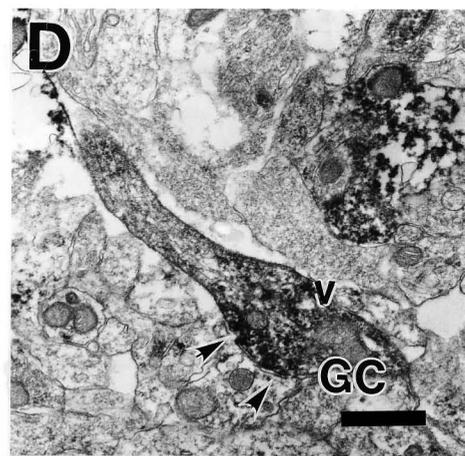
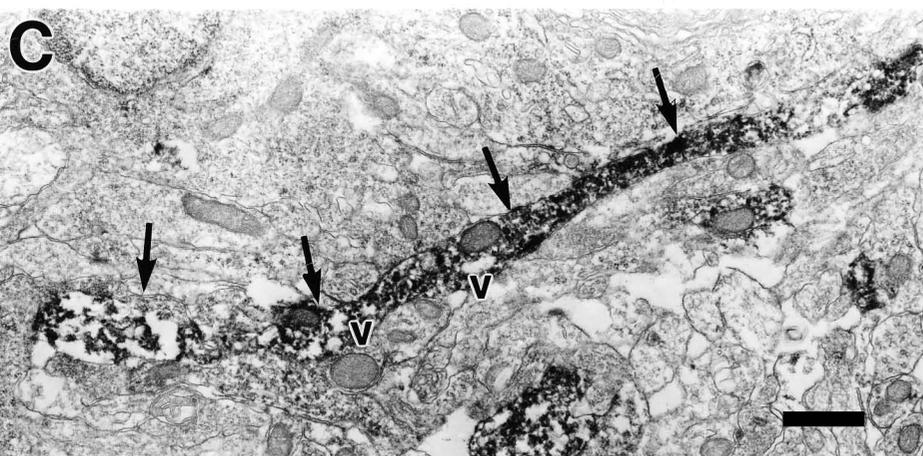
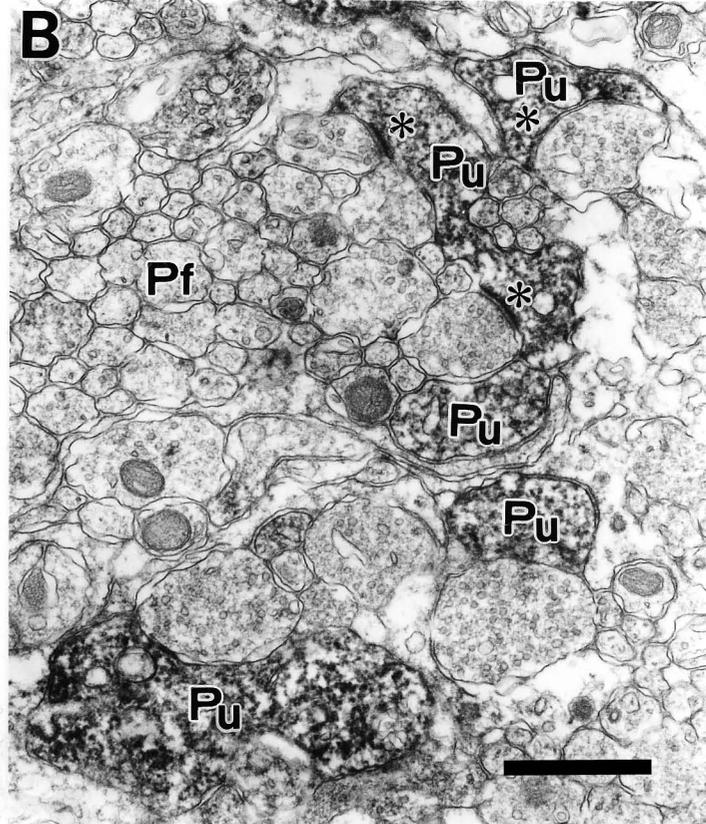
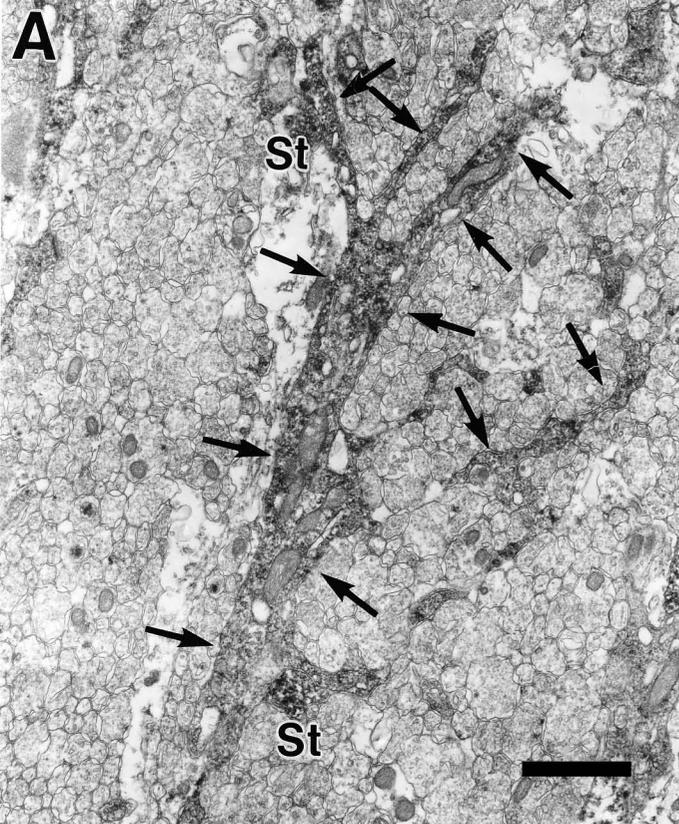
- A**
- β -Ala
 - Asp
 - GABA
 - Glu
 - Gly

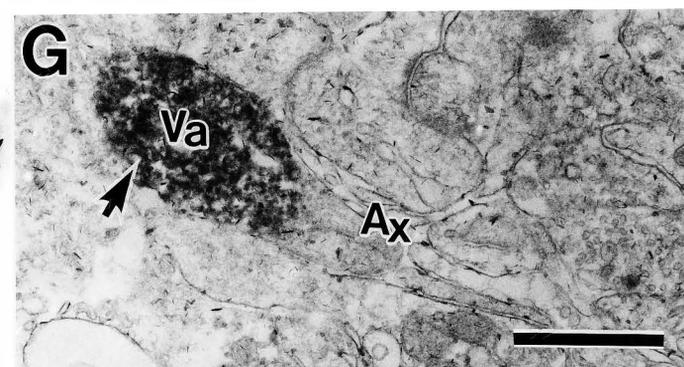
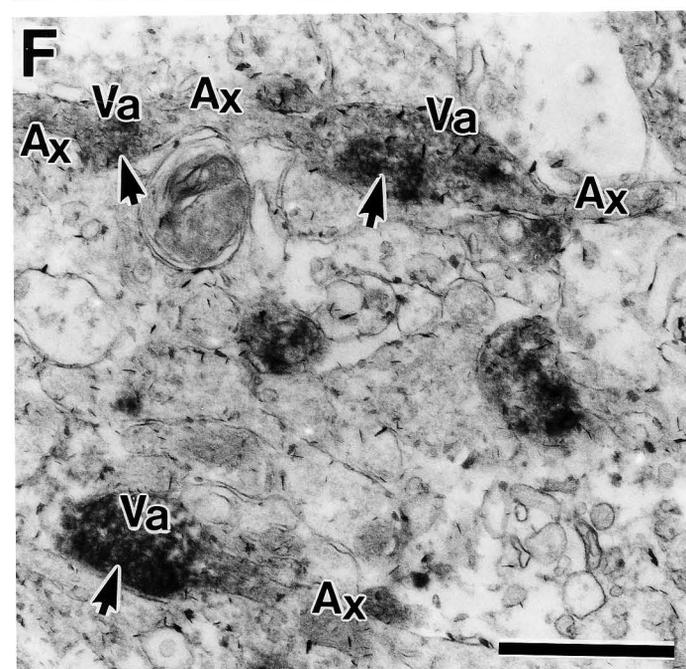
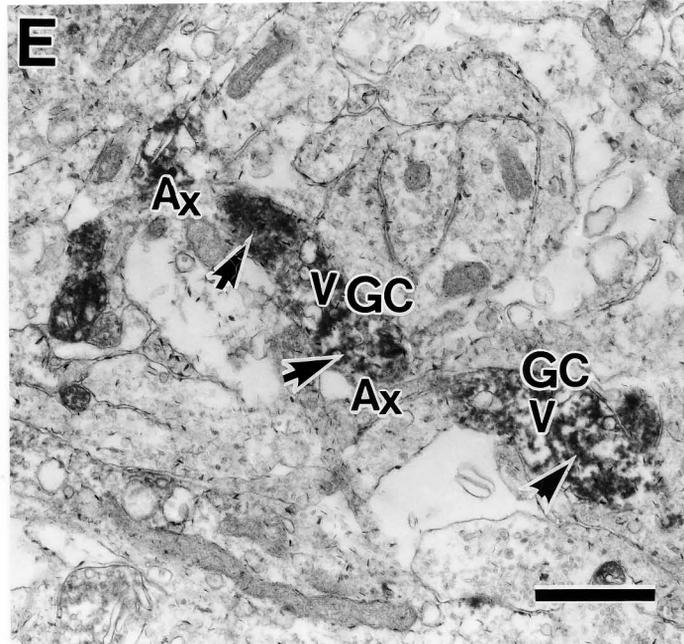
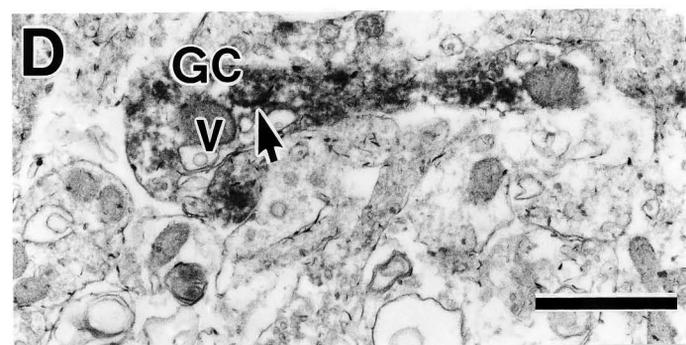
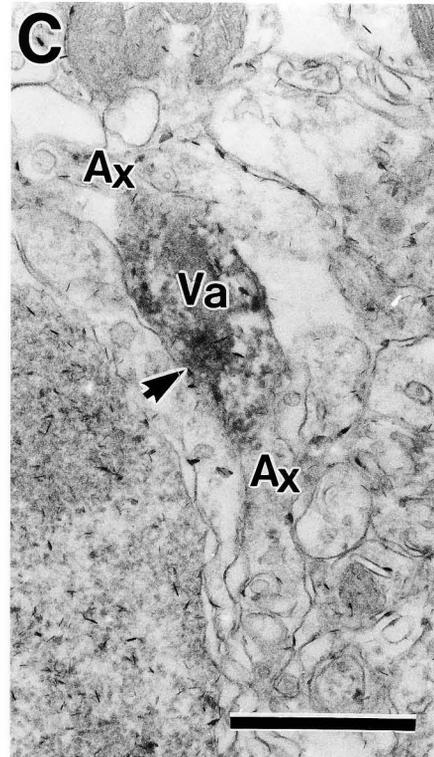
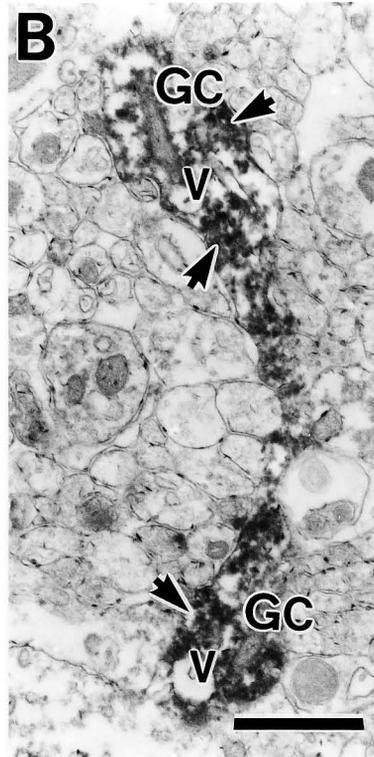
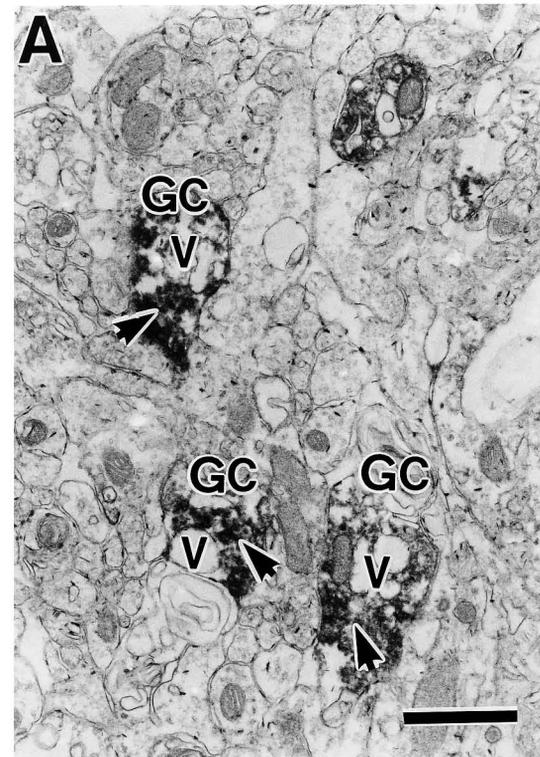


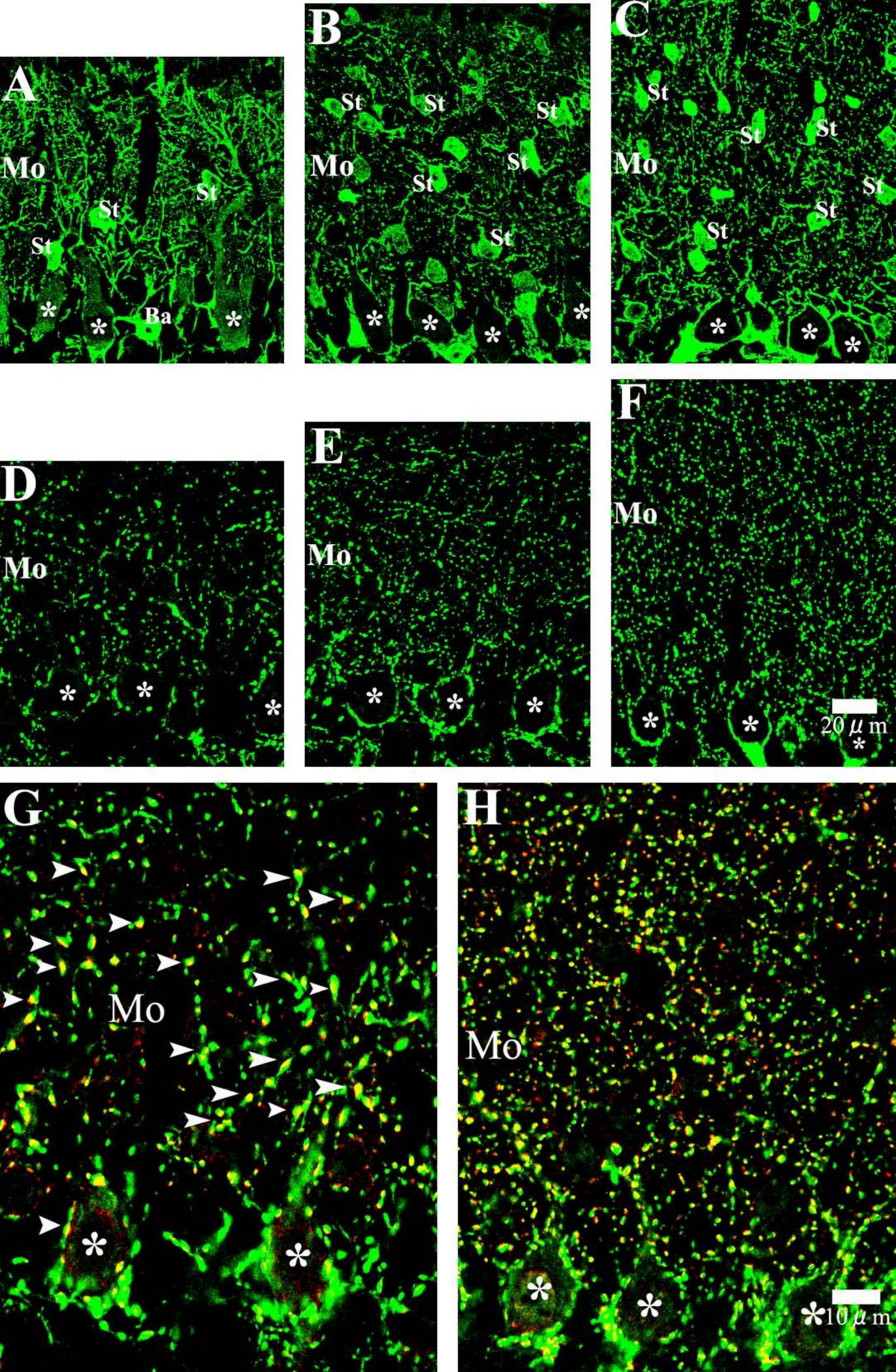
- D**
- a*-VGAT
a-VGAT+pep
- 97.4kD
- 68kD
- 43kD
- 29kD



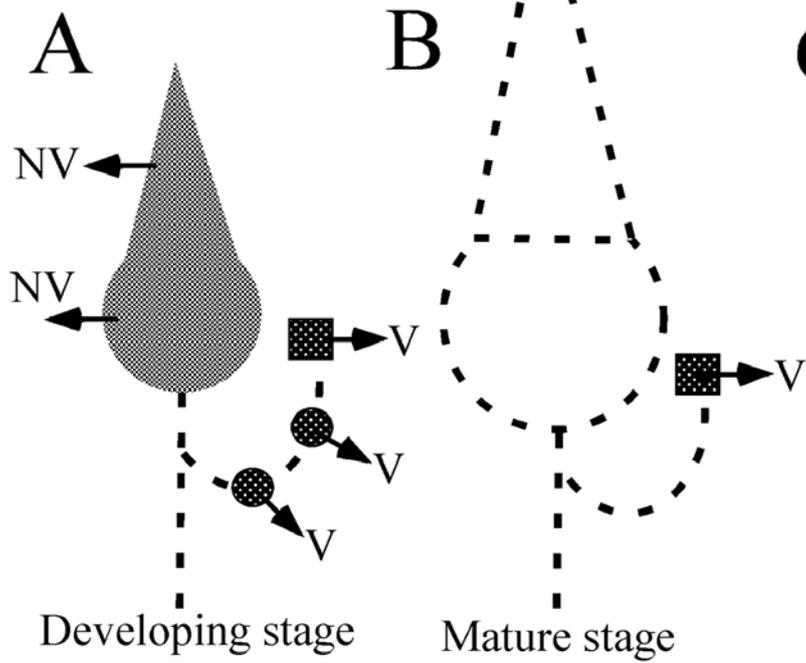








Purkinje cells



Golgi cells other GABAergic neurons

