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**Morphological development and maturation of the GABAergic synapses in the mouse cerebellar granular layer**

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**Abstract**

In the adult central nervous system (CNS),  $\gamma$ -amino butyric acid (GABA) is a predominant inhibitory neurotransmitter, which regulates glutamatergic activity. Recent studies have revealed that GABA serves as an excitatory transmitter in the immature CNS, and is involved in brain morphogenesis. To elucidate how GABA exerts its effect on immature neurons and how GABAergic synapses are formed, we examined both development of pre- and post-synaptic elements of the GABAergic synapses formed between granule and Golgi cells in the mouse cerebellar granular layer. Immunohistochemistry for glutamic acid decarboxylase (GAD) demonstrated that GABA was localized throughout the Golgi cells before postnatal day 7 (P7), and became confined to the axon terminals during the second postnatal week. Electron microscopic analysis demonstrated that GABAergic synapses were clearly detected at P10. *In situ* hybridization and immunohistochemistry for the GABA<sub>A</sub> receptor  $\alpha$ 1 and  $\alpha$ 6 subunits, which are mainly involved in inhibitory synaptic transmission, demonstrated that both subunits appeared at P7. Distribution of both subunits expanded in the granular layer with special reference to the development of GABAergic synapses. Furthermore, the majority of the subunits accumulated adjacent to the GABAergic terminals. These results suggested that in the granular layer, GABA might be non-synaptically secreted from Golgi cell axons and dendrites during the first postnatal week. From the second postnatal week, GABA is synaptically released and begins to mediate inhibitory transmission. Furthermore, it was suggested that GABAergic innervation could initiate expression and trafficking of the GABA<sub>A</sub> receptors containing the  $\alpha$ 1 and  $\alpha$ 6 subunits.

**Classification Terms**

**Theme A:** Development and Regeneration

**Topic:** Neurotransmitter systems and channels

**Key words:** synapse-formation, glutamic acid decarboxylase, GABA<sub>A</sub> receptor, synaptic glomerulus, Golgi cell, granule cell,

## 1 Introduction

In the adult central nervous system (CNS),  $\gamma$ -amino butyric acid (GABA) is a predominant neurotransmitter, which mediates fast inhibitory synaptic transmission and regulates glutamatergic activity [33,42,52,53]. Recent studies have revealed that GABAergic signaling changes developmentally. In the immature CNS, GABA serves as an excitatory transmitter, and acts as a trophic factor for development, such as changes in cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death [5,8,10,33,43,53,68]. Nevertheless, it is still unclear how GABA exerts its effect on neurons, and how functional GABAergic synapses are established.

Synaptic glomeruli in the cerebellar granular layer provide an ideal system for the investigation of GABAergic synapse development for the following reasons. The cerebellar granular layer consists of only two major types of neurons, granule cells and Golgi cells. The synaptic architecture in the granular layer is quite simple [29,40,41,54]. The majority of the excitatory synapses are formed only between mossy fiber terminals and granule cell dendrites and inhibitory synapses are formed only between granule cell dendrites and Golgi cell axon terminals within synaptic glomeruli, which have characteristic shapes. GABAergic synapses and their pre- and post-synaptic elements are easily detected by light- and electron microscopy [2,49,54]. Formation of GABAergic synapses begins after birth [27,28,37].

Several electron microscopic investigations demonstrated ontogeny of the GABAergic synapses [2,27,28,45]. Sequential to excitatory synapse assembly, inhibitory synapses appear at the end of the first postnatal week and the number of GABAergic synapses increases during the second and third postnatal week in the rat cerebellar granular layer [2,27,28]. During the same period, intracellular localization of glutamic acid decarboxylase (GAD), which is a synthetic enzyme of GABA [57,58,60], drastically changes [45].

Furthermore, *in situ* hybridization and immunohistochemistry for the GABA<sub>A</sub> receptor subunits demonstrated that subunit composition drastically changes during development, with specific and distinct subunits expressed in, and localized at, the postsynapse in the cerebellum [3,11,22,23,38,39,50,51,55,67,75,76]. We previously demonstrated that immature granule cells express the GABA<sub>A</sub> receptor  $\alpha 2$  subunit, and that the  $\alpha 2$  subunit in the GABA<sub>A</sub> receptors shift to  $\alpha 1$  and  $\alpha 6$  subunits during the synaptogenesis [62]. This result suggests that GABA could activate GABA<sub>A</sub> receptors containing the  $\alpha 2$  subunit and mediate differentiation and maturation of granule cells.

In the present study, we examined both development of the pre- and post-synaptic elements of the GABAergic synapses formed between granule cell dendrites and Golgi cell axon terminals in the cerebellar granular layer of mice and tested their spatial and temporal relationship. First, to reveal how GABA exerts its effect on maturing granule cells in the granular layer, we investigated development of the presynaptic elements, which synaptically and non-synaptically release GABA by immunohistochemistry for GAD, and demonstrated where GABA is secreted from in the developing cerebellar granular layer. Second, to reveal how functional GABAergic synapses are formed, we examined the ontogeny of the GABAergic synapses by electron microscopic analysis, and developmental changes in expression, and localization of the GABA<sub>A</sub> receptor  $\alpha 1$  and  $\alpha 6$  subunit mRNAs and proteins by *in situ* hybridization and immunohistochemistry. Both subunits appear during postnatal development and are thought to be involved in inhibitory synaptic transmission [11,23,39,47,65,73,76].

In this study, we found that GAD is localized throughout the Golgi cells, including growth cones, during the first postnatal week, and accumulation of GAD to axon terminals, formation of GABAergic synapses, and distribution of GABA<sub>A</sub> receptors at the synaptic site

occurred almost simultaneously during the second postnatal week. These results suggested that in the granular layer, GABA might be extrasynaptically secreted from Golgi cells during the first postnatal week. From the second postnatal week, GABA is synaptically released and begins to mediate inhibitory transmission. Furthermore, it was suggested that GABAergic innervation could initiate expression and trafficking of the GABA<sub>A</sub> receptors containing the  $\alpha 1$  and  $\alpha 6$  subunits.

## 2 Materials and Methods

### 2.1 Animals

We examined mice from the C57BL/6CrSlc strain at postnatal day 0, 3, 5, 7, 10, 12, 14, 21 and 90 (as adults). At each postnatal stage, three mice were sacrificed for *in situ* hybridization, and five mice were used for immunohistochemistry.

### 2.2 *In situ* hybridization

We used 45-mer oligonucleotide probes for the detection of GABA<sub>A</sub> receptor subunit mRNAs; 3'- GAC TAA AAG AAC CTA TTG ATG GGG TGT GGG GGC TTT TAG CTG AGG -5' and 3'- CAG ATG GTA CTT GGA GTC AGA ATG CAC AAC AAT CTC CGC TTC CAG -5', which are complementary to nucleotide residues 1385-1429 of the mouse  $\alpha 1$  subunit [72] and 1276-1320 of the mouse  $\alpha 6$  subunit [34], respectively. The specificity of these probes was verified in a previous report [61]. *In situ* hybridization histochemistry was performed as described in a previous paper [61].

### 2.3 Immunohistochemistry

#### 2.3.1 Immunohistochemistry for glutamic acid decarboxylase (GAD)

Under deep ether anesthesia, 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. Cerebella were cut sagittally at a thickness of 50 $\mu$ m with a microslicer (Dosaka, Kyoto, Japan). After treatment with 3% normal goat serum in PB, sections were immersed in a mixed primary antibody solution containing GAD65 (diluted 1:2000, Chemicon International Inc. AB5082, USA) and GAD67 antiserum (diluted 1:4000, Chemicon International Inc. AB108, USA) overnight at room temperature [35]. In the immature and mature CNS, GABA is synthesized by two isoenzymes, glutamic acid decarboxylase 65 (GAD65) and GAD67. Two isoforms of GAD differ in their intracellular distribution [35]. In the adult CNS, GAD65 is mainly localized at axon terminals, whereas GAD67 is abundantly detected in cell bodies of GABAergic neurons. Both enzymes, however, are involved in producing GABA *in vivo*. To observe the whole area where GABA is synthesized and localized, both antibodies were mixed and used as a primary antibody. Peroxidase staining was performed using the ABC method (Histofine SAB-PO(R) Kit, Nichirei Co. Japan). For light microscopic observation, stained sections were mounted on glass slides precoated with gelatin. For electron microscopic analysis, sections were post-fixed with 1% glutaraldehyde in PB and 1% OsO<sub>4</sub> 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 culmen were prepared and observed under an electron microscope H-800 (Hitachi, Japan).

### 2.3.2 Immunohistochemistry for GABA<sub>A</sub> receptor $\alpha 1$ and $\alpha 6$ subunits

Specificity of the antibodies against the  $\alpha 1$  and  $\alpha 6$  subunits and the procedure for the immunohistochemical staining were described in a previous report [61].

These experiments were permitted by The Animal Care and Use Committee of

Hokkaido University School of Medicine.

### **3 Results**

#### *3.1 Postnatal change in localization of GAD in the cerebellar granular layer*

Development of the presynaptic elements, which could synaptically and extrasynaptically release GABA, and formation of the GABAergic synapses were examined in the granular layer of the culmen (lobules IV+V) in the mouse cerebellum by immunohistochemistry for GAD.

The perikaryon and short dendrites of Golgi cells were immunolabeled between the Purkinje cell layer and prospective white matter at P0 (Fig. 1A). Subsequently, Golgi cell axons with varicosities (arrows), which formed plexi or nets, became clearly discernible in the internal granular layer at P3 (Fig. 1B), P5 (Fig. 1C), and P7 (Fig. 1D). The labeling pattern gradually changed during the second postnatal week. The immunolabeling in axons became weaker, and varicosities were more clearly stained at P10 (Fig. 1E). Axons themselves became almost negative, and GAD-positive dots, presumably Golgi cell axon terminals, were arrayed as ring-shaped profiles (arrowheads) at the peripheral part of synaptic glomeruli at P12 (Fig. 1F). At P14 (Fig. 1G), P21 (Fig. 1H) and P90 (Fig. 1I), Golgi cell bodies were still positive, and ring-shape profiles consisting of fine dots were clearly stained.

By electron microscopic analysis, immature mossy terminals with a smooth surface had already formed asymmetric synapses (asterisks) with relatively large differentiating granule cell dendrites at P5 (Fig. 2A). In contrast, GAD-positive profiles, containing large vacuoles and a smooth endoplasmic reticulum, which were considered to be growth cones and elongating axons of Golgi cells [18,36], had not yet formed obvious symmetric synapses with granule cell dendrites at the same postnatal day (Fig. 2A-C). At P7 (Fig. 2D), GAD-positive

profiles often attached to granule cell dendrites, but only a few protosynaptic contacts or preliminary synapses were observed. In addition, growth cones and axons, containing vacuoles and a smooth endoplasmic reticulum, were often labeled around developing mossy terminals (Fig. 2E). At P10, cerebellar glomeruli became more complex and sealed by astrocyte processes (Fig. 3A). GAD-positive profiles, presumably Golgi cell axon terminals, often formed synapses (arrowheads) with GAD-negative granule cell dendrites and dendritic digits (Fig. 3B-F). At the same postnatal day, substantial asymmetric synapses (asterisks) had been already formed between mossy terminals and granule cell dendrites (Fig. 3A-F). At P12 (Fig. 3G) and P14 (Fig. 3H), growth cone-like structures disappeared from the granular layer, and immunolabeled Golgi axon terminals often formed synapses (arrowheads) with granule cell dendrites. These results are quite similar to that in the rat cerebellum [45].

These results demonstrated that GAD was distributed throughout the Golgi cells during the first postnatal week. The immunolabeling became confined to the axon terminals during the second postnatal week while GABAergic synapses were being formed.

### *3.2 Developmental change in expression and localization of the GABA<sub>A</sub> receptor $\alpha$ 1 and $\alpha$ 6 subunits*

Development of the postsynaptic elements for the GABAergic synapses was examined by *in situ* hybridization and immunohistochemistry for the GABA<sub>A</sub> receptor  $\alpha$ 1 and  $\alpha$ 6 subunits.

In the internal granular layer, both  $\alpha$ 1 (Fig. 4A-H) and  $\alpha$ 6 (Fig. 5A-H) subunit mRNAs of GABA<sub>A</sub> receptors appeared at P7, and their signals similarly increased in intensity during cerebellar development. Weak signals for the  $\alpha$ 1 subunit mRNA were localized in the Purkinje cell layer and cerebellar nuclei at P0 (Fig. 4A, E). At P7 (Fig. 4B, F), hybridization

signals for  $\alpha 1$  accumulated at the Purkinje cell bodies, and appeared in the deeper half of the internal granular layer. At P14 (Fig. 4C, G) and P21 (Fig. 4D, H), the signals were localized in the molecular, Purkinje and internal granular layer. The external granular layer, however, was negative throughout the cerebellar development. Hybridization signals for the  $\alpha 6$  subunit mRNA appeared at P7 in the internal granular layer (Fig. 5A, B, E, F), and the intensity of the signals increased during development (Fig. 5B-D, F-H). The external granular layer, molecular layer, and Purkinje cell layers were negative throughout cerebellar development (Fig. 5E-H). These results indicated that granule cells start to express both  $\alpha 1$  and  $\alpha 6$  subunits after settling in the internal granular layer.

In the internal granular layer, both  $\alpha 1$  and  $\alpha 6$  subunit proteins appeared at P7, and the immunolabeling pattern similarly changed during cerebellar development. At P5 (Fig. 6A), immunoreactivity for the  $\alpha 1$  subunit was observed in the molecular and Purkinje cell layers, whereas it was negative in the internal granular layer. Subsequently,  $\alpha 1$ -immunolabeling appeared in the bottom of the internal granular layer (long white arrows) at P7 (Fig. 6B, G), but the  $\alpha 1$ -positive tiny dots were sparse, and ring-shaped profiles were not yet formed (long white arrows, Fig. 6B). At P10 (Fig. 6C, H), ring-shaped profiles (thick white arrows) became discernible in the internal granular layer by  $\alpha 1$ -immunohistochemistry. During the second and third postnatal week, the intensity and density of the  $\alpha 1$ -immunolabeling gradually increased, and ring-shaped profiles (thick white arrows) became more clearly labeled in the granular layer (Fig. 6D-F, I-K). At a higher magnification of the granular layer,  $\alpha 1$ -immunoreactivity revealed finer dots throughout cerebellar development (Fig. 6G-K). The finer dots were arrayed as ring-shaped profiles (arrowheads) after P10 (Fig. 6H-K). Immunoreactivity for the  $\alpha 6$  subunit (long white arrows) appeared in the deeper half of the internal granular layer at P7

(Fig. 7A, B, G). Subsequently,  $\alpha 6$ -immunolabeling was detected in the whole internal granular layer, showing ring-shaped profiles (thick white arrows) at P10 (Fig. 7C, H). After P12, density and intensity of the  $\alpha 6$ -immunolabeling gradually increased, and ring-shaped profiles became more visible (Fig. 7D-F, I-K). At a higher magnification,  $\alpha 6$ -immunohistochemistry also exhibited numerous finer dots, and the finer dots formed ring-shaped profiles after P10 (Fig. 7G-K).

Furthermore, the spatial and temporal relationship between the localization of the GABA<sub>A</sub> receptors and the GABAergic synapses was examined by double labeling for GAD and the  $\alpha 1$  or  $\alpha 6$  subunit. At P7 (Fig. 8A), sparse red  $\alpha 1$ -immunopositive dots (long white arrows) were localized within the green GAD-positive plexi or nets, consisting of Golgi cell axons, axon varicosities and terminals. Subsequently,  $\alpha 1$ -immunohistochemistry often exhibited rings (thick white arrows), and attached to the GAD-positive plexi, nets and rings at P10 (Fig. 8B). At P14 (Fig. 8C) and P90 (Fig. 8D),  $\alpha 1$ -positive ring-shaped profiles merged with GAD-positive rings (arrowheads) in the synaptic glomeruli. The developmental change in the relationship between both immunohistochemical localization of the  $\alpha 6$  subunit and GAD (Fig. 8E-H) was similar to that between the  $\alpha 1$  subunit and GAD (Fig. 8A-D). The immunoreactivity for the  $\alpha 6$  subunit appeared within the GAD-positive profiles at P7 (Fig. 8E) and exhibited ring-shaped profiles at P10 (Fig. 8F), P14 (Fig. 8G) and P90 (Fig. 8H). Although the majority of the  $\alpha 6$  subunits were localized adjacent to the GAD-positive profiles during development (Fig. 8E-H), little of the  $\alpha 6$  subunit protein was localized other than at the GAD-positive profiles (long arrows, Fig. 8G, H) as reported previously [51].

These results demonstrated that granule cells started to express both  $\alpha 1$  and  $\alpha 6$  subunits during the second postnatal week when GABAergic synapses were formed with

Golgi cell axon terminals. The distribution of both subunits expanded in the granular layer with special reference to the development of GABAergic synapses. The majority of the subunit-proteins were localized adjacent to the GAD-positive profiles.

#### **4 Discussion**

In the present study, we investigated the development and maturation of GABAergic synapses in the cerebellar granular layer. The results are summarized in Figure 9. At the early developmental stage, GAD is localized throughout the Golgi cells including dendrites, cell bodies, axons, axon varicosities, and growth cones (Fig. 9A). During the formation of GABAergic synapses, GAD becomes confined to the cell bodies and axon terminals, granule cells start to express the GABA<sub>A</sub> receptor  $\alpha 1$  and  $\alpha 6$  subunits, and the subunit-proteins are targeted to the synaptic sites (Fig. 9B, C). In the adult cerebellum, GAD accumulates in the terminals and two subunits are localized at the postsynaptic sites (Fig. 9D).

##### *4.1 Developmental changes in the GABA-releasing mechanism*

Immunohistochemistry for GAD demonstrated that GABA is synthesized in the Golgi cells from the early developmental stage, and its localization alters with special reference to formation of GABAergic synapses. This result suggested that the GABA releasing system might developmentally undergo a change from a non-synaptic to a synaptic mechanism. In the immature cerebellum, GABA is distributed throughout the Golgi cells in mice as well as rats [45]. The vesicular GABA transporter (VGAT), which is a membrane protein of GABAergic vesicles that transports cytosolic GABA into the vesicles [14,19,21,56,59], is also detected in the granular layer from early postnatal days (data not shown). GAD is often localized around synaptic vesicles, and this result indicates that GABA

is localized in both cytoplasm and vesicles in GABAergic neurons although synapses were not formed. Physiological and biochemical studies have demonstrated that non-vesicular forms of GABA are also secreted via the plasma membrane by the reverse transporter action of GABA transporters (GATs) [4,7,9,25,30,63,64,68]. Taken together, in the developing brain, GABA may be non-synaptically released from Golgi cells via the plasma membrane by vesicular and non-vesicular mechanisms. Non-synaptically released GABA activates GABA<sub>A</sub> receptors, and plays various roles in brain development [5,6,8,10,33,53,68,74]. At the mature stage, in contrast, GABA is synthesized mainly around axon terminals (Fig. 9D), transported into synaptic vesicles, and released at the synaptic site [21,44,45,56,68]. Synaptically released GABA activates GABA<sub>A</sub> receptors, including the  $\alpha 1$  and  $\alpha 6$  subunits, on the postsynaptic membrane, mediates inhibitory synaptic transmission, and regulates glutamatergic excitability activity [33,42,52,53].

#### *4.2 Formation and maturation of GABAergic synapses in the cerebellar granular layer*

Synapse formation is considered to be a multi-step process [15,48,69]. While exploring their environment, axonal growth cones lead elongating axons to their proper targets and make contact with the dendrites and cell bodies of target neurons. Initial contact is followed by the establishment of stable synapses. In the pre-synapse, synaptic vesicles accumulate in the nerve terminals and dock near the active zone. In the postsynapse, GABA receptors are targeted to, and clustered at, an appropriate synaptic site opposite the GABA-releasing site and mediate inhibitory synaptic transmission [33,42,52]. At the early postnatal stage in the cerebellar granular layer, GAD was localized in the developing axons and growth cones, which had not yet formed synapses with granule cell dendrites. Subsequently, GAD-positive profiles often attached to the granule cell dendrites, and few

preliminary synapses or protosynapses were detected at P7, as in the rat cerebellum [45].

During the second postnatal week, GABAergic synapses increased in number, while GAD-positive growth cones gradually disappeared. During the same period, GABA<sub>A</sub> receptor  $\alpha$ 1 and  $\alpha$ 6 subunits appeared, and distribution of the subunit proteins expanded in the cerebellar granular layer. Moreover, the majority of both  $\alpha$ 1 and  $\alpha$ 6 subunit proteins accumulated adjacent to the Golgi cell axon terminals. These results demonstrated that (1) GAD-positive growth cones and varicosities probably undergo a change to GABAergic terminals [26,48], (2) GABAergic synapses were massively formed during the second postnatal week [2,28,31,37], and (3) GABAergic innervation might initiate the expression of the GABA<sub>A</sub> receptor  $\alpha$ 1 and  $\alpha$ 6 subunit genes, as well as trafficking and clustering of proteins to the synaptic site [1,8,12,13,16,17,20,24,46,48,61].

The cerebellum is deeply involved in learning motor skills [29,40,41]. The inhibitory synapses between Golgi cells and granule cells suppress the excitation of granule cells by mossy fiber inputs, ultimately modulating the neuronal activity of Purkinje cells, which are the major cerebellar cortical output. During the second postnatal week, mice open their eyes and start moving around. Eye opening and increasing motor activity after the second postnatal week in the mouse imply extensive development of motor control, coordination and learning. Our study demonstrated that during the same developmental period, functional GABAergic synapses are formed synapse and GABAergic inhibition starts in the cerebellar glomeruli [32,66,70,71]. Overall, these results suggested that the start of GABAergic inhibition is crucial for acquisition of motor learning.

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## Figure Legends

### **Figure 1** Developmental Changes in the distribution of GAD in the cerebellar granular layer.

Postnatal changes in the pattern of GAD-immunohistochemistry in the granular layer was examined in mice at P0 (A), P3 (B), P5 (C), P7 (D), P10 (E), P12 (F), P14 (G), P21 (H) and P90 (I). Golgi cell bodies (Go) were positive throughout development (A-I). Before P7, axons and axon varicosities of Golgi cells (small arrows) were positive (B-D), whereas the immunoreactivity was confined at the terminals and varicosities, exhibiting ring-shaped profiles (arrowheads) at the peripheral part of the glomeruli after P10 (E-I).

### **Figure 2** Electron microscopic localization of GAD in the developing granular layer at P5 (A-C) and P7 (D, E).

Immunoreactivity of GAD was observed within axons and growth cones (GC), containing numerous smooth endoplasmic reticulum and vacuoles (v). Mossy terminals (MF) had already formed asymmetric synapses (asterisks) with granule cell dendrites (Gr), whereas no obvious synaptic contact was detected between GAD-positive profiles and granule cell dendrites.

### **Figure 3** Electron microscopic localization of GAD in the developing granular layer at P10 (A-F), P12 (G), and P14 (H).

Mossy terminals (MF) formed asymmetric synapses (asterisks) with granule cell dendrites (Gr). Immunoreactivity for GAD was observed within the Golgi cell terminals and varicosities (Go), which often formed symmetric synapses (arrowheads) with granule cell dendrites and dendritic digits at the peripheral part of glomeruli sealed with astrocyte processes (As).

**Figure 4** Developmental changes in expression of the  $\alpha 1$  subunit mRNA in the cerebellum.

(A-D) Dark field micrographs showing expression of the  $\alpha 1$  mRNA in the cerebellum at P0 (A), P7 (B), P14 (C) and P21 (D).

(E-H) Bright field microscopic photographs showing localization of the  $\alpha 1$  mRNA in the cortex of the culmen consisting of an external granular layer (EGL), Purkinje cell layer (PL) and internal granular layer (IGL) or granular layer (GL) at P0 (E), P7 (F), P14 (G) and P21 (H).

**Figure 5** Developmental changes in expression of the  $\alpha 6$  subunit mRNA in the cerebellum.

(A-D) Dark field micrographs showing expression of the  $\alpha 6$  mRNA in the cerebellum at P0 (A), P7 (B), P14 (C) and P21 (D).

(E-H) Bright field micrographs showing localization of the  $\alpha 6$  mRNA in the cerebellar cortex of the culmen consisting of an external granular layer (EGL), Purkinje cell layer (PL) and internal granular layer (IGL) or granular layer (GL) at P0 (E), P7 (F), P14 (G) and P21 (H).

**Figure 6** Developmental changes in localization of the  $\alpha 1$  subunit in the cerebellar cortex.

(A-F) Immunofluorescence histochemistry for the  $\alpha 1$  in the cerebellar cortex at P5 (A), P7 (B), P10 (C), P12 (D), P14 (E) and P21 (F). Asterisks show the position of Purkinje cell bodies in the Purkinje cell layer (PL). In the granular layer (GL), the immunoreactivity first appeared at P7 (long white arrows) and subsequently formed rings (thick white arrows) after P10.

(G-K) Immunofluorescence histochemistry for the  $\alpha 1$  subunit in the cerebellar cortex at P7 (G), P10 (H), P12 (I), P14 (J) and P21 (K) at a higher magnification. Finer dots were sparse at

P7 (long white arrows) and gradually exhibited ring-shaped profiles after P10 (arrowheads).

**Figure 7** Developmental changes in localization of the  $\alpha 6$  subunit in the cerebellar cortex.

(A-F) Immunofluorescence histochemistry for the  $\alpha 6$  subunit in the cerebellar cortex at P5

(A), P7 (B), P10 (C), P12 (D), P14 (E) and P21 (F). The immunoreactivity first appeared in the granular layer (GL) at P7 (long white arrows) and subsequently formed rings (thick white arrows) after P10.

(G-K) Immunofluorescence histochemistry for the  $\alpha 6$  subunit in the cerebellar cortex at a higher magnification at P7 (G), P10 (H), P12 (I), P14 (J) and P21 (K). Finer dots were sparse at P7 (white arrows) and gradually formed ring-shaped profiles after P10 (arrowheads).

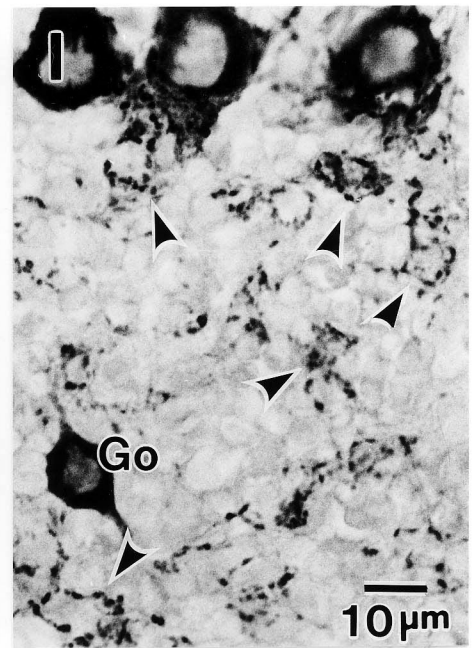
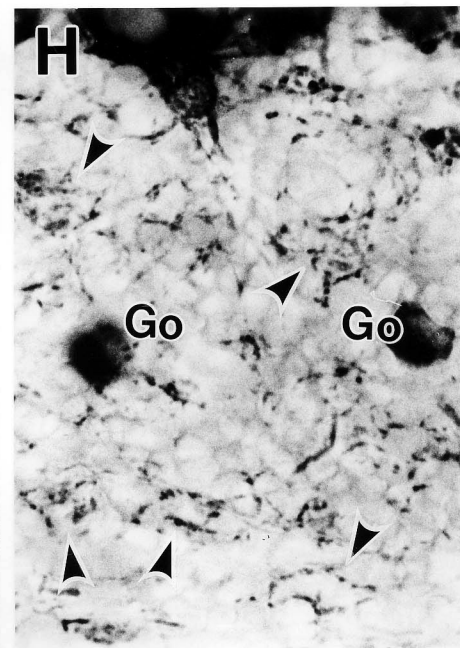
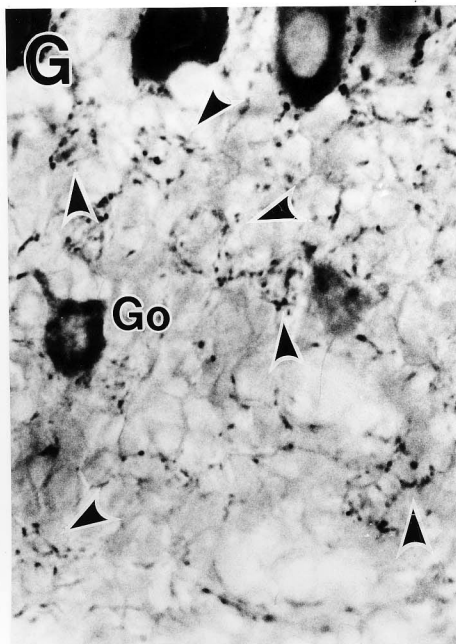
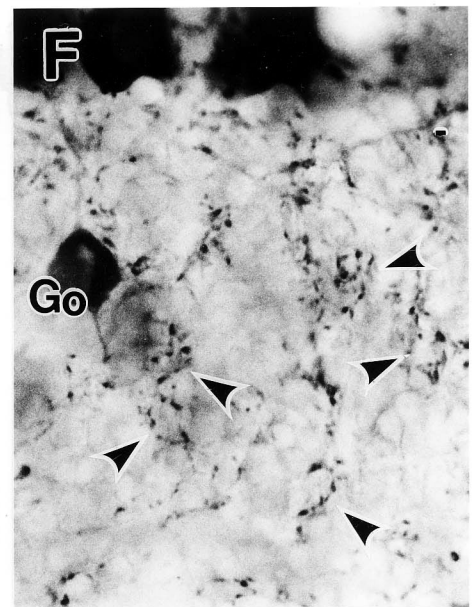
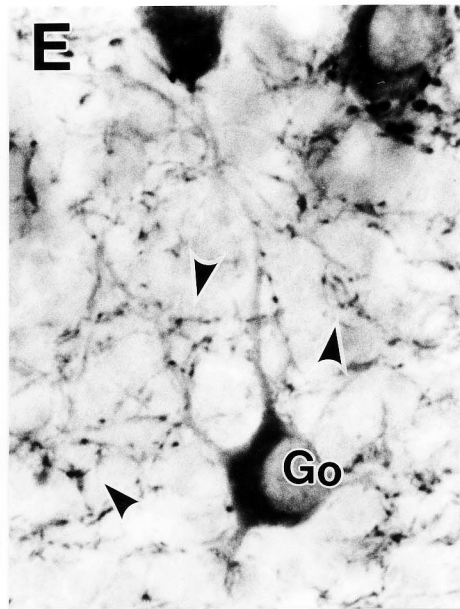
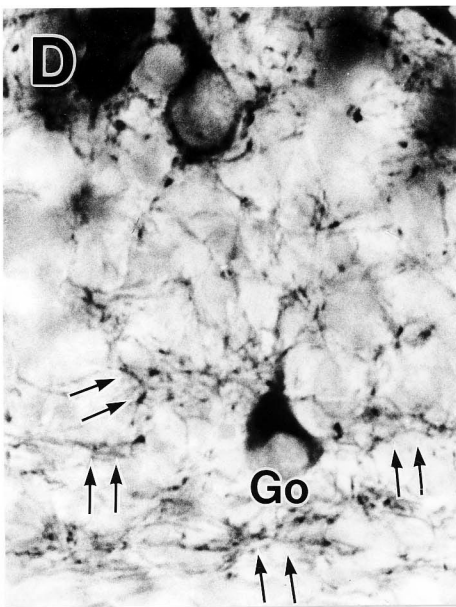
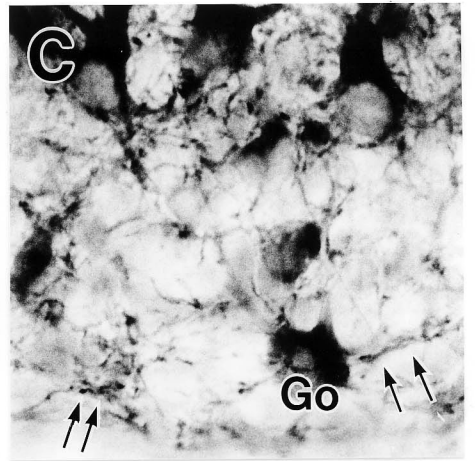
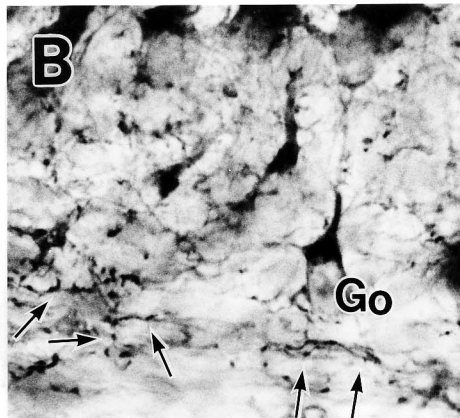
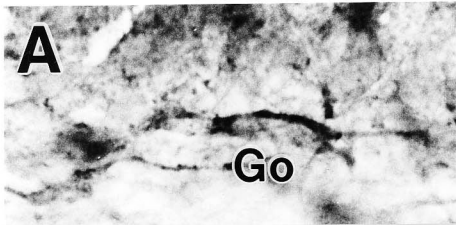
**Figure 8** Spatial and temporal relationship between development of the GABAergic network and localization of the  $\alpha 1$  and  $\alpha 6$  subunits in the cerebellar granular layer.

(A-D) Double labeling of GAD (FITC, green) and the  $\alpha 1$  subunit (Cy3<sup>TM</sup>, red) at P7 (A), P10 (B), P14 (C) and P90 (D). At P7,  $\alpha 1$ -immunoreactivity appeared within the GAD-positive plexus (long white arrows, A). Subsequently,  $\alpha 1$ -immunoreactivity exhibited ring-shaped profiles (thick white arrows, B) at P10.  $\alpha 1$ -positive rings (arrowheads) merged with the GAD-positive rings at P14 (C) and in adults (D).

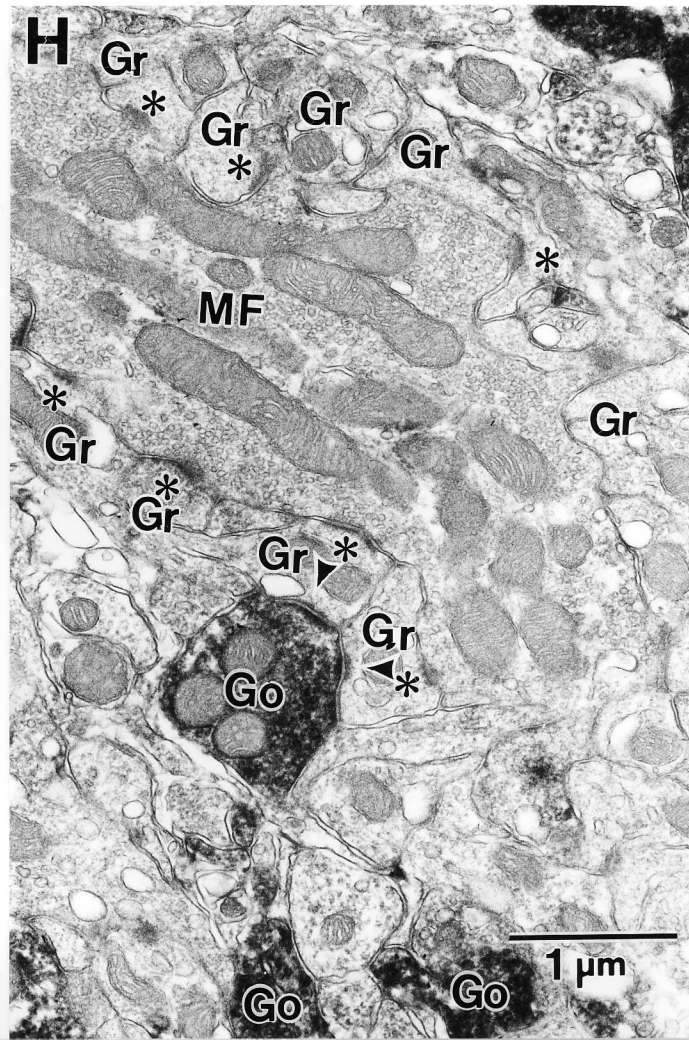
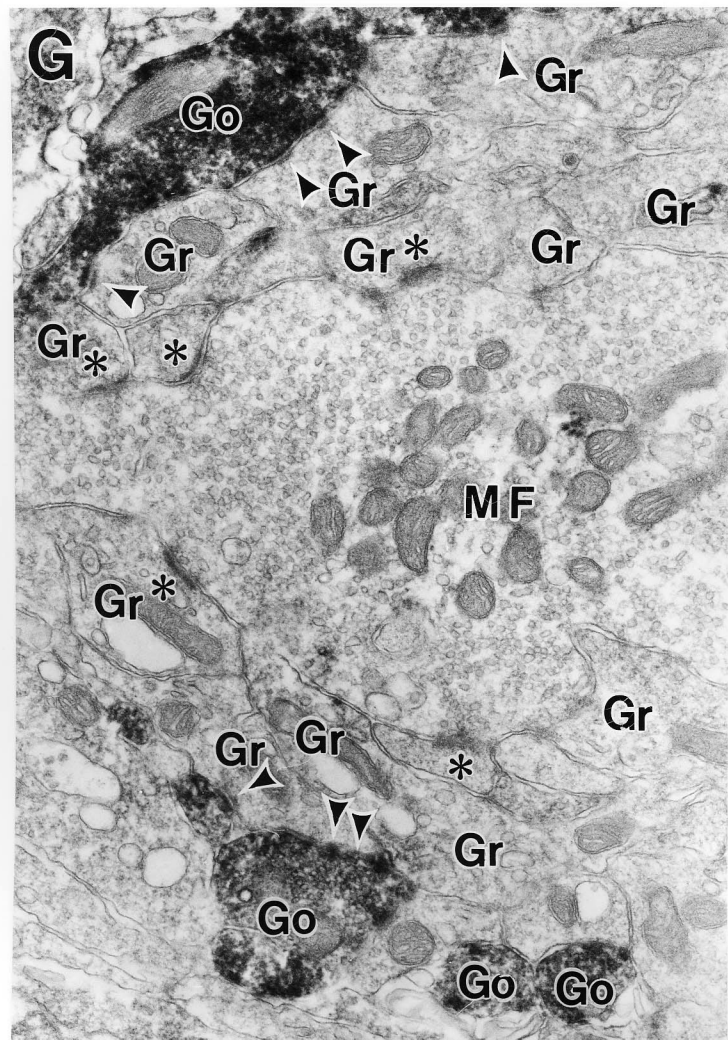
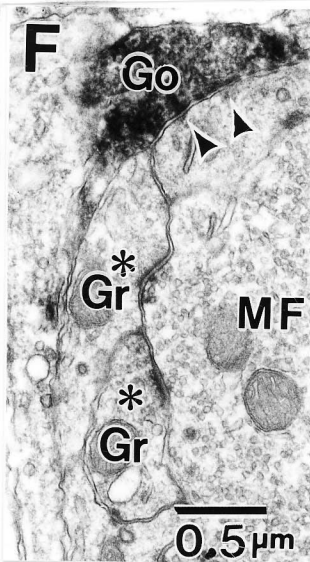
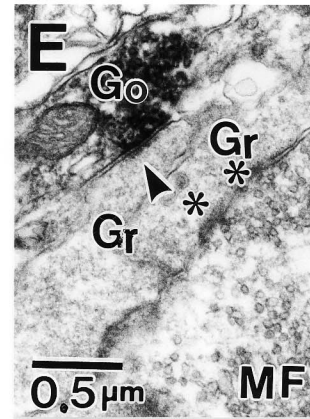
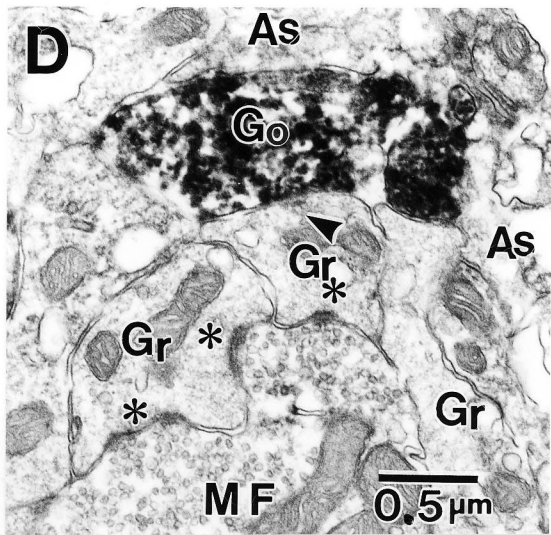
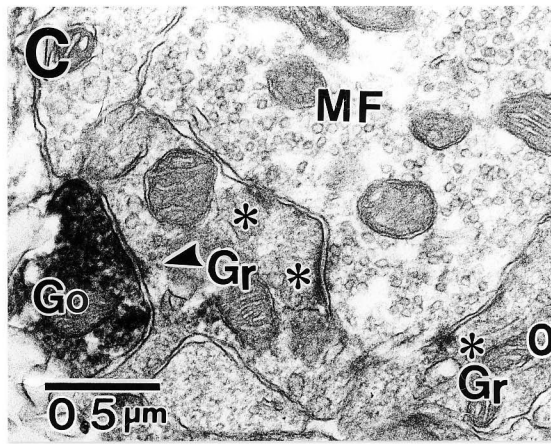
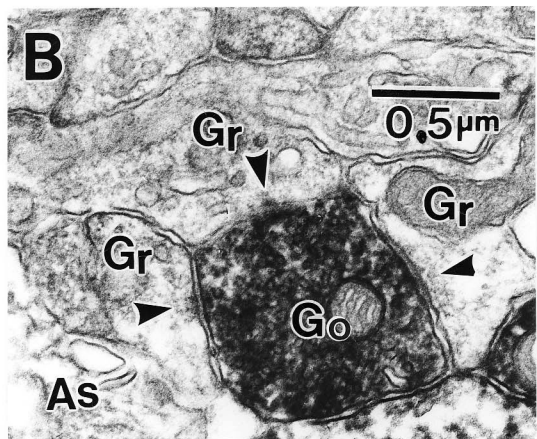
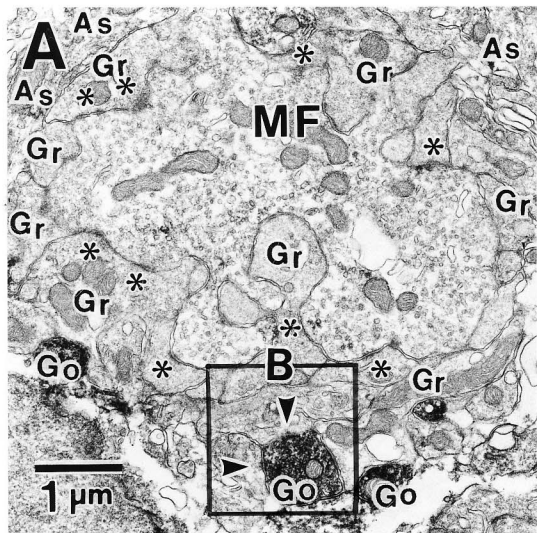
(E-H) Double labeling of GAD (FITC, green) and the  $\alpha 6$  subunit (Cy3<sup>TM</sup>, red) at P7 (E), P10 (F), P14 (G) and P90 (H). Changes in immunohistochemistry were similar to those of GAD and the  $\alpha 1$  subunit. In addition, the  $\alpha 6$ -immunoreactivity often localized independently of GAD-immunoreactivity (arrows).

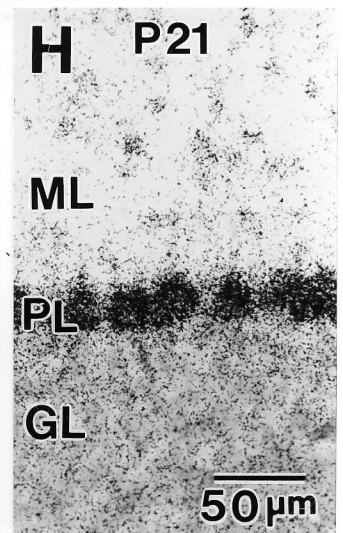
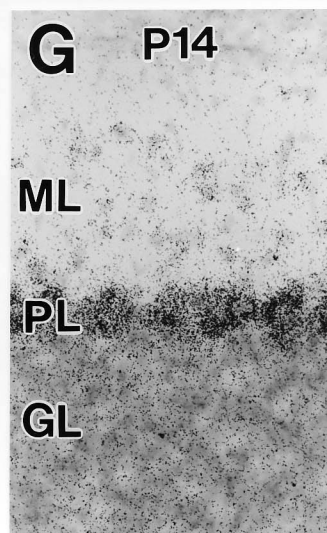
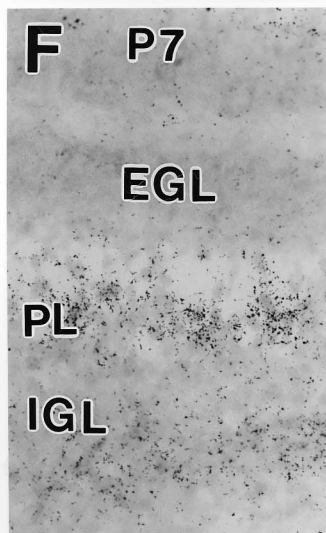
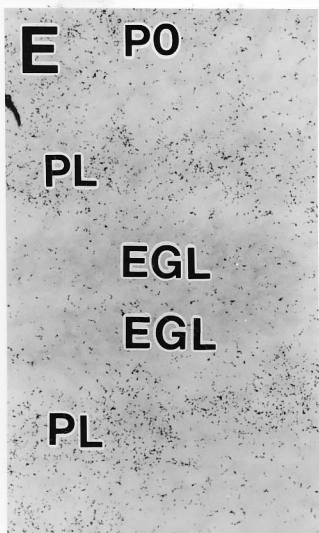
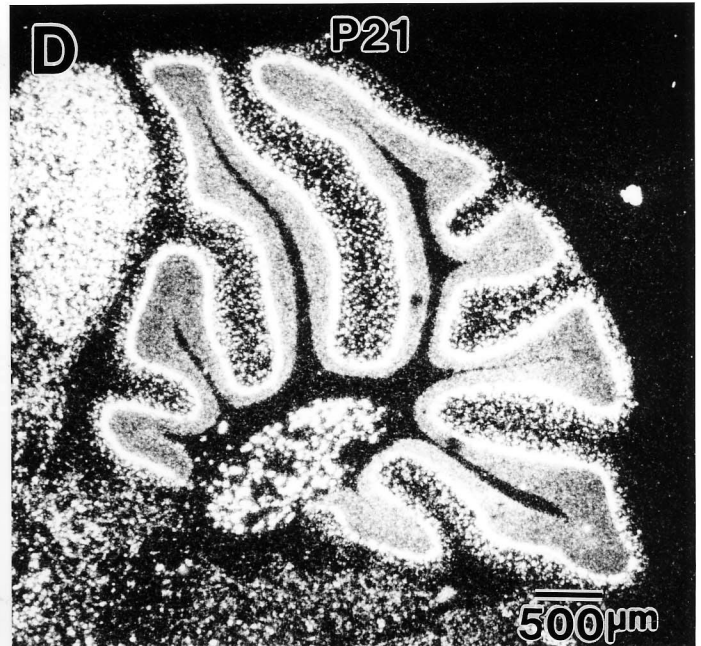
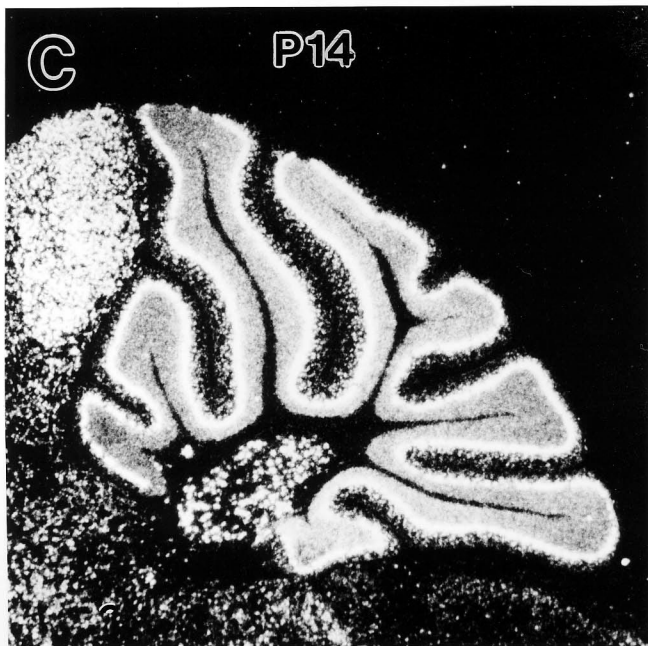
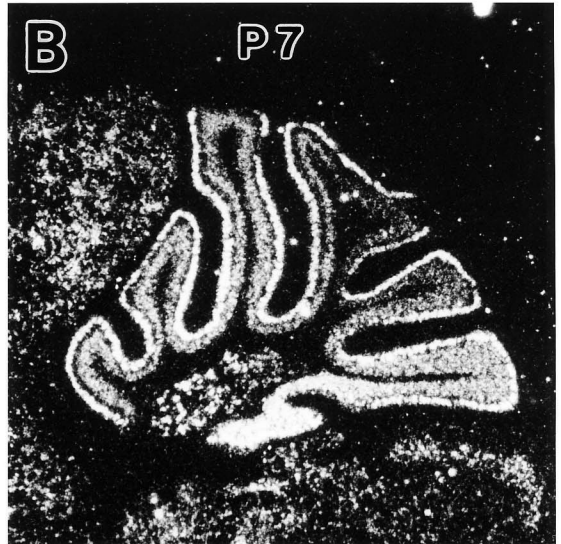
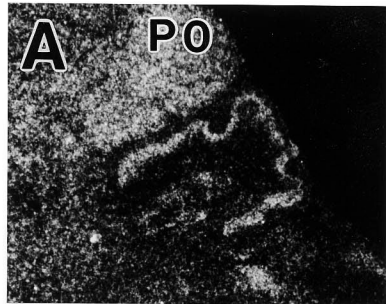
**Figure 9** Schematic illustration of the development of pre- and post-synaptic elements for GABAergic transmission in the cerebellar granular layer.

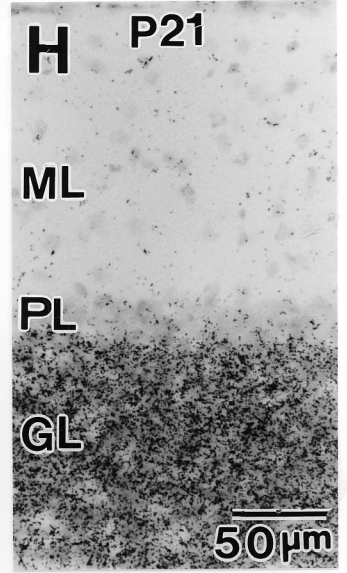
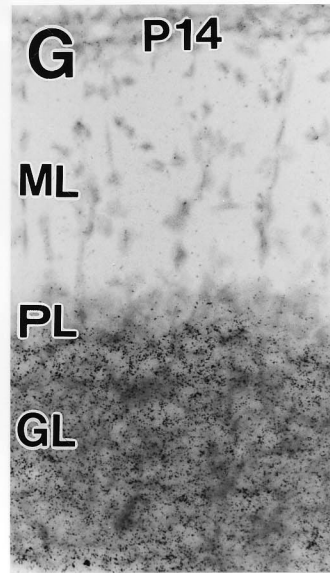
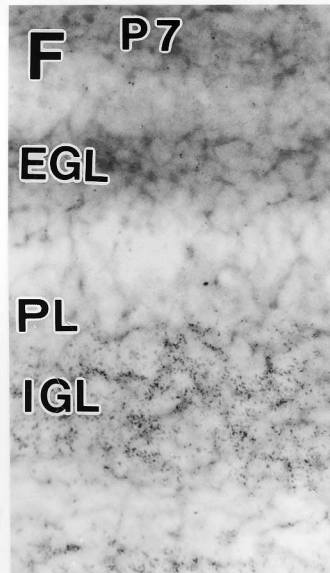
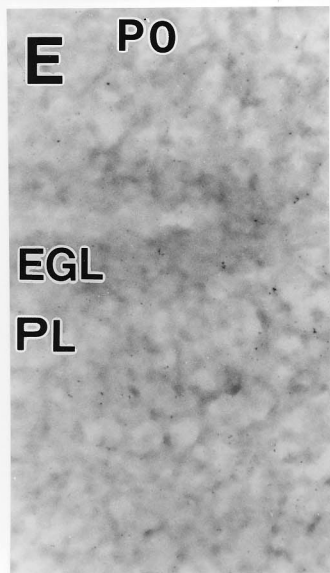
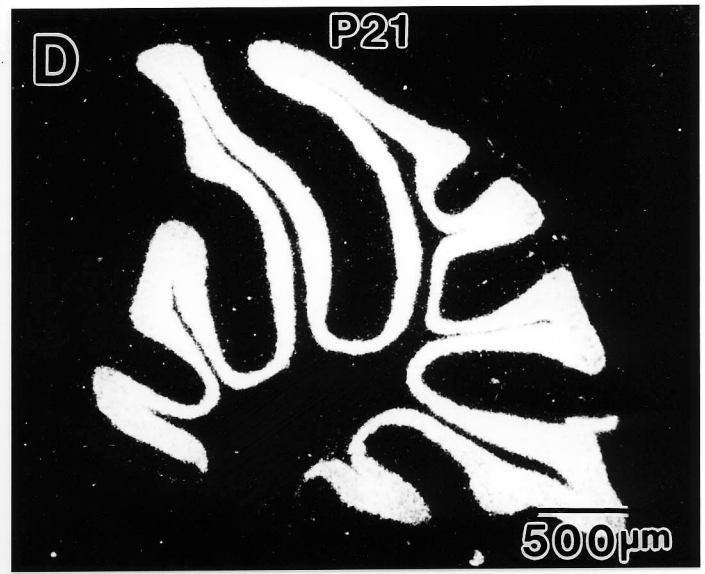
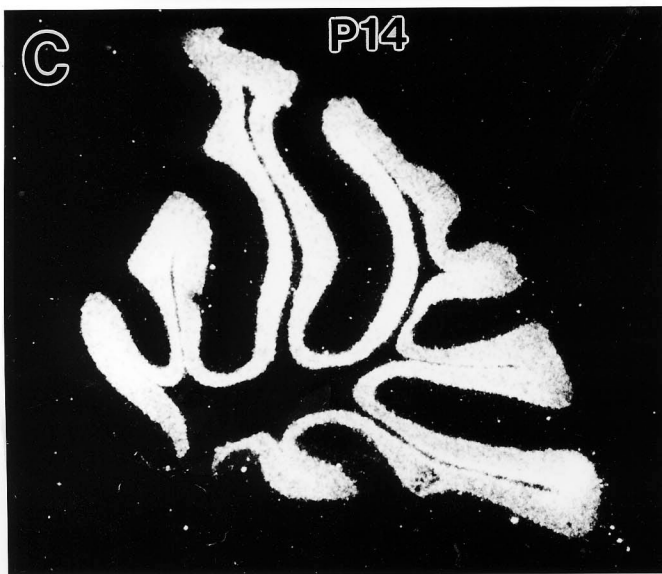
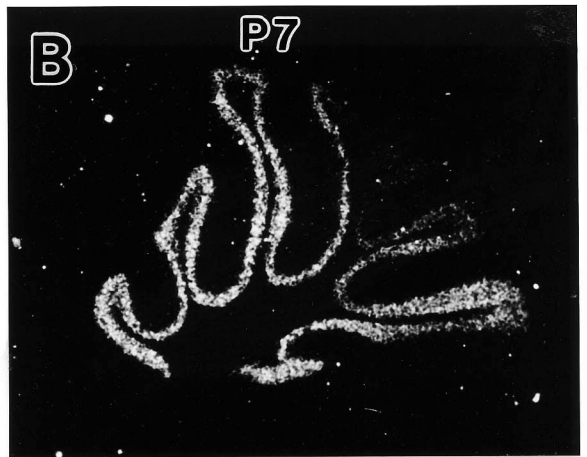
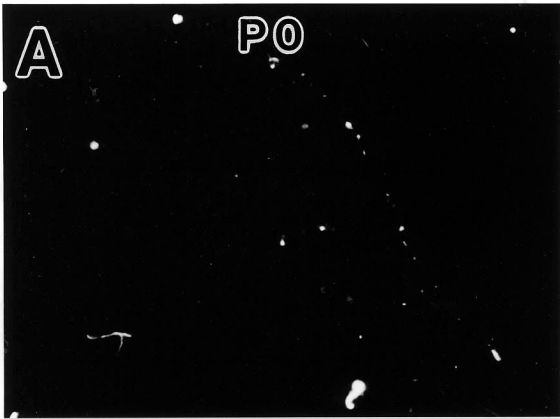
Before inhibitory synapses are formed, GAD-immunoreactivity, which is considered to show the localization of GABA, is widely localized in the Golgi cells including cell bodies (square), axons (lines), axon varicosities (black ovals), and growth cones (pentagons) (A). Granule cells start to express two GABA<sub>A</sub> receptor subunits,  $\alpha 1$  and  $\alpha 6$  ( $\alpha$ ), when GABAergic synapses appear (B). While GABAergic synapses are being formed, GAD accumulates at the axon terminals and varicosities, and the subunit-proteins are targeted to the synaptic sites (B, C). In the adult cerebellum, GAD-immunoreactivity was confined to the terminals and two subunits also localized at the postsynaptic sites (D).

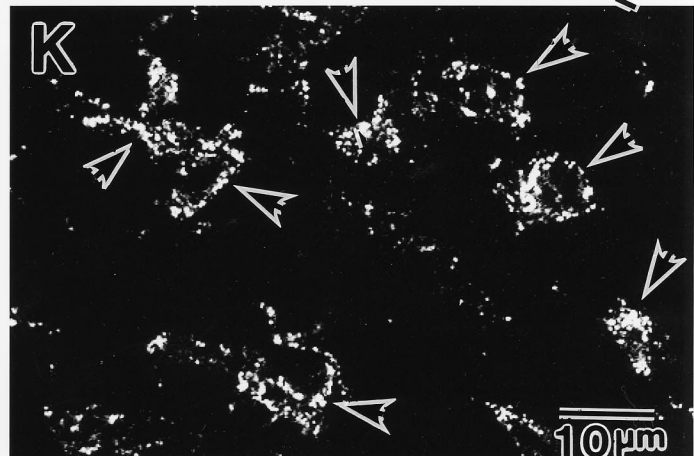
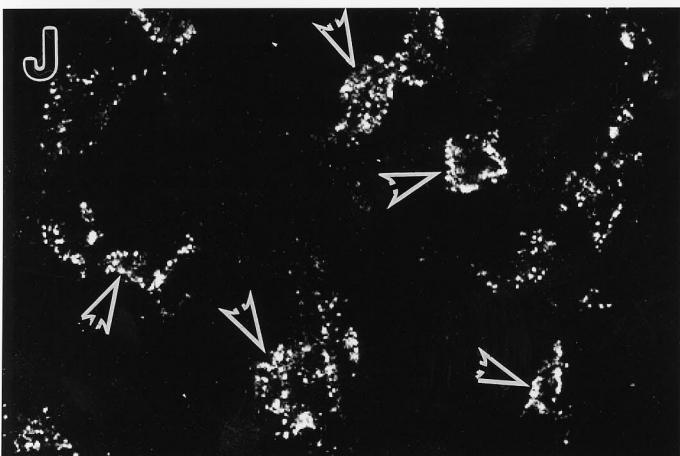
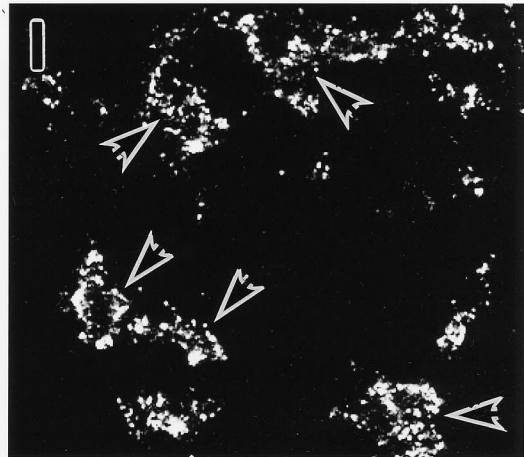
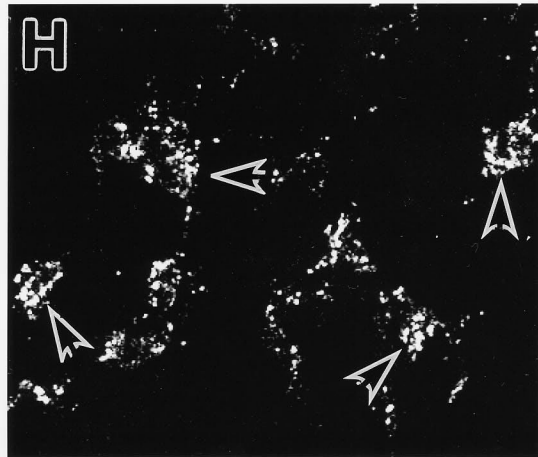
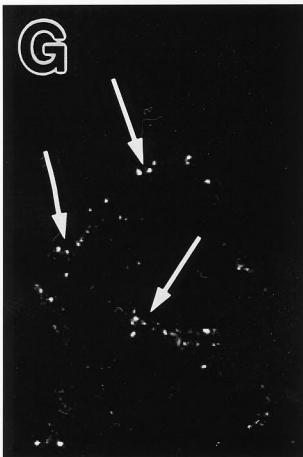
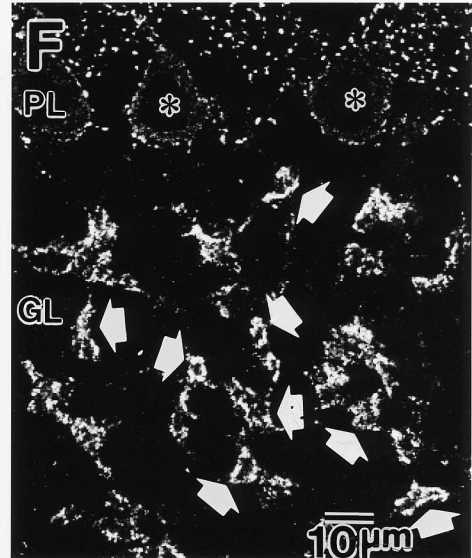
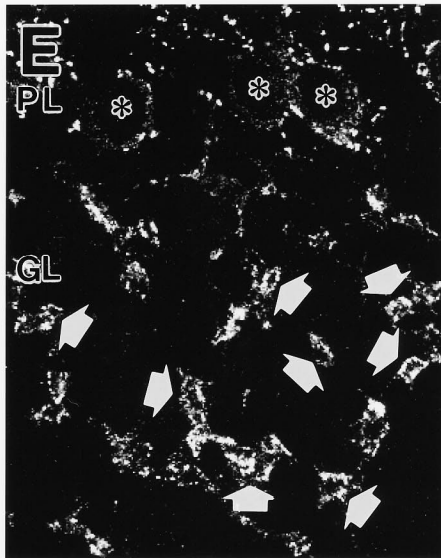
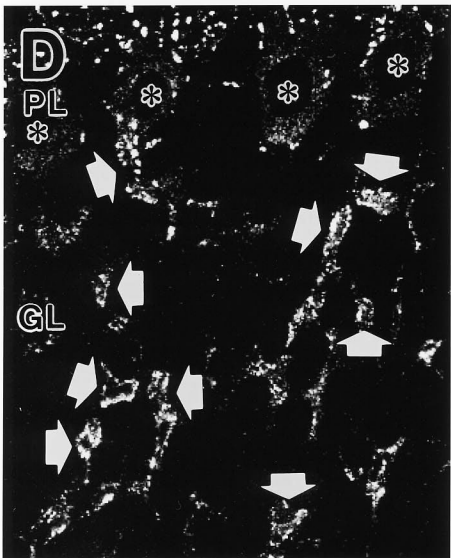
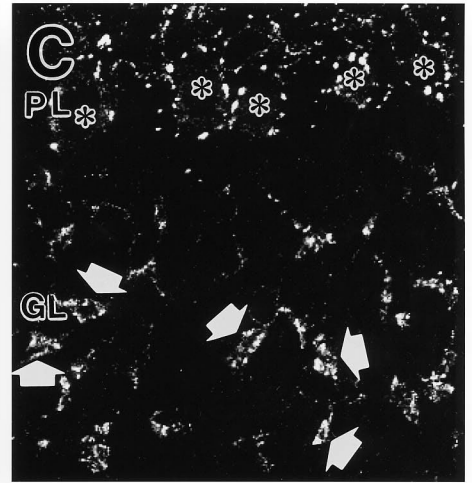
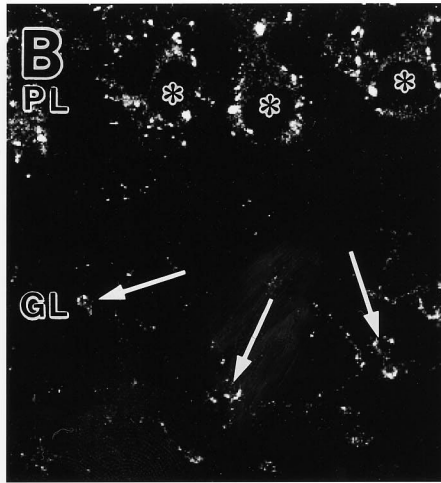
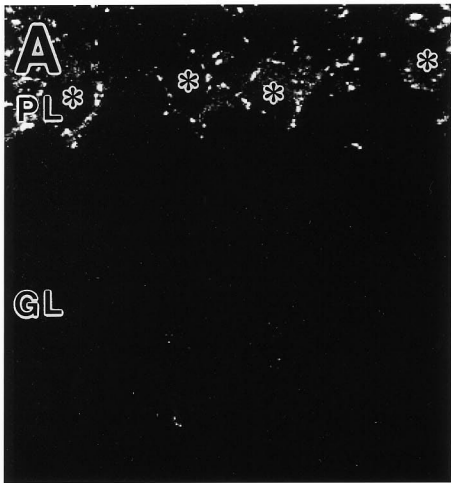


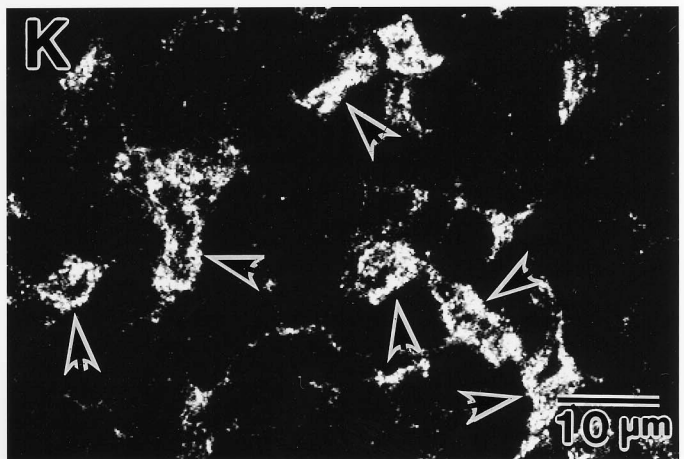
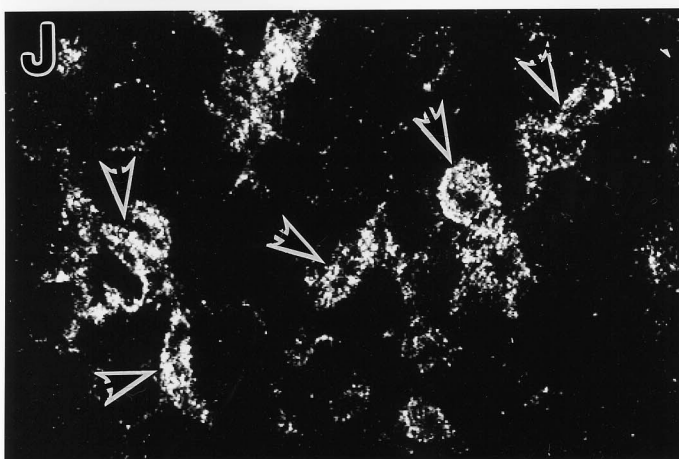
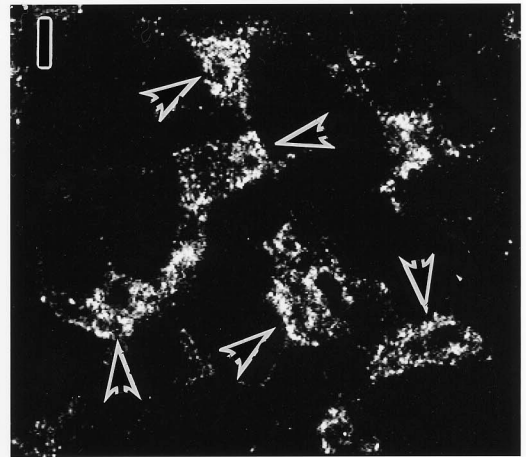
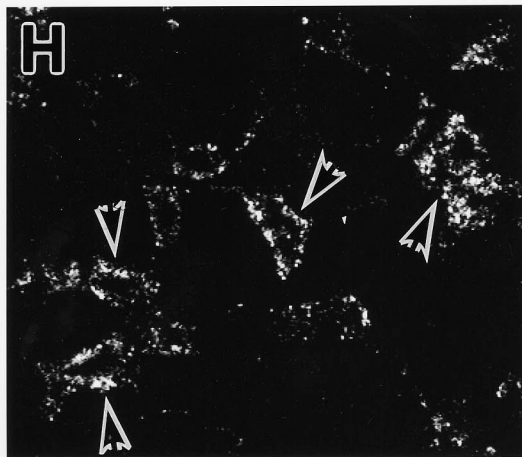
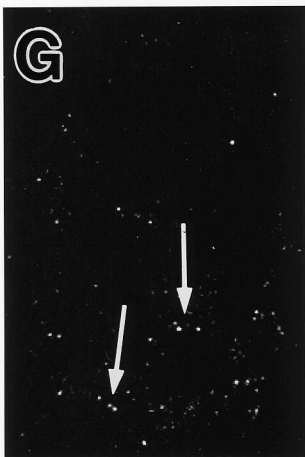
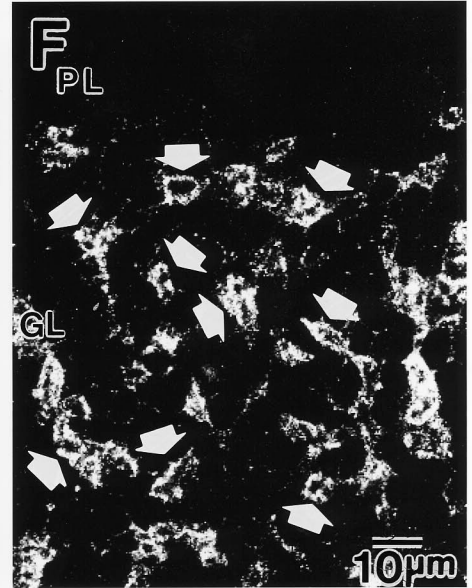
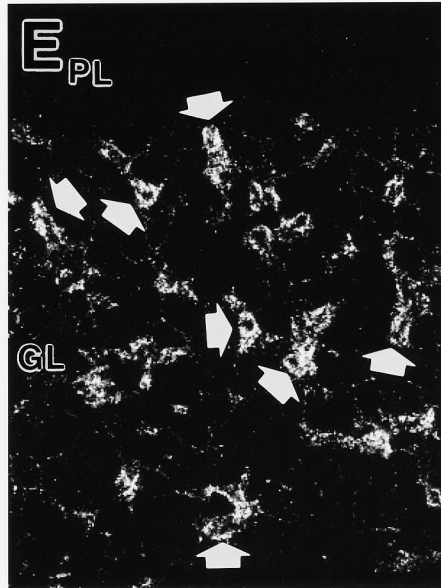
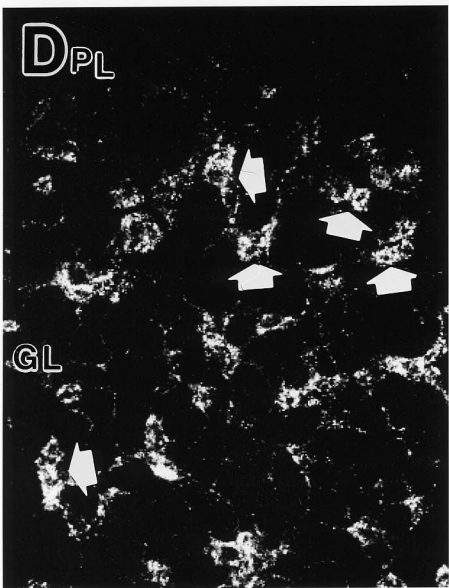
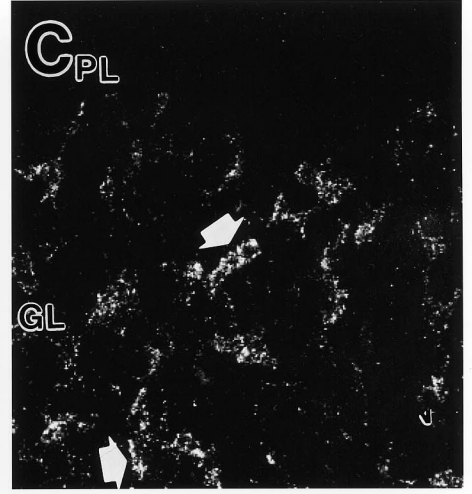
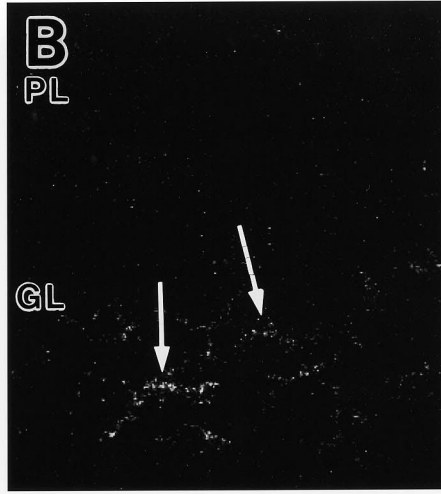
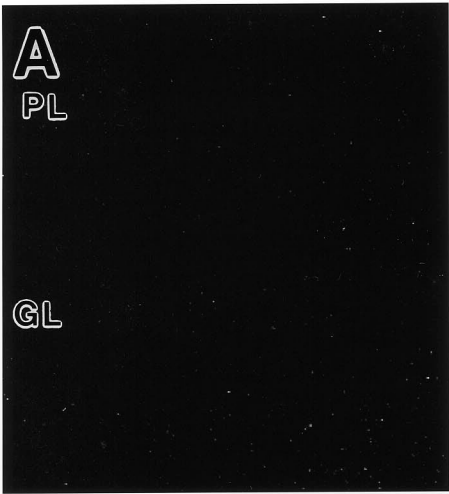


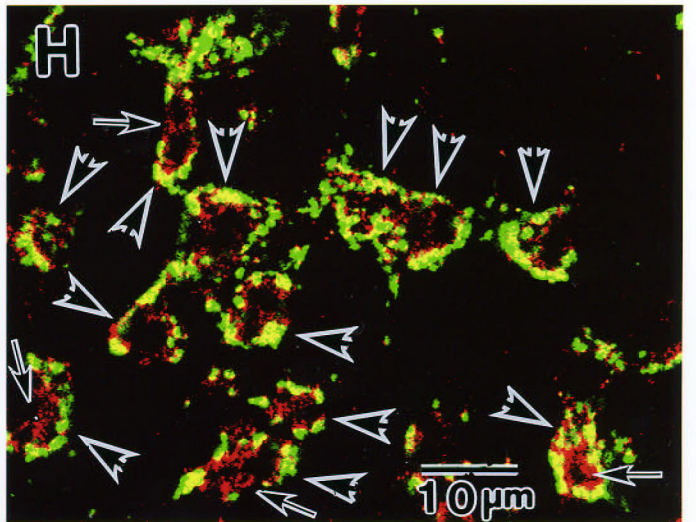
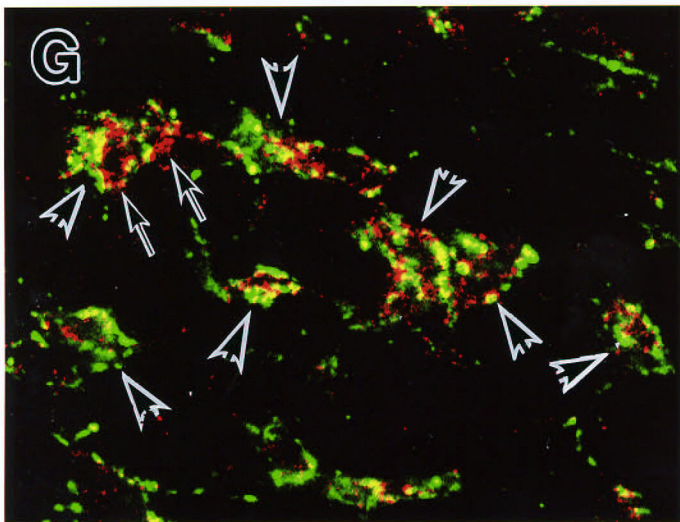
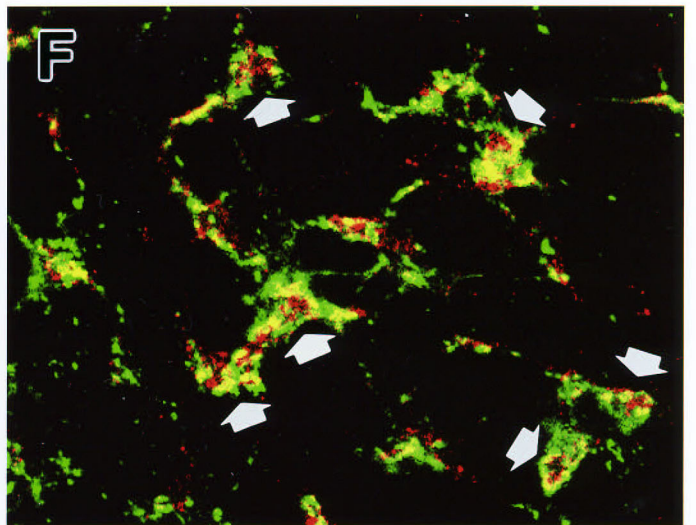
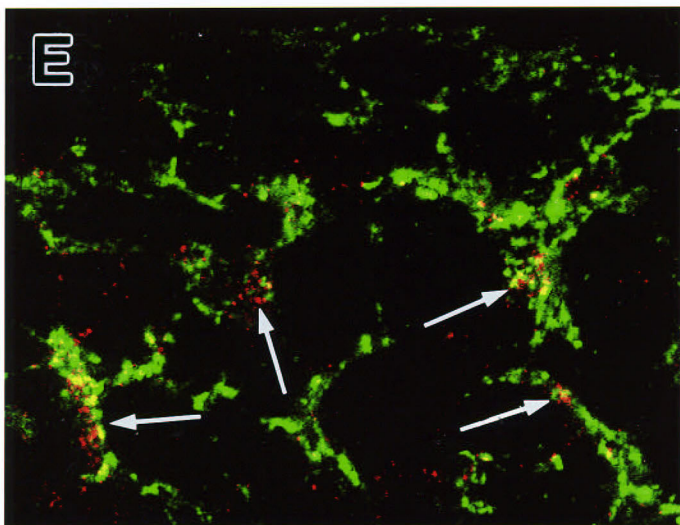
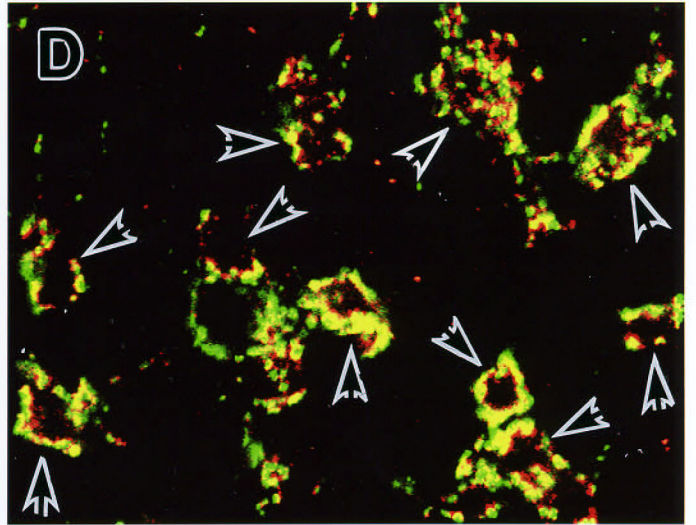
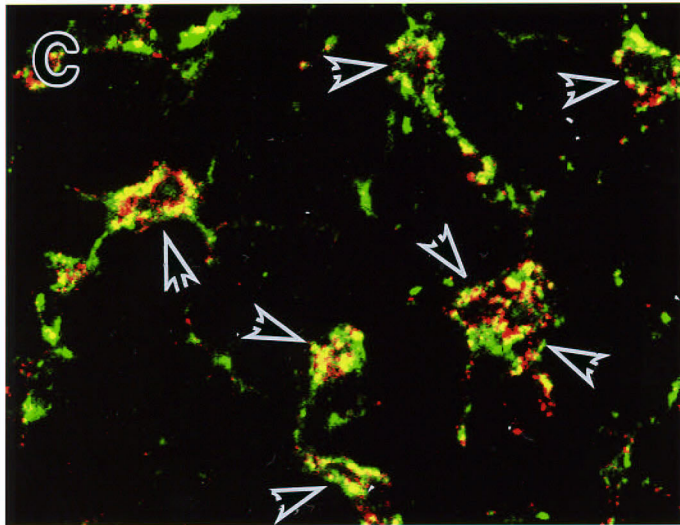
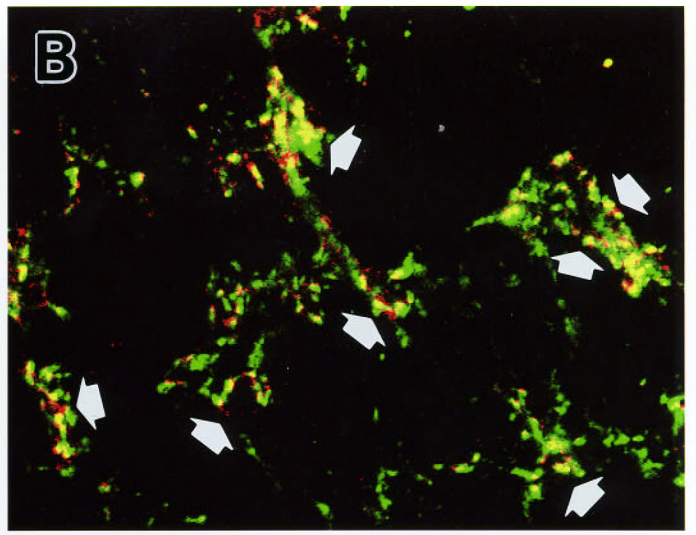
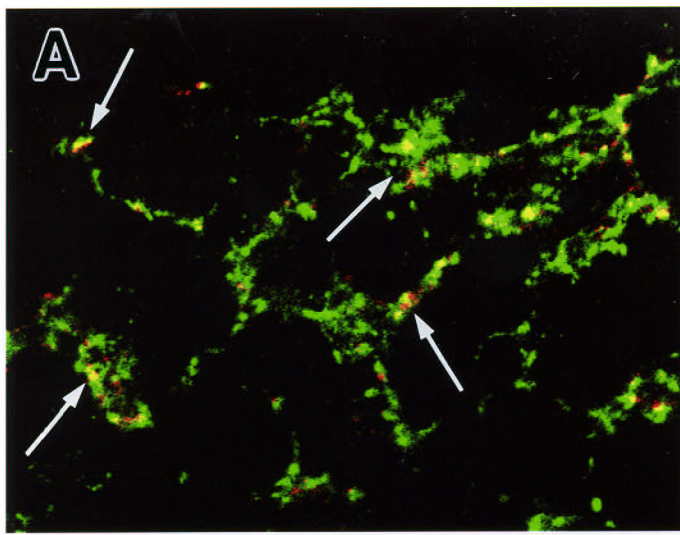




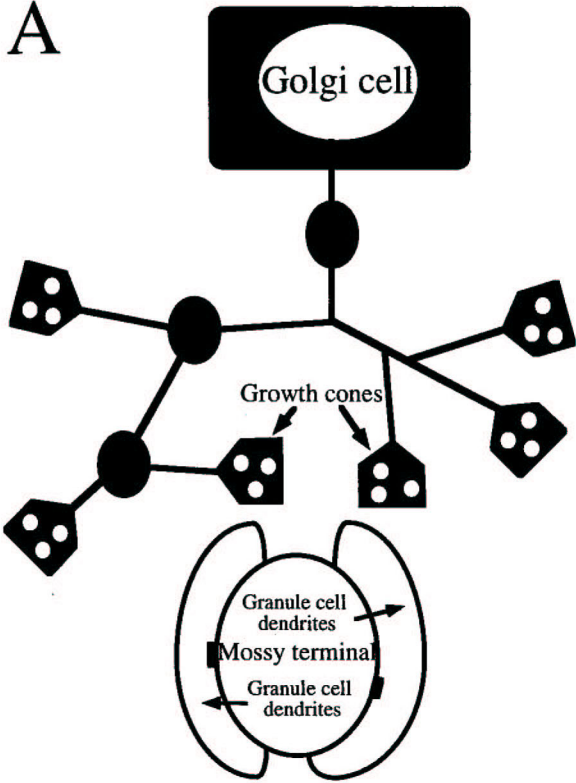




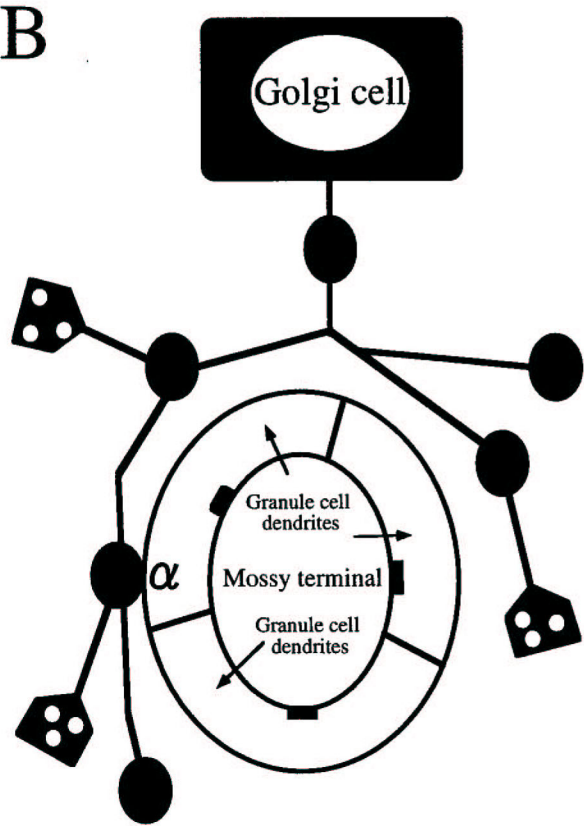




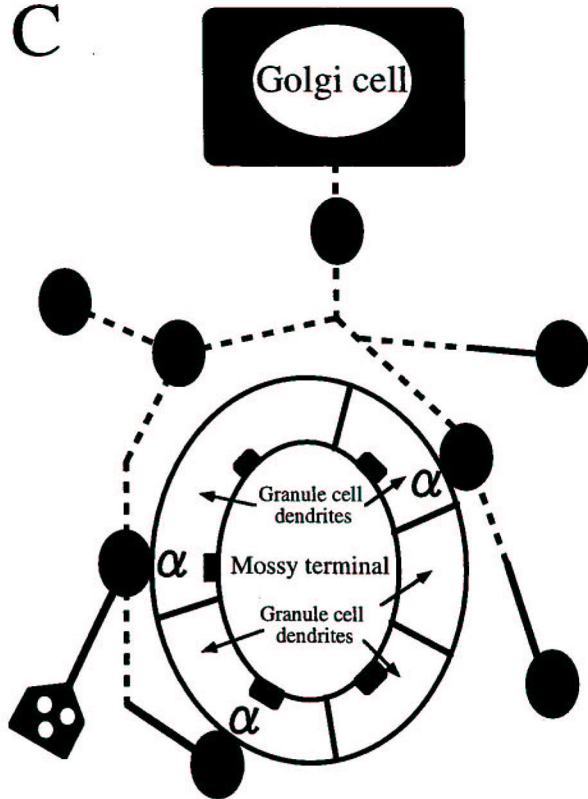
A



B



C



D

