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**Review****GABAergic signaling in the developing cerebellum**

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**Running title:** Development of GABAergic signaling

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## Abstract

In the adult central nervous system (CNS),  $\gamma$ -amino butyric acid (GABA) is a predominant inhibitory neurotransmitter, and regulates glutamatergic activity. Recent studies have revealed that GABA serves as an excitatory transmitter in the immature CNS, and acts as a trophic factor for brain development. Furthermore, synaptic transmission by GABA is also involved in expression of the higher brain functions, such as memory, learning, and anxiety. These results indicate that GABA plays various roles in the expression of brain functions, and the GABAergic roles developmentally change in accordance with an alteration of GABAergic transmission and signaling. We have morphologically investigated the developmental change in GABAergic transmission system and the key factors for the formation of GABAergic synapses and networks using the mouse cerebellum, which provides an ideal system for the investigation of brain development. Here, we focus on GABA and GABA<sub>A</sub> receptors in the developing cerebellum, and address the processes how GABA exerts its effect on the developing neurons and the mechanisms underlying formation of functional GABAergic synapses.

**Key words:** GABA<sub>A</sub> receptor, GABAergic synapse, GABAergic vesicle, morphogenesis, inhibitory transmission

## Introduction

In the mammalian central nervous system (CNS), GABA is a predominant neurotransmitter, and plays various roles in the expression of brain functions by the activation of ionotropic and metabotropic GABA receptors. In the adult CNS, GABA mediates inhibitory synaptic transmission, regulates glutamatergic activity and prevents hyperexcitation, such as seizures (Macdonald & Olsen, 1994; Olsen & Avoli, 1997; Kardos, 1999). In the immature CNS, GABA regulates brain morphogenesis, such as changes in cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death (Barker *et al.*, 1998; Belhage *et al.*, 1998; Kardos, 1999; Varju *et al.*, 2001; Ben-Ari, 2002; McCarthy *et al.*, 2002; Owens & Kriegstein, 2002). Furthermore, during the maturation period, GABAergic transmission controls the experience-dependent plasticity in the visual cortex, induces long-term potentiation, which is an electrophysiological basis of memory and learning (Wolff *et al.*, 1993; Ben-Ari *et al.*, 1997; Freund & Gulyas, 1997; McBain & Maccaferri, 1997; Hensch *et al.*, 1998; Paulsen & Moser, 1998; Kardos, 1999; Fagiolini & Hensch, 2000), modulates anxiety (Nutt *et al.*, 1990; Pratt, 1992), and generates circadian rhythms (Turek & Van Reeth, 1988; Nutt *et al.*, 1990; Pratt, 1992; Wagner *et al.*, 1997).

It is thought that developmental shift in the action of GABA is based on an alteration of GABAergic transmission and signaling. During development, GABA transmitter system undergoes a change from non-synaptic to synaptic mechanisms (Taylor & Gordon-Weeks, 1991; Attwell *et al.*, 1993; Fon & Edwards, 2001; Varju *et al.*, 2001; Owens & Kriegstein, 2002), and subunit compositions and localization of ionotropic GABA receptors drastically change (Gambarana *et al.*, 1990; Araki *et al.*, 1992; Laurie *et al.*, 1992b; Poulter *et al.*, 1992; Fritschy *et al.*, 1994; Ma & Barker, 1995; Maric *et al.*, 1997; Owens *et al.*, 1999). Environmental changes, such as decreasing of intracellular chloride concentration, influence

on the response of GABA receptors (Cherubini *et al.*, 1991; Rohrbough & Spitzer, 1996; Perkins & Wong, 1997; Serafini *et al.*, 1998; Ganguly *et al.*, 2001; Ben-Ari, 2002; Owens & Kriegstein, 2002). In the first half, we review the developmental change in GABAergic signaling, and discuss how GABA exerts its effect on immature neurons during brain development.

Establishment of GABAergic synapses is crucial for the expression of normal and higher brain functions such as memory and learning. In the second half, we address the key factors for the formation of functional GABAergic synapses, and discuss the mechanisms underlying the formation of GABAergic synapses and networks.

## **GABA and GABA receptors**

In the CNS, GABA is produced from glutamate by two isoforms of glutamic acid decarboxylase (GAD65 and GAD67) (Martin & Rimvall, 1993; Barker *et al.*, 1998; Varju *et al.*, 2001), and is loaded into vesicles by vesicular GABA transporter (VGAT) (McIntire *et al.*, 1997; Reimer *et al.*, 1998; Fon & Edwards, 2001). In response to nerve stimulation, GABA is released by the fusion of vesicles with the presynaptic membrane at the nerve terminals, and activates GABA receptors. GABAergic signals are terminated by reuptake of neurotransmitter into nerve terminals or surrounding glia by the plasma membrane GABA transporters (GATs) (Cherubini & Conti, 2001).

GABA receptors are classified into three groups on the basis of pharmacology and biochemistry; GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub>. Among them, fast synaptic transmission is mediated by ionotropic GABA receptors, GABA<sub>A</sub> and GABA<sub>C</sub> receptors (Macdonald & Olsen, 1994; Kaupmann *et al.*, 1998; Mehta & Ticku, 1999; Bormann, 2000). GABA<sub>A</sub> receptor is a member of ligand-gated ion channel receptor families, and considered to be

composed of heteromeric five subunits belonging to seven different subunit families,  $\alpha$ 1-6,  $\beta$ 1-3,  $\gamma$ 1-3,  $\delta$ ,  $\varepsilon$ ,  $\pi$ , and  $\theta$  (Olsen & Tobin, 1990; Macdonald & Olsen, 1994; Nayeem *et al.*, 1994; Sieghart, 1995; Tretter *et al.*, 1997; Mehta & Ticku, 1999; Sieghart *et al.*, 1999). Native GABA<sub>A</sub> receptors contain at least one  $\alpha$ -, one  $\beta$ -, and one  $\gamma$ -subunits with the  $\delta$ -,  $\varepsilon$ -,  $\pi$ -, and  $\theta$ -subunits to substitute for the  $\gamma$  subunit (Pritchett *et al.*, 1989; Sieghart, 1995; McKernan & Whiting, 1996; Sieghart *et al.*, 1999). The subunit compositions drastically change during brain development (Gambarana *et al.*, 1990; Araki *et al.*, 1992; Laurie *et al.*, 1992a; Laurie *et al.*, 1992b; Poulter *et al.*, 1992), and exhibit pharmacological and electrophysiological properties. Among seven subunit families,  $\alpha$  subunits may mainly reflect the functional diversity of the GABA<sub>A</sub> receptors. The expression and localization of each type of  $\alpha$  subunit have remarkable regional differences and drastically change during brain development (Laurie *et al.*, 1992a; Laurie *et al.*, 1992b; Fritschy *et al.*, 1994; Hornung & Fritschy, 1996). The variety of  $\alpha$  subunits in the GABA<sub>A</sub> receptors influences the electrophysiological characteristic and pharmacological sensitivity to GABA, benzodiazepine families, neurosteroids, and ethanol (Pritchett *et al.*, 1989; Luddens *et al.*, 1990; Olsen & Tobin, 1990; Macdonald & Olsen, 1994; Sieghart, 1995; Kardos, 1999). GABA binding opens the pore of GABA receptors and induces influx or efflux of anions such as chloride ions (Olsen & Tobin, 1990; Macdonald & Olsen, 1994; Sieghart, 1995; Kardos, 1999). GABA<sub>C</sub> receptor is also an ion-channel type receptor, which is composed of only single or multiple  $\rho$  subunits, and is considered as pharmacological variants of GABA<sub>A</sub> receptors (Bormann & Feigenspan, 1995; Mehta & Ticku, 1999; Bormann, 2000). GABA<sub>B</sub> receptor, which includes three isoforms, R1a, R1b, and R2 (Kaupmann *et al.*, 1997; Kaupmann *et al.*, 1998), is a metabotropic receptor, activates G proteins, negatively regulates the second messenger system, responds slow acting inhibition of channel and receptor functions (Bormann, 1988; Connors *et al.*, 1988; Nicoll,

1988; LeVine, 1999).

### **Extrasynaptic GABA release in the developing cerebellum**

GABA appears in the GABAergic neurons long before the onset of synaptogenesis, (Lauder *et al.*, 1986; Lipton & Kater, 1989; Van Eden *et al.*, 1989; Lauder, 1993; Liu *et al.*, 1997; Fairen *et al.*, 1998) and its subcellular localization gradually changes during brain development (McLaughlin *et al.*, 1975; Behar *et al.*, 1993; Takayama & Inoue, 2004a). Before GABAergic synapses are formed, GABA is distributed widely in the GABAergic neurons, including cell bodies, dendrites, axons, axon varicosities, and growth cones (Fig. 1A). The VGAT, which is a membrane protein of GABAergic vesicles and transports cytosolic GABA into the vesicles (Chaudhry *et al.*, 1998; Reimer *et al.*, 1998; Dumoulin *et al.*, 1999; Takamori *et al.*, 2000; Fon & Edwards, 2001), accumulates at the axon varicosities and growth cones where GABAergic synapse are not yet formed (Fig. 1C, E). This result indicates that GABA is localized in both cytoplasm and vesicles throughout GABAergic neurons. During the synapse formation, GABA becomes confined to the axon terminals, and gradually disappears from axons themselves and dendrites. After finishing synapse formation, GABA is almost co-localized with VGAT at the synaptic sites where the GABA<sub>A</sub> receptor α1 subunit accumulates (Fig. 1B, D, F). This result indicates that majority of GABA is exclusively localized in the synaptic vesicles within the axon terminals in the matured cerebellum.

Physiological and biochemical studies have demonstrated that non-vesicular form of GABA is also secreted via the plasma membrane by reverse transporter actions of GATs (Jaffe & Vaello, 1988; Taylor *et al.*, 1990; Taylor & Gordon-Weeks, 1991; Attwell *et al.*, 1993; Behar *et al.*, 1993; Belhage *et al.*, 1993; Gao & van den Pol, 2000; Varju *et al.*, 2001). Therefore, in the developing brain, cytosolic GABA might be extrasynaptically released from

dendrites, axons and cell bodies via the plasma membrane by GATs (Yan & Ribak, 1998), and GABA in the vesicles might be also extrasynaptically released from the axon varicosities and growth cones (Varju, *et al.*, 2001). These results as a whole suggest that dendrites of Purkinje cells and axons of stellate and basket cells in the molecular layer and dendrites and axons of Golgi cells in the granular layer could supply extracellular GABA which mediates morphogenesis in the immature cerebellum. In the mature cerebellum, GABA is exclusively transported into the synaptic vesicles by VGAT at the axon terminals, is synaptically released, and might mediate inhibitory transmission.

### **GABAergic signaling before synapse formation**

#### *GABAergic roles in the developing brain*

During brain development, extrasynaptically released GABA diffuses in the extracellular space and activates GABA receptors on neighboring neurons. The activation of GABA<sub>A</sub> receptors depolarizes membrane potential, since the Cl<sup>-</sup> reversal potential of the neuronal membrane is elevated (Cherubini *et al.*, 1991; Rohrbough & Spitzer, 1996; Perkins & Wong, 1997; Serafini *et al.*, 1998; Leinekugel *et al.*, 1999; Ben-Ari, 2002; Owens & Kriegstein, 2002). In the immature CNS, Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> co-transporter 1 (NKCC1), which raises the concentration of intracellular chloride ion, [Cl<sup>-</sup>]<sub>i</sub>, is predominantly expressed, and elevates the equilibrium potential of Cl<sup>-</sup>. Under the high [Cl<sup>-</sup>]<sub>i</sub> condition, the activation of GABA<sub>A</sub> receptors generates efflux of chloride ion and depolarization of membrane potential (Fig.2A). In contrast, K<sup>+</sup>-Cl<sup>-</sup> co-transporter 2 (KCC2), which lowers the [Cl<sup>-</sup>]<sub>i</sub>, becomes a predominant chloride co-transporter in the mature CNS, and the GABA induces hyperpolarization of membrane potential and inhibition of excitability (Fig. 2B). GABA<sub>A</sub>-receptor-mediated depolarization in the immature CNS, activates voltage-dependent

$\text{Ca}^{++}$  channels (VDCC) (Fig. 2A) and/or N-methyl-D-aspartate (NMDA) type glutamate receptors, and elevates cytosolic  $\text{Ca}^{++}$  ion (Connor *et al.*, 1987; Yuste & Katz, 1991; Reichling *et al.*, 1994; Leinekugel *et al.*, 1995; Brickley *et al.*, 1996; Obrietan & van den Pol, 1996; Ben-Ari *et al.*, 1997; Serafini *et al.*, 1998; Eilers *et al.*, 2001). The elevation of cytosolic calcium effects various steps of CNS development, such as (1) cell proliferation, (2) cell migration, and (3) neuronal maturation, including synaptogenesis (Barker *et al.*, 1998; Belhage *et al.*, 1998; Kardos, 1999; Varju *et al.*, 2001; Ben-Ari, 2002; McCarthy *et al.*, 2002; Owens & Kriegstein, 2002). GABA acts as an anti-proliferation molecule, reduces the DNA synthesis in the proliferating precursor cells, and depresses the rate of cellular proliferation by the activation of  $\text{GABA}_A$  receptors and other  $\text{GABA}_A$ -receptor related molecules (LoTurco *et al.*, 1995; Haydar *et al.*, 2000). GABA modulates neuronal migration by ‘chemotaxis’ and ‘chemokinesis’ at the femtomolar ( $10^{-15}\text{M}$ ) and micromolar ( $\mu\text{M}$ ) level, respectively (Behar *et al.*, 1994; Behar *et al.*, 1995; Behar *et al.*, 1996). Activation of  $\text{GABA}_B$  and  $\text{GABA}_C$  receptors promotes the migration out of the proliferating layer, whereas that of  $\text{GABA}_A$  receptor slows or almost stops the movement in the cortical plates (Behar *et al.*, 2000). Furthermore, exposure of neurons to GABA or  $\text{GABA}_A$  receptor agonists induces the synthesis of specific molecules such as neuron-specific enolase (NSE) and neural cell adhesion molecules (NCAMs), enhances the growth rate of neuronal processes, and facilitates synapse formation by inducing the expression and targeting of GABA receptor subunits, which mediate synaptic transmission (Wolff *et al.*, 1978; Meier & Jorgensen, 1986; Meier *et al.*, 1987; Spoerri, 1988; Abraham & Schousboe, 1989; Barbin *et al.*, 1993; Kim *et al.*, 1993; Elster *et al.*, 1995; Mitchell & Redburn, 1996; Carlson *et al.*, 1997; Belhage *et al.*, 1998; Carlson *et al.*, 1998; Mellor *et al.*, 1998; Gao & van den Pol, 2000; Moss & Smart, 2001). In the case of interneurons and their networks, GABA might stimulate the expression of

neurotrophins, such as brain derived neurotrophic factors (BDNF) and their receptors, such as trks, and enhance the growth of neurons and synapses (Berninger *et al.*, 1995; Marty *et al.*, 1996; Vicario-Abejon *et al.*, 1998; Rico *et al.*, 2002).

### ***GABA<sub>A</sub> receptor expression in the developing cerebellum***

The subunit compositions of GABA<sub>A</sub> receptors drastically change during brain development (Gambarana *et al.*, 1990; Araki *et al.*, 1992; Laurie *et al.*, 1992b; Poulter *et al.*, 1992; Maric *et al.*, 1997; Owens *et al.*, 1999; Takayama & Inoue, 2004a; Takayama & Inoue, 2004b). We investigated the developmental changes in expression and localization of the GABA<sub>A</sub> receptor  $\alpha$  subunits, which may mainly reflect the functional diversity of the GABA<sub>A</sub> receptors, in the cerebellum. Proliferating cells in the ventricular zone adjacent to the fourth ventricle and the upper half of the external granular layer expressed no  $\alpha$  subunits, which are indispensable for the functional GABA<sub>A</sub> receptors. After finishing cell proliferation, cerebellar neurons start to express the functional GABA<sub>A</sub> receptors. Differentiating Purkinje cells express the  $\alpha 3$  subunits, migrating and maturing granule cells express the  $\alpha 2$  subunit, and both subunits disappear from the cerebellar cortex after finishing synapse formation (Table 1) (Takayama & Inoue, 2004b). In addition, the  $\beta 3$ ,  $\gamma 1$ , and  $\gamma 3$  subunits are also abundantly expressed in the developing cerebellum (Laurie, *et al.*, 1992b). These results suggest that extrasynaptically released GABA could activate GABA<sub>A</sub> receptors consisting of above restricted subunits and be involved in the regulation of proliferation, neuronal migration and maturation in the cerebellum.

### **Formation of GABAergic synapses**

Assembly of GABAergic synapses is crucial for normal and higher brain functions. Synapse formation is considered to be a multi-step process (Vaughn, 1989; Cherubini & Conti,

2001; Moss & Smart, 2001). While exploring their environment, axonal growth cones lead elongating axons to their proper targets and make contact with dendrites and cell bodies of target neurons. Initial contact is followed by the establishment of stable synapses. In the presynapse, synaptic vesicles accumulate to the nerve terminals and docked near the active zone. In the postsynapse, GABA receptors which mediate inhibitory synaptic transmission are targeted to and clustered at an appropriate synaptic site opposite the GABA-releasing site (Macdonald & Olsen, 1994; Olsen & Avoli, 1997; Kardos, 1999). At the same time, GABA<sub>A</sub> receptors, which are involved in brain morphogenesis, disappear from postsynaptic neurons (Takayama & Inoue, 2004a; Takayama & Inoue, 2004b).

### ***Target determination***

It is not fully understood how GABAergic neurons search, recognize and determine their target neurons. To reveal the mechanisms how GABAergic neurons determine their targets and form synapses, we employed the cerebellar mutant mice.

First, we examined the specificity of neuron-to-neuron connection in the mutant cerebellum. In the normal cerebellar cortex, five major types of neurons innervate distinct types of target neurons (Llinas & Hillmann, 1969; Palay & Chan-Palay, 1974; Ito, 1984). Stellate and basket cells form synapses with Purkinje cell dendritic shafts and cell bodies, respectively. Golgi cell axons form synapses not with Purkinje cells but with granule cell dendrites at the peripheral part of synaptic glomeruli. These specific innervation patterns, however, are not preserved in the abnormal environment of the reeler and weaver cerebellum. Golgi cells directly innervate to Purkinje cells in the central mass of the reeler cerebellum, and weaver cerebellum (Rakic, 1976; Mariani *et al.*, 1977; Caviness & Rakic, 1978; Sotelo & Privat, 1978; Wilson *et al.*, 1981; Takayama, 1994). In both regions, granule cells were few or absent. Thus, Golgi cell axons form synapses with neighboring neurons instead of granule

cells. This result indicates that targets of Golgi cells are not strictly determined but are influenced by the environment.

Next, we examined the changes in synaptic architecture in the cerebellar mutant mice. Inhibitory input significantly increases in the cortical surface of reeler cerebellum and the cerebellum of Wriggle mouse sagami (WMS) (Kokubun, 1991; Inoue *et al.*, 1993; Takayama, 1994). In both regions, synaptic connections between parallel fibers and Purkinje dendrites decrease, and GABAergic synapses are formed at the dendritic spines, where excitatory synapses are usually formed. This result indicates the relationship between GABAergic and glutamatergic inputs.

These results suggested that targets of GABAergic neurons plastically alter according to the environment, and GABAergic innervation is influenced by the glutamatergic excitatory inputs. Furthermore, recent investigations revealed that GABAergic innervation (Kapfer *et al.*, 2002; Kim & Kandler, 2003; Kandler, 2004).

### ***Change in subunit compositions***

As shown in a previous paragraph, expression of the GABA<sub>A</sub> receptor  $\alpha$  subunits in the cerebellum developmentally changes especially during the GABAergic synapse formation (Takayama & Inoue, 2004a; Takayama & Inoue, 2004b). While the expression of the  $\alpha 2$  and  $\alpha 3$  subunits is decreasing, the  $\alpha 1$  and  $\alpha 6$  subunits appear and increase in their expression (Laurie *et al.*, 1992b; Poulter *et al.*, 1992; Tia *et al.*, 1996; Wisden *et al.*, 1996; Maric *et al.*, 1997; Mellor *et al.*, 1998). Therefore, the  $\alpha$  subunits in the GABA<sub>A</sub> receptors shift from the  $\alpha 2$  and  $\alpha 3$  subunits to the  $\alpha 1$  and  $\alpha 6$  subunits during the development. This result suggests that two pieces of evidences, disappearance of the subunits, which are involved in morphogenesis, and appearance of the subunits, which mediate inhibitory synaptic transmission, are crucial for the GABAergic synapse formation.

To test the mechanism underlying the change in subunit compositions, we investigated their relationship with neuronal maturation such as migration, axonal and dendritic extension, and formation of excitatory and inhibitory synapses using reeler mutant mice. In the reeler cerebellum, maturation of malpositioned Purkinje cells assumed to be arrested in terms of the synaptic architecture and dendritic arborization (Rakic, 1976; Mariani *et al.*, 1977; Caviness & Rakic, 1978; Sotelo & Privat, 1978; Wilson *et al.*, 1981; Takayama, 1994). Parallel fibers and axons from stellate and basket cells do not innervate the Purkinje cells in the central cerebellar mass. Moreover, multiple innervations from climbing fibers remain in the adult reeler cerebellum. Instead, Purkinje cells directly form synapses with mossy fibers and Golgi cell axons. Dendrites of Purkinje cells are poorly developed and extend almost randomly. The  $\alpha 3$  subunit, however, is almost negative as in the normal mature cerebellum (Fig. 3E, F) (Takayama & Inoue, 2003), and malpositioned Purkinje cells abundantly express the  $\alpha 1$  subunit (Fig. 3A, B) (Frostholm *et al.*, 1991; Takayama & Inoue, 2003). These results indicate that developmental change in subunit composition is independent of neuronal maturation, such as settling in the normal neuronal position, maturation of excitatory networks. Absence of normal inhibitory synapse with stellate and basket cell axons nor heterologous input from Golgi cells do not affect the developmental change in subunit composition. Previous in vitro studies have indicated that GABAergic stimulation induces the low-affinity type GABA receptor expression, which is involved in inhibitory synaptic transmission (Meier *et al.*, 1984; Belhage *et al.*, 1986; Kim *et al.*, 1993; Elster *et al.*, 1995; Gao & Fritschy, 1995; Carlson *et al.*, 1997; Belhage *et al.*, 1998; Carlson *et al.*, 1998; Mellor *et al.*, 1998; Raetzman & Siegel, 1999; Schousboe, 1999). The change in subunit composition simultaneously occurred during the GABAergic synaptogenesis (Table 1). These results suggest that innervation of GABAergic fibers may be important for the

change in subunit composition even if the synapses are heterologous and ectopic, and GABAergic innervation might initiate and/or accelerate the changes in subunit composition.

### ***Specific subunit expression***

In the CNS, distinct types of subunits are expressed at distinct synapses (Laurie *et al.*, 1992a; Persohn *et al.*, 1992; Wisden *et al.*, 1992). In the normal cerebellum, GABAergic transmission between stellate cell axons and Purkinje cell dendrites is mediated by GABA<sub>A</sub> receptors containing only the  $\alpha 1$  subunit but not the remaining five  $\alpha$  subunits (Laurie *et al.*, 1992a; Persohn *et al.*, 1992; Wisden *et al.*, 1992; Wisden *et al.*, 1996). In contrast, inhibitory transmission between Golgi cell axons and granule cell dendrites is mediated by GABA<sub>A</sub> receptors containing both  $\alpha 1$  and  $\alpha 6$  subunits (Laurie *et al.*, 1992a; Persohn *et al.*, 1992; Wisden *et al.*, 1992; Nusser *et al.*, 1995; Wisden *et al.*, 1996; Nusser *et al.*, 1998).

To test the relationship between types of presynapse and subunits in the postsynapse, we examined the expression of subunits in the reeler cerebellum. In the central cerebellar mass of the reeler cerebellum, Purkinje cells directly form synapses with Golgi cell axons (Rakic, 1976; Mariani *et al.*, 1977; Caviness & Rakic, 1978; Sotelo & Privat, 1978; Wilson *et al.*, 1981; Takayama, 1994). If presynaptic neurons determine the type of receptor subunits in the postsynaptic neurons, GABAergic innervation from Golgi cells would induce Purkinje cells to express the  $\alpha 6$  subunit in the central cerebellar mass. Nevertheless, Purkinje cells in the central cerebellar mass do not express the  $\alpha 6$  nor  $\alpha 2$  subunits (Fig. 3C, D, G, H) (Takayama & Inoue, 2003). This result indicates that Golgi cell innervation does not induce expression of the  $\alpha 6$  subunit in the Purkinje cells, and suggests postsynaptic self-autonomous manners, not presynaptic neurons, determine the types of subunits.

### ***Synaptic targeting and clustering of GABA<sub>A</sub> receptor proteins***

Synaptic targeting and clustering of GABA<sub>A</sub> receptors are mediated by the

interaction of the subunit proteins with subsynaptic cytoskeleton, and it is thought that diversity of subunits in the GABA<sub>A</sub> receptors is important for subcellular localization (Barnes, 2000; Moss & Smart, 2001). Most of single subunits are retained within the endoplasmic reticulum (Connolly *et al.*, 1996; Gorrie *et al.*, 1997; Taylor *et al.*, 2000). Specific subunits such as the  $\gamma 2$  subunit can lead the assembled GABA<sub>A</sub> receptors to the cell surface and synaptic site (Connolly *et al.*, 1999) in conjunction with a range of diverse molecules such as gephyrin (Craig *et al.*, 1996; Essrich *et al.*, 1998; Kneussel *et al.*, 1999; Sassoë-Pognetto & Fritschy, 2000), GABA<sub>A</sub>-receptor associated protein (GABARAP) (Wang *et al.*, 1999), microtubule-associated proteins, transporters, protein kinases and so on (Moss & Smart, 2001). Furthermore, anchoring proteins such as gephyrin and GABARAP are also involved in clustering of the receptor proteins (Barnes, 2000; Moss & Smart, 2001).

## Conclusion

Developmental change in GABAergic signaling is summarized in Figure 4. At the early cerebellar developmental stage, GABA is synthesized and localized throughout the GABAergic neurons including dendrites (black curved lines), cell bodies (gray circles), axons (black lines), axon varicosities (black circles), and growth cones (black triangles). In this stage, GABAergic neurons extrasynaptically secrete GABA by vesicular and non-vesicular mechanisms (brown arrows) into extracellular space. Functional GABA<sub>A</sub> receptors appear after leaving the proliferating zone. Granule cells express the GABA<sub>A</sub> receptors containing  $\alpha 2$  subunit. Extrasynaptically secreted GABA may activate GABA<sub>A</sub> receptor on the developing and maturing granule cells in the external granular and molecular layers and regulate proliferation and migration. In the internal granular layer (IGr), GABA is released from axons and dendrites of Purkinje and Golgi cells, and might mediate network formation, including

extension of neuritis and dendrites and synapse formation. Attachment of GABAergic axons to the targeting neurons might induce the synapse formation, such as changing in subunit composition of GABA<sub>A</sub> receptors ( $\alpha 2 \rightarrow \alpha 1/6$ ). In the mature cerebellum, GABA is synthesized exclusively in cell bodies and axon terminals, transported into synaptic vesicles, and released at the synaptic site. GABA, which is synaptically released, 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 activity.

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## Figure legends

**Figure 1** Immunohistochemical localization of GABA (A, B), VGAT (C, D) and GABA<sub>A</sub> receptor α1 subunit (E, F) in the cerebellar cortex at postnatal day 7 (P7) (A, C, E) and postnatal one month (P1M) (B, D, F).

During the development, GABAergic localization drastically changes. In the immature cerebellum, GABA is localized widely in the GABAergic neurons, including dendrites, cell bodies, and axons (A). VGAT-immunohistochemistry shows that some of GABA is loaded to vesicles in the axon varicosities (C), and the other is localized in the cytoplasm (A). At the same stage, functional GABAergic synapses, however, are quite few by the immunohistochemistry for GABA<sub>A</sub> receptor α1 subunit (E). These results indicate that GABA is localized at both cytoplasm and vesicles where GABAergic synapses are not yet formed. In contrast, in the mature cerebellum, majority of GABA is localized in the GABAergic vesicles at the axon terminals (B, D) at GABAergic synapses (F).

Abbreviations and symbols, Mo: molecular layer, Pu: Purkinje cell layer, Gr: granular layer, asterisks: Purkinje cell bodies.

**Figure2** Schematic illustrations of the developmental change in GABA actions.

A) In the immature CNS, opening of the GABA<sub>A</sub> receptors (GABAR) generates efflux of chloride ion and depolarization of membrane potential, since intracellular chloride concentration, [Cl-]<sub>i</sub>, is relatively high by a dominant action of sodium-potassium-chloride co-transporter 1 (NKCC1). GABA-inducing depolarization activates the voltage dependent calcium channel (VDCC) and mediates calcium influx.

B) In the mature CNS, GABA mediates influx of chloride ion, since potassium-chloride co-transporter 2 (KCC2) lowers the intracellular chloride concentration. Influx of chloride ion

mediates hyperpolarization of membrane potential.

**Figure 3** Distinct expression of the GABA<sub>A</sub> receptor  $\alpha 1$  (A, B),  $\alpha 2$  (C, D),  $\alpha 3$  (E, F) and  $\alpha 6$  (G, H) subunits in the normal (A, C, E, G), and reeler (B, D, F, H) cerebella.

The specific expression of  $\alpha$  subunit mRNAs in each neuronal type was preserved in the reeler cerebellum. Furthermore, abnormal expression of  $\alpha$  subunits was not detected, although GABAergic networks were altered and neuronal maturation is severely disturbed. Abbreviations: IC: inferior colliculus, Mo: molecular layer, Pu: Purkinje cell layer, Gr: granular layer, Nu: cerebellar nucleus, WM: white matter, asterisks: central cerebellar mass beneath the granular layer, CM: central cerebella mass under the white matter.

**Figure 4** Schematic illustration of developmental change in GABAergic signaling on the granular cells

In the external granular layer (EGr), proliferating granule cells (red neurons) contain no functional GABA<sub>A</sub> receptors ( $\phi$ ). Granule cells start to express the GABA<sub>A</sub> receptors containing the  $\alpha 2$  subunit when they leave the proliferating zone and move into the premigratory zone. In the molecular layer (Mo), vertically migrating granule cells also express the  $\alpha 2$  subunit. In the internal granular layer (IGr), the subunit composition of the GABA<sub>A</sub> receptors changes. While granule cells were maturing during migration, dendritic elongation, and synapse formation, they continue to express the  $\alpha 2$  subunit. Subsequently, the  $\alpha 1$  and  $\alpha 6$  subunits appear in the granule cells and the  $\alpha 2$  subunit disappears from the granular layer. In the adult cerebellum, granule cells heavily express the  $\alpha 1$  and  $\alpha 6$  subunits.

During the differentiation and maturation of granule cells, GABA, localized widely in the GABAergic neurons (gray neurons with black neurites), is extrasynaptically released

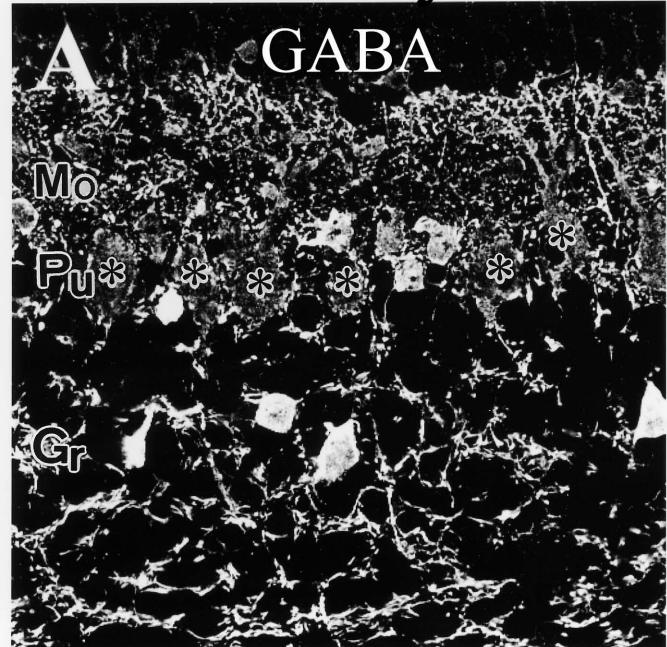
from Purkinje (Pu), stellate (St), Golgi (Go) cells, and mediates trophic actions (brown arrows), such as “stop proliferation”, “migration” and “synapse formation”. After cerebellar maturation, GABA is confined to the axon terminals, is exclusively released at the synaptic site (blue arrows), and mediates inhibitory transmission.

**Table 1**

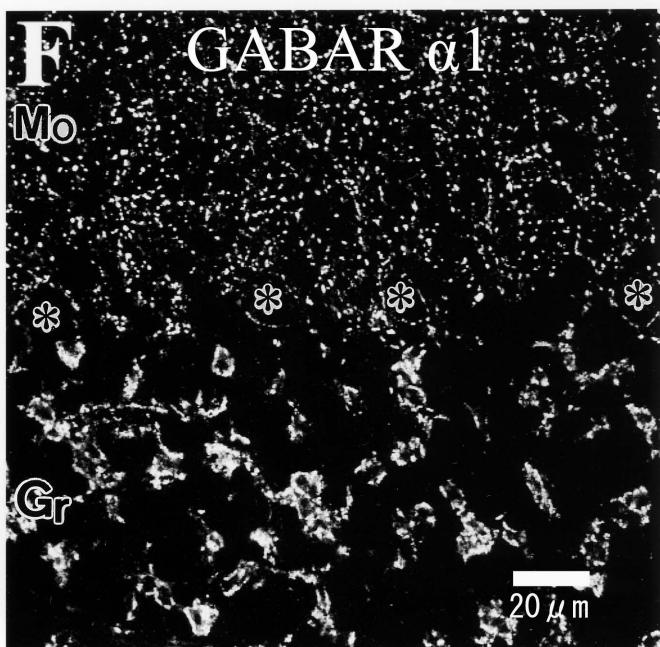
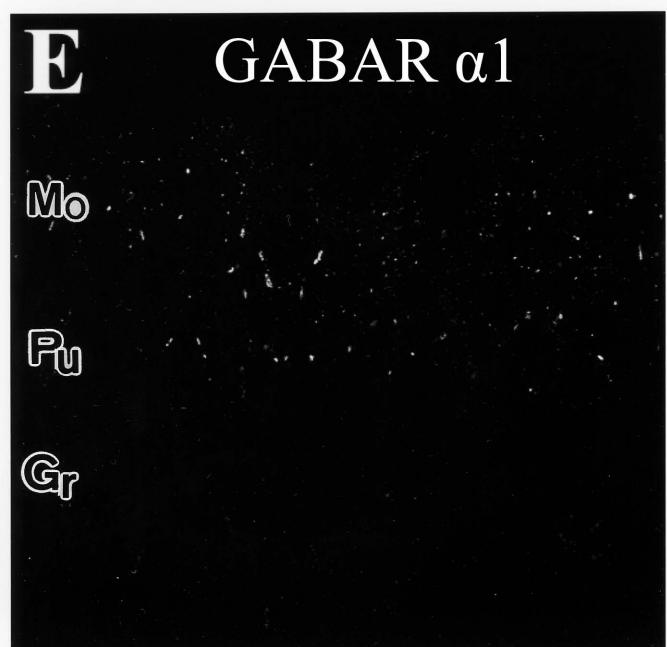
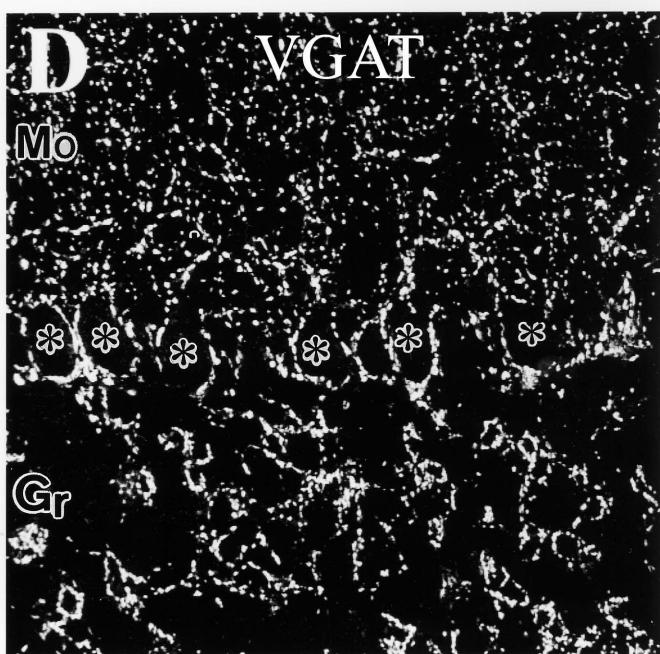
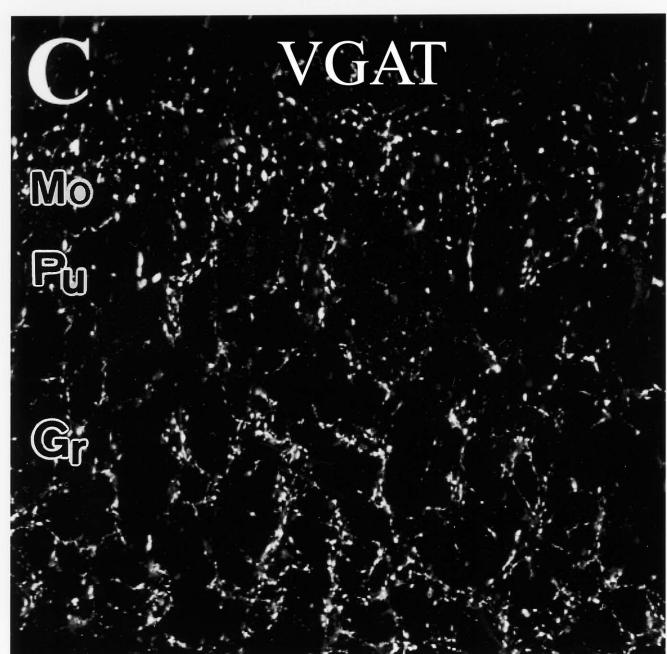
Changes in expression of the predominant  $\alpha$  subunits in the cerebellar cells

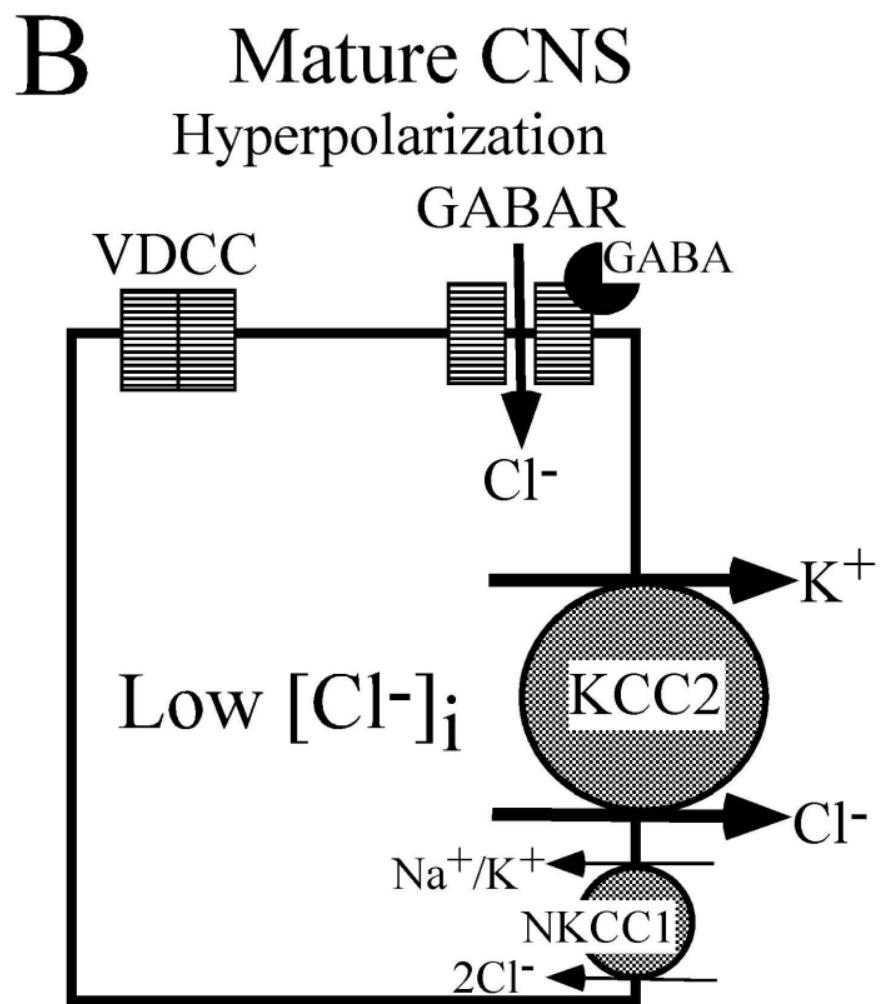
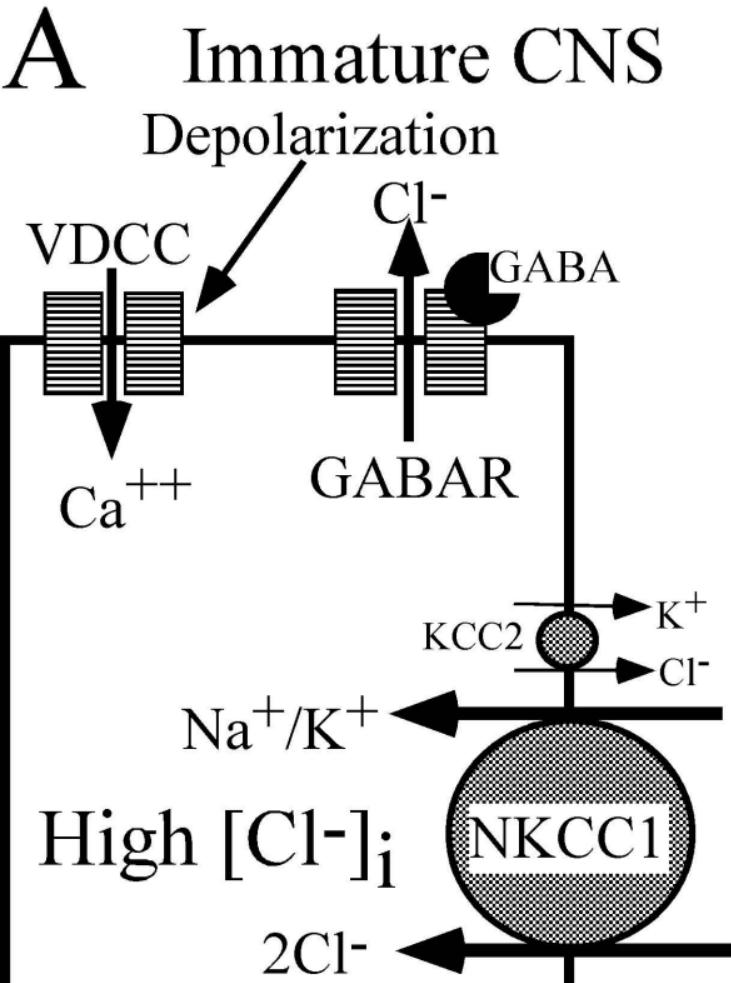
	Proliferating stage	Migrating and Differentiating stage	Matured stage
Purkinje cells	negative	$\alpha 3$ subunit	$\alpha 1$ subunit
Granule cells	negative	$\alpha 2$ subunit	$\alpha 1$ and $\alpha 6$ subunits
Nucleus neurons	negative	$\alpha 2$ and $\alpha 3$ subunits	$\alpha 1$ subunit

P 7day

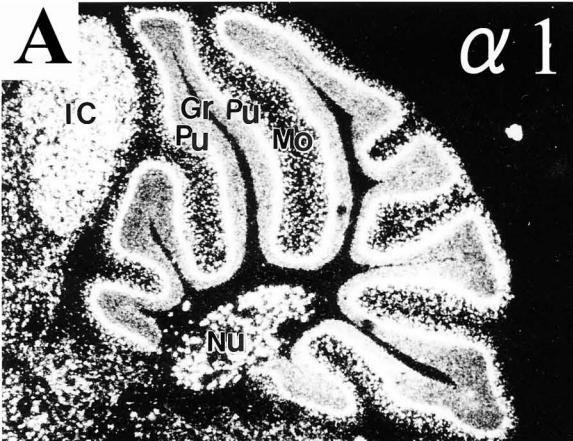


P 1month

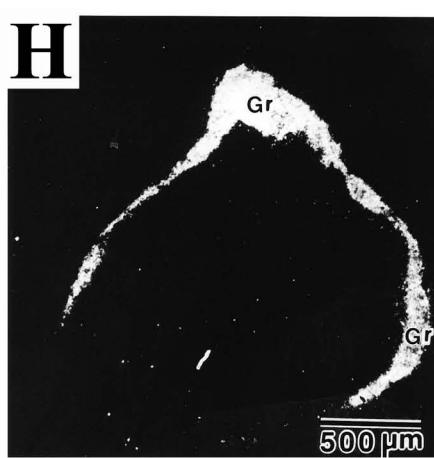
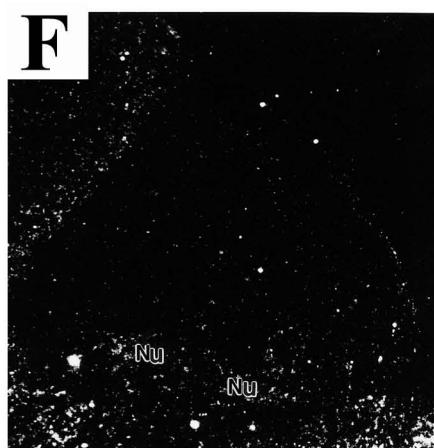
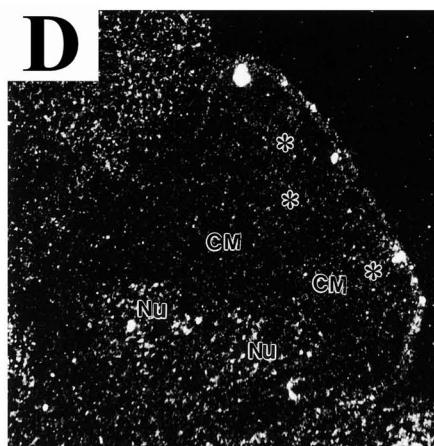
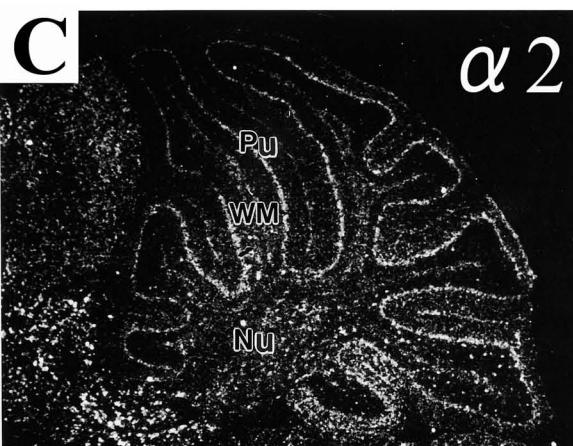
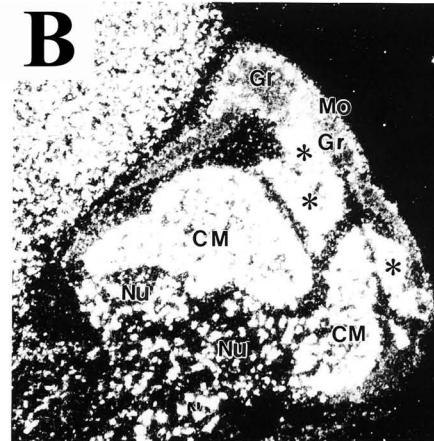




# Normal



# Reeler



500  $\mu$ m

500  $\mu$ m

